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A case study of neurodevelopmental risks from combined exposures to lead, methyl-mercury, inorganic arsenic, polychlorinated biphenyls, polybrominated diphenyl ethers and fluoride

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ABSTRACT

We performed a mixture risk assessment (MRA) case study of dietary exposure to the food contaminants lead, methylmercury, inorganic arsenic (iAs), fluoride, non-dioxin-like polychlorinated biphenyls (NDL-PCBs) and polybrominated diphenyl ethers (PBDEs), all substances associated with declines in cognitive abilities measured as IQ loss. Most of these chemicals are frequently measured in human biomonitoring studies. A component-based, personalised modified reference point index (mRPI) approach, in which we expressed the exposures and potencies of our chosen substances as lead equivalent values, was applied to perform a MRA for dietary exposures. We conducted the assessment for four different age groups (toddlers, children, adolescents, and women aged 18–45 years) in nine European countries. Populations in all countries considered exceeded combined tolerable levels at median exposure levels. NDL-PCBs in fish, other seafood and dairy, lead in grains and fruits, methylmercury in fish and other seafoods, and fluoride in water contributed most to the combined exposure. We identified uncertainties for the likelihood of co-exposure, assessment group membership, endpoint-specific reference values (ESRVs) based on epidemiological (lead, methylmercury, iAs, fluoride and NDL-PCBs) and animal data (PBDE), and exposure data. Those uncertainties lead to a complex pattern of under- and overestimations, which would require probabilistic modelling based on expert knowledge elicitation for integration of the identified uncertainties into an overall uncertainty estimate. In addition, the identified uncertainties could be used to refine future MRA for cognitive decline.

Abbreviations: ESRVs, endpoint-specific reference values; iAs, inorganic arsenic; IQ, intelligence quotients; LB, lower bound; LOD, level of detection; LOQ, level of quantification; metHg, methyl mercury; MRA, mixture risk assessment; mRPI, modified reference point index; NDL-PCBs, non-dioxin-like polychlorinated biphenyls; PBDEs, polybrominated diphenyl ethers; POD, point of departure; SF, scaling factor; UB, upper bound; UF, uncertainty factor.

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1. Introduction

Populations are exposed to unintentional mixtures of chemicals via their diet, drinking water, inhaled air, dust or contact with consumer products. Until recently, the risks of chemicals to human populations were frequently assessed on a chemical-by-chemical basis and for single exposure routes only. However, increasing awareness of the potential risks from mixtures shifted the focus towards combined exposures to multiple chemicals and routes. Depending on the regulatory framework or region, different terminologies are used, such as cumulative risk assessment (e.g. used by the European Food Safety Authority, EFSA, for combined exposure to pesticides, EFSA 2020a; b, 2022), cumulative impact assessment (e.g. United States Environmental Protection Agency; US EPA, 2022) or mixture risk assessment (MRA). Such an assessment can focus on the combined exposure to chemicals only (e.g. pesticides; EFSA 2020a; b, 2022) or include non-chemical stressors (e.g., social determinants as in US-EPA cumulative impact assessment; US EPA, 2022) In this paper, we will focus on the combined exposure to chemicals only and use the wording MRA.

Considerable efforts have gone into developing concepts, methods, and guidance for MRA ((e.g. Boobis et al., 2008; EFSA, 2007, 2008, 2019, 2021a; Fox et al., 2017; WHO, 2008; Bopp et al., 2018; OECD, 2018). To harmonise MRA within the European Union, EFSA developed two pieces of guidance for human risk assessment of combined exposure to multiple chemicals (EFSA 2019, 2021a). In the 2019 report, EFSA elaborated a tiered approach for several aspects of mixture risk assessment across EFSA's domains (EFSA 2019). The EFSA 2021 report developed criteria for the grouping of chemicals for MRA (EFSA 2021a). Mechanistic information (common mode of action or adverse outcome pathway) through a structured weight of evidence approach is regarded as the gold standard. When such mechanistic data are not readily available, EFSA proposes that grouping may be performed using a common adverse outcome (phenomenon) or a common target organ/-system. EFSA used these grouping principles for dietary exposures to pesticides and proposed common assessment groups derived for chronic effects on the thyroid and for those that have acute effects on the nervous system (EFSA, 2020a; EFSA, 2020b, EFSA et al., 2022).

Biomonitoring studies have shown that humans are exposed to mixtures of contaminants from different chemical classes, such as heavy metals and persistent organic pollutants (Haug et al., 2018; Buekers et al., 2021; Julvez et al., 2021). Despite these findings, MRA is often limited to groups of structurally related contaminants, such as dioxins and dioxin-like polychlorinated biphenyls (PCBs), phthalates or polyfluorinated alkyl substances. We therefore became interested in making a leap to a MRA for chemicals that transcend groups of closely related substances and that would facilitate future scientifically based risk management decisions.

In this paper, we present the results of a MRA case study of developmental neurotoxicants in food in which we applied the EFSA approach to assess possible risks of reduced cognitive function in children. Applying this approach to external dietary exposure allows for identification of risk-driving chemical substance combinations. We focused on chemicals with a high occurrence in human biomonitoring matrices of approximately 1300 English, French, Spanish, Lithuanian, Norwegian and Greek mothers and children of the Early-Life Exposome (HELIX) cohorts, and associations with IQ loss in children after maternal or early childhood exposures, as identified by Grandjean and Landrigan (2006, 2014). Accordingly, we selected the food contaminants lead, methyl mercury, inorganic arsenic, non-dioxin-like polychlorinated biphenyls (NDL-PCBs), and polybrominated diphenyl ethers (PBDEs). Fluoride was added to this list because recent evidence suggests it also may affect cognitive development (Grandjean 2019, 2022). In some European countries, fluoride is added to drinking water (EFSA, 2013). The aim of the paper is 1) to investigate the feasibility of MRA for chemicals from different classes that are associated with IQ loss and 2) to identify challenges and major uncertainties in the input data. The results should not be regarded as formal national risk assessments.

2. Methods

2.1. Cumulative assessment group

Food contaminants were included in the assessment group based on the following criteria:

1. A high occurrence rate in human biomonitoring matrices of approximately 1300 English, French, Spanish, Lithuanian, Norwegian and Greek mothers and children of HELIX cohorts, defined as quantifiable in >50% of blood and urine samples, as shown by Haug et al. (2018). Polychlorinated organic pollutants, brominated flame retardants, per- and polyfluoroalkyl substances, heavy metals, phthalate metabolites, phenols, and organophosphates met this criterion.
2. Evidence of associations with cognitive declines, measured as IQ loss as identified by Grandjean and Landrigan (2006, 2014). Lead, methyl mercury, inorganic arsenic, PCBs, PBDEs and organophosphate pesticides fulfilled this criterion. Although not measured in the study of Haug et al. (2018), fluoride was included because high intake levels are associated with IQ loss (Grandjean 2019, 2022).
3. Sufficient data to derive a point of departure (POD) and an endpoint-specific reference value (ESRV) from epidemiological studies. This criterion was met by lead and methyl mercury as their health-based guidance value is based on IQ loss (EFSA 2010a; US EPA 2001). ESRV for PBDEs are extrapolated from developmental neurotoxicity (locomotion and total activity) in rodents. The available epidemiological data for PCBs, inorganic arsenic and fluoride allowed estimations of POD and ESRV, but the data basis for organophosphates was judged to be insufficient. They were therefore not included in the present assessment. Accordingly, the cumulative assessment group for this study was composed of lead, methyl mercury, inorganic arsenic, PCBs, fluoride and PBDEs.

2.2. Estimation of PODs and ESRVs

For all the substances included in the assessment group, we collated quantitative dose estimates for declines in IQ scores and related ESRV for developmental neurotoxicity. The ESRV is defined as the POD of the substance divided by its uncertainty factor (UF) and is used to calculate external exposure. As much as possible, ESRVs were retrieved from existing evaluations of competent authorities (lead, methyl mercury, PBDE). In some cases, however, it was necessary to conduct separate reviews to derive the respective ESRV *de novo* (fluoride, inorganic arsenic). To make the mixture risk assessment as consistent as possible, we attempted to relate all ESRVs to the same effect magnitude, IQ losses by 1 point. However, some studies derived exposures associated with 5-point IQ losses. In such cases, we extrapolated to a 1-point loss. In addition, for some substances an additional UF was applied to take other uncertainties into account.

Table 1 provides an overview of the data used for the derivation of ESRVs. For each substance, details of the derivation of the ESRV are provided below. It should be noted that these ERSVs, unless they are health-based guidance values (e.g. lead), do not have the normative character of such values and should only be used for the purpose of a MRA. Except for iAs, the ESRVs were derived for expected mothers. The same ESRVs were used for all age groups, regardless they were derived from mothers or children.

2.3. Lead

We followed the considerations of EFSA's CONTAM panel (EFSA 2010a). Based on the study by Lanphear et al. (2005) the Panel estimated that a blood lead level of 12 μg/L in children aged 5–10 years old

Table 1
Chemicals in the assessment group, their endpoint-specific reference value (ESRV) used for the scaling factor (SF) calculation and data used for the derivation of the reference dose.

Chemical	Effect	IQ test	Reference point	Sex	Reference type	Species	Conversion to intake dose	Uncertainty factor	ESRV	SF
Lead[a]	IQ loss in children (0–7 years) of exposed mother	FSIQ	1 point IQ loss related to 12 μg/L Pb in blood	Boys and girls	$BMDL_{01}$	Human	Expectant mothers: foetal/maternal Pb blood ratio ~ 0.9	–	0.54 μg/kg bw/d	1
Inorganic arsenic[b]	IQ loss in exposed children, contemporaneous exposure	Raw verbal IQ	2.6 points IQ loss in girls for every 100 μg/L urine	Girls	LOAEL	Human	Conversion to 1 IQ point by linear extrapolation	–	1.3 μg/kg bw/d	0.42
Methyl mercury[c]	IQ loss in children of exposed mothers	Several cognitive test, including FISQ	5 points IQ loss related to 4–25 ppm in maternal hair	Boys and girls	$BMDL_{05}$	Human	Via estimation of blood levels, then kinetic modelling and extrapolation	10	0.1 μg/kg bw/d	5.4
Fluoride[d]	IQ loss in children of exposed mothers	General cognitive index, FSIQ.	0.1–0.2 mg/L urine	Boys and girls	$BMDL_{01}$	Human	With Rugg-Gunn et al., 2011; daily excretion of F at BMDL = 0.1–0.4 mg/d; equivalent to 2.4–12 μg/kg	–	9 μg/kg bw/d	0.06
NDL-PCBs[e]	IQ loss in children of exposed mothers	FSIQ	5 points IQ loss related to 0.63–0.71 μg/g lipid in mother's milk	Boys and girls	$BMDL_{05}$	Human	Via estimation of body burden, kinetic model Factor 2 applied for conversion from 5 to 1 IQ point loss.	2	15 ng/kg bw/d	36
PBDE[f]	developmental neurotoxicity (locomotor, total activity)	–	PBDE-47: 309 μg/kg bw		$BMDL_{10}$	Mice	Via critical body burden in mice and humans to an external dose taking into account kinetic information, except for PBDE 209 since toxicokinetics are assumed to be similar in mice and man[f]. For PBDE-209 the external dose in mice was extrapolated to humans.	PBDE-47: 2.5	PBDE-47: 68.8 ng/kg bw/d	PBDE-47: 7.9
			PBDE-99:12 μg/kg bw/d					PBDE-99: 2.5	PBDE-99:1.68 ng/kg bw/d	PBDE-99: 318
			PBDE-153: 83 μg/kg bw/d					PBDE-153: 2.5	PBDE-153: 3.84 ng/kg bw/d	PBDE-153: 142
			PBDE-209: 1700 μg/kg bw/d					PBDE-209: 100	PBDE-209: 17 μg/kg bw/d	PBDE-209: 0.032

See section 2.2 for explanation. Abbreviations: IQ intelligence quotient; FSIQ full scale intelligence quotient; BMDL Benchmark dose lower limit; LOAEL lowers observed adverse effect level; Pb-lead; F-fluoride; NDL-PCBs non-dioxin-like PCBs; PBDE polybrominated diphenyl ethers; kg kilogram; d day.

[a] Data were retrieved from EFSA (2010a) and were based on Lanphear et al. (2005).
[b] Data were retrieved form Tsuji et al. (2015) and based on Hamadani et al. (2011).
[c] Data were retrieved from Rice et al. (2003).
[d] Data were retrieved from Grandjean (2019).
[e] Data were retrieved from EFSA (2005) and based on Jacobson et al. (2002).
[f] Data were retrieved from EFSA (2011a) and Martin et al. (2017).

Table 2
Description of the food consumption data of nine different European countries, including method of food consumption survey, year(s) in which the food consumption survey was conducted, the name of the survey, the population addressed, the total number of individuals and consumptions days included in the study, and the subpopulation groups and number of individuals included in the cumulative exposure assessment for chemicals relevant for IQ loss.

Country	Food consumption survey						Subpopulation in study	
	Method	Years	Name	Population[a] (years of age)	N total[b]	Consumption days	Subpopulation[c] (years of age)	N[d]
Austria (AT)	24-h dietary recall	2018	ADOLESCENTS-2018-2	10–17	657	2	10–17	657
		2016		18–64	2250	2	F 18-45	1013
		2018	NATIONAL-2016 PREGNANT-2018-2	Pregnant F 19-47	302	2	19–45	299
Croatia (HR)	24-h and 48-h dietary recall	2011–2012	NIPNOP-HAH-2011-2012	18–64	2002	3	F 18-45	629
Cyprus (CY)	24-h dietary recall	2014–2017	2014-2017-LOT1	0–9	848	3	1–2	279
			2014-2017-LOT2	10–76	1016	3	3–9	300
							10–17	274
							F 15-45	287
Czech Republic (CZ)	24-h dietary recall	2003–2004	SISP04	4–64	2353	2	4–9	389
							10–17	298
							F 15-45	419
Denmark (DK)	Food record	2006–2007	IAT 2006-07	0–3	1743	7	1–2	894
		2005–2008	DANSDA 2005-08	4–75	2700	7	3	23
							4–9	298
							10–17	377
							F: 18-45	570
France (FR)	FPQ[e] and 24 h dietary recall	2014–2015	INCA3	General population[f]	4874	3	Toddlers[f]	149
							Other children	921
							Adolescents	1221
Italy (IT)	Food record	2005–2006	INRAN SCAI 2005-06	0–97	3323	3	1–2	36
							3–9	193
							10–17	247
							F 15-45	703
Netherlands (NL)	Food record, 24 h dietary recall	2012–2016	FCS2016_Core	1–80	4313	2	1–2	440
							3–9	853
							10–17	870
							F 18-45	485
Slovenia (SI)	24 h dietary recall	2018	SI.MENU-2018	0.25–75	1981	2	1–2	344
							10–17	493
							F18-45	113

[a] Indicates the age range of the population included in the food consumption survey.
[b] Indicates the number of subjects included in the food consumption survey.
[c] Indicates the age range of the subpopulation included in the case study: toddlers (1–2 years of age), other children (3–9 years of age), adolescents (10–17 years of age) or women in their childbearing age 18–45 years). Unless otherwise stated, the subpopulation included males and females. F means females.
[d] Indicates the number of subjects included per subpopulation in the case study.
[e] FPQ: Food propensity questionnaire.
[f] Due to privacy reasons the French food consumption data contained age groups instead of individual ages.

is associated with an IQ loss of 1 point. By toxicokinetic modelling, EFSA converted this blood lead level into a daily intake of 0.5 μg/kg. Taking account of a foetal/maternal blood lead ratio of 0.9, this is equivalent to a daily intake of 0.54 μg/kg d by expectant mothers, which was used as ESRV in our study. No uncertainty factors were applied.

2.4. Methyl mercury

For our case study, we used the ESRV derived for methyl mercury as described by Rice et al. (2003). Evidence of declines in cognitive ability after maternal methyl mercury exposure during pregnancy comes from three main epidemiological cohorts, those in the Faroe Islands, New Zealand and the Seychelles. Reviewing data from these three cohorts, Rice et al. (2003) used benchmark dose modelling for in-utero exposure for all cognitive effects, including IQ scores of the Faroes cohort and estimated that maternal hair mercury levels of between 4 and 25 ppm are associated with IQ losses by 5 points in their children. Toxicokinetic modelling assuming a hair to blood ratio of 250 and a one compartment model assuming 1) 95% of oral methyl mercury being absorbed, 2) 5.9% of absorbed methyl mercury present in blood, 3) a blood volume of 5 L, 4) an elimination rate of 0.014 day^{-1}, and 5) a fixed body weight of 67 kg for pregnant women (Rice et al., 2003; US EPA 2001) revealed that these hair levels resulted from maternal daily methyl mercury intakes of between 0.447 and 1.9 μg/kg d. It should be noted that this approach assumes a ratio of 1:1 between maternal and cord blood (Rice et al., 2003). By application of an UF of 10 (to account for differences in maternal toxicokinetics and -dynamics), Rice et al. estimated a daily intake of 0.1 μg/kg d as tolerable. We employed this value in our case study. However, it is unclear whether the UF of 10 also caters for an extrapolation to exposures associated with 1 IQ point loss.

2.5. iAs

We adopted the values used by Tsuji et al. (2015) in their systematic review of arsenic-induced developmental neurotoxicity and risk assessment. Tsuji et al. evaluated several epidemiological studies that described associations between inorganic arsenic exposure and verbal IQ scores and rated the data from the Matlab cohort (Bangladesh) communicated by Hamadani et al. (2011) as most suitable for quantitative risk assessments. Hamadani et al. observed a decrease in cognitive ability by 2.6 IQ points in girls for every 100 μg/L increase in speciated urinary arsenic levels. This was related to contemporaneous arsenic exposures; a window of vulnerability for inorganic arsenic and developmental neurotoxicity is poorly defined. Conversion to an IQ loss by 1 point is associated with an increase by 38.5 μg/L speciated urinary arsenic levels. By application of a one-compartment toxicokinetic model, and assuming a urinary excretion rate of 0.4 L/day, 70–90% of oral dose excreted in urine (estimated from monkeys), and a body

weight of 14.9 kg (mean of Matlab cohort) Tsuji et al. (2015) estimated that such urinary arsenic levels result from daily intakes of between 1.1 and 1.47 μg/kg d. We selected the midpoint of this range (1.3 μg/kg d) as ESRV in our study. No UF was applied because the POD was based on human data.

2.6. Fluoride

Grandjean et al. (2022) recently presented a benchmark modelling for IQ losses associated with fluoride exposures in which they used data from two prospective birth cohort studies, the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) cohort in Mexico and the Maternal-Infant Research on Environmental Chemicals (MIREC) cohort in Canada. Assuming a benchmark response of 1 IQ point loss, they derived benchmark concentrations (BMCs) of maternal urinary fluoride and benchmark concentration levels (BMCLs). The BMC for maternal urinary fluoride associated with a 1-point decrease in IQ scores of preschool-aged boys and girls was 0.31 mg/L (BMCL, 0.19 mg/L). The BMD was 0.33 mg/L (BMCL 0.20 mg/L) when pooling the IQ scores from the older ELEMENT children and the MIREC cohort. From these two prospective studies the joint data showed BMCL results about 0.2 mg/L.

Assuming a 24 h urine volume of 1.5 L, this urinary fluoride levels would lead to a daily maternal fluoride excretion of 0.3 mg/d. Rugg--Gunn et al. (2011) have recorded the relationship between total fluoride intake and daily urinary fluoride excretion. Based on 8 studies among adults with a total of 269 data pairs (Fig 3 in Rugg-Gunn et al., 2011) it can be estimated that a daily excretion of 0.3 mg fluoride is to be expected with daily intakes of 0.6 mg. Assuming a body weight of 65 kg, this converts to an intake of 9 μg/kg d which we adopted as ESRV in our study. No UFs were applied.

2.7. NDL-PCBs

From the study of IQ loss in children of PCB-exposed mothers by Jacobson et al. (2002), a benchmark concentration of 0.63–0.71 μg/g lipid in mother's milk is associated with a benchmark response of 5% in terms of full-scale IQ loss (benchmark dose, lower limit, see Table 3 in Jacobson et al.). This value applies to all PCBs. To estimate daily intakes from PCB lipid levels, we followed the assumptions made in EFSA (2005): Adipose tissue constitutes 20% of an adult's body weight, the overall biological half-life of the most persistent PCB congeners is 10 years (3650 days) and the absorbed fraction is 0.9. Based on these assumptions, the daily PCB maternal intakes that will give rise to such PCB lipid levels at steady state can be estimated as 26–30 ng/kg d. For this, the following formula was used: intake [ug/kg/d] = serum lipid level [ug/kg lipid] * 0.138/T1/2 [d]/f, where T1/2 is half-life of excretion, 0.138 a composite of ln 2 and 0.2, and f the absorbed fraction.

Considering that the benchmark concentrations given by Jacobson et al. do not correspond to IQ losses of 1 point, we lowered these values and chose 15 ng/kg d as the ESRV in our study by applying an UF of 2.

PCBs can be split into 12 dioxin-like congeners (DL-PCBS) and 197 NDL-PCBs. We included only NDL-PCBs in our case study for two reasons: 1) According to EFSA, information on neurodevelopmental effects of DL-PCBs is too limited for risk assessment (EFSA 2018) and 2) human body burden in of PCBs in human biomonitoring is frequently assessed based on the sum of three indicator congeners PCB-138, -153 and 180, multiplied by two for inclusion of three additional PCB congeners −28, −52 and −101 (Kraft et al., 2017), which are all NDL-PCBs (EFSA 2005; JECFA 2016).

2.8. PBDEs

We adopted the congener-specific values for PBDE 47, 99, 153 and 209 which EFSA (2011a) used for margin of exposure considerations related to developmental neurotoxicity, applying an UF of 2.5 to PBDE-47, -99 and −153 to account for inter-species difference in toxicodynamics. For PBDE-209 an UF of 100 was applied (Martin et al., 2017). EFSA regarded the available data for other congeners as too unreliable to establish similar values. Therefore, those congeners were not included in the case study. The ESRVs for these PBDE congeners are: PBDE 47–68.8; PBDE 99–1.68; PBDE 153–3.84; PBDE 209–17,000 ng/kg d.

It is noted that these values are derived from motor activity effects observed in a developmental neurotoxicity study in rodents. There is no information how these would relate to IQ loss in humans. However, for the purpose of the present exercise these values were taken as the doses that would lead to 1 IQ point loss in humans.

2.9. Scaling factors

To be able to sum exposures, scaling factors (SF) were used to describe the toxicity of a substance *s* in terms of the toxicity of an index compound and can be used to combine exposures of substances in an assessment group. The SF of substances included in our case study was obtained by dividing the ESRV of lead (Pb) by that of the substance of interest by using the following equation:

$$SF_s = \frac{ESRV_{Pb}}{ESRV_s} \quad \text{(Equation 1)}$$

It should be noted that the scaling factor by definition differs from a relative potency factor (RPF), which is also used to describe the toxicity of a substance s in terms of the toxicity of an index compound to enable combining exposures of substances in an assessment group. Scaling factors can only be called RPFs if chemicals 1) act via a common mode of action; 2) differ only in potency (i.e., their individual dose–response curves should be parallel on log–dose scale), and 3) do not interact (Bosgra et al., 2009; EFSA 2019; Bil et al., 2021). Since this information is lacking for the substances in our case study, we used scaling factors.

2.10. Food consumption data

Food consumption data were obtained from the EFSA data warehouse[1] upon approval of data owners. Consumption data were obtained from nine European Member States and were derived from national food consumption surveys. Table 2 provides an overview of the characteristics of the food consumption data use in this study. Food consumption data were received using the non-hierarchical coding of the harmonized food coding system FoodEx1 (EFSA 2011b) and re-coded in the hierarchical FoodEx1 codes to allow for extrapolation of concentration data.

In their assessments for regulatory purposes, EFSA subdivided the population into age groups, i.e. infants, toddlers, other children, adolescents, adults, elderly and very elderly (EFSA 2011c). In our study, we mirrored the EFSA age groups for children and adolescents as close as possible (i.e. toddlers aged 1–2 years, other children aged 3–9 years, adolescents aged 10–17 years), but selected women aged 18–45 years as proxy for pregnant women. Infants (below the age of 12 months) were not included because the limited availability of food consumption data for this age group among the countries.

2.11. Chemical concentration data in food

Chemical concentration data from the years 2014–2018 were obtained for NDL-PCBs, PBDEs, lead, inorganic arsenic, methyl mercury, and fluoride from the ESFA data warehouse. Data were obtained from 15 European Member States that agreed to share data: Austria, Cyprus, the Czech Republic, Denmark, Estonia, Finland, France, Croatia, Hungary, Ireland, Italy, Luxembourg, the Netherlands, Sweden and Slovenia. Data were formatted according to EFSA standard sample descriptions (SSD1; EFSA 2010b), with food items coded according to the harmonized

[1] https://www.efsa.europa.eu/en/microstrategy/food-consumption-survey.

Table 3
Personalised modified reference point index) for substances relevant to loss of intelligence scores (lead, methyl mercury, inorganic arsenic, fluoride, non-dioxin-like polychlorinated biphenyls and polybrominated diphenyl ethers) calculated for toddlers (1–2 years), children aged 3–9 years, adolescents (10–17 years) and women in their childbearing age (18–45 years).

	Toddlers		Other children		Adolescents		Women child bearing age	
	LB[b]	UB[c]	LB	UB	LB	UB	LB	UB
P50								
AT[a]	–	–	–	–	1.6 (1.5–1.8)[f]	2.9 (2.7–3.2)	2.0 (1.8–2.1)	3.5 (3.2–3.8)
CY	4.7 (4.3–5.3)	9.2 (8.6–9.8)	3.3 (3.0–3.7)	6.3 (5.9–6.9)	1.8 (1.6–2.0)	3.4 (3.1–3.8)	1.5 (1.3–1.7)	2.6 (2.6–3.2)
CZ	–	–	3.6 (3.0–3.6)	6.0 (5.6–6.4)	2.3 (2.1–2.5)	4.1 (3.8–4.4)	1.6 (1.4–1.7)	2.7 (2.5–3.0)
DK	5.4 (4.8–6.0)	9.7 (9.2–10.5)	3.7[d] (3.2–4.0) 4.3[d] (3.2–6.7)	6.9[e] (6.4–7.5) 7.8[e] (6.8–10)	1.9 (1.7–2.2)	3.6 (3.3–3.9)	2.0 (1.8–2.1)	3.4 (3.2–3.7)
FR	4.5 (3.8–5.4)	9.0 (7.8–10.5)	4.2 (3.7–4.7)	7.3 (6.8–7.8)	2.1 (1.8–2.3)	3.7 (3.4–4.1)	–	–
HR	–	–	–	–	–	–	1.4 (1.2–1.5)	2.7 (2.5–3.0)
IT	7.6 (4.1–11)	12 (8.6–16)	4.7 (4.2–5.6)	8.2 (7.2–9.2)	2.7 (2.3–3.1)	4.7 (4.1–5.2)	2.3 (2.0–2.6)	4.0 (3.6–4.7)
NL	4.1 (3.5–5.1)	8.3 (7.4–9.4)	2.6 (2.4–2.9)	5.4 (5.2–5.9)	1.6 (1.4–1.8)	3.2 (3.0–3.5)	1.5 (1.4–1.7)	2.9 (2.6–3.2)
SI	3.4 (3.1–3.8)	7.1 (6.6–7.6)	–	–	1.5 (1.3–1.6)	2.8 (2.5–3.0)	1.3 (1.1–1.5)	2.5 (2.1–2.8)
P95								
AT	–	–	–	–	4.4 (3.5–5.7)	6.5 (5.8–7.7)	6.0 (4.7–7.6)	7.8 (6.8–9.5)
CY	15 (11–18)	21 (17–25)	11 (9.0–14)	15 (13–20)	6.4 (5.8–8.2)	8.8 (7.9–9.9)	6.5 (5.3–9.4)	8.6 (7.3–11)
CZ	–	–	15 (12–20)	20 (15.4–23.2)	11 (8.1–14)	13.1 (9.9–16.6)	7.9 (6.7–9.9)	9.7 (8.2–12)
DK	21 (18–26)	27 (23–31)	13[d] (9.9–16) 14[d] (9.3–20)	17[e] (14–21) 18[e] (12–25)	5.1 (4.0–6.8)	7.3 (6.3–9.1)	5.0 (4.3–6.1)	7.1 (6.5–8.0)
FR	14 (11–21)	20 (16–29)	13 (11–17)	18 (15–22)	6.8 (5.8–8.4)	9.5 (8.5–11)	–	–
HR	–	–	–	–	–	–	5.0 (4.2–6.3)	6.9 (6.0–8.4)
IT	17 (12–23)	24 (19–31)	14 (11–17)	18 (15–22)	9.7 (8.4–12.9)	12 (10.0–15.3)	10 (8.1–13)	12 (10–15)
NL	11 (8.6–14)	16 (14–19)	8.8 (6.8–10)	12 (11–15)	5.0 (4.0–6.4)	7.2 (6.2–8.8)	5.6 (4.5–7.3)	7.4 (6.3–9.5)
SI	11 (8.5–15)	15 (13–20)	–	–	6.9 (5.5–9.4)	8.5 (7.3–11)	6.7 (4.4–12)	8.0 (6.0–14)

[a] AT-Austria, CY-Cyprus, CZ-Czech Republic, DK-Denmark, FR-France, HR-Croatia, IT-Italy, NL-Netherlands, SI-Slovenia.
[b] LB is lower bound scenario. In this scenario analytical values below the limit of detection or limit of quantification were assumed to equal 0.
[c] UB is upper bound scenario. In this scenario analytical values below the limit of detection or limit of quantification were assumed to equal the value of the particular limit.
[d] DANSDA 2005–08 food consumption survey; children aged 4–9 years old.
[e] IAT 2006–07 food consumption survey; children aged 3-years old.
[f] Values between brackets indicate the upper and lower boundaries of the uncertainty interval quantified for uncertainties in food consumption and food occurrence data due limited sample sizes.

FoodEx1 coding system (EFSA 2011b). Wherever possible, food concentration data were used at FoodEx1 level 4, the most detailed level. For example, if sufficient concentration data, defined as at least 50 measurements, were available at the FoodEx1 level 4 code 'cow milk, <1% fat (skimmed milk)' concentration data at this level were used. If sufficient concentration data were not available, concentration data were grouped at a less detailed level. For example, 'cow milk, <1% fat (skimmed milk)' was then recoded into to cow's milk (level 3), liquid milk (level 2) or milk and dairy products (level 1), wherever relevant.

Data with empty cells in any important field of the SSD file, such as level of detection, level of quantification and analytical value, or invalid concentration units, were omitted. If a FoodEx code was missing, but the product name was available, the corresponding FoodEx code was added manually. Only data obtained from random sampling and convenient data were included. For each substance and composite food combination in the dataset it was decided to use the analytical data as such or to convert the food into its ingredients (see matching food consumption and concentration data). Complexity of the food (e.g. the FoodEx1 code represents a broad range of composite foods rather than a single food, such as meat-based dishes), availability of recipe data, and number of measurements for the composite food and its ingredients were important criteria for this decision. Once decided to convert a composite food into its ingredients, the analytical data of the composite food were removed from the data set. Supplemental material A provides information on the decisions made for the foods, and Supplemental material B shows the FoodEx1 level used for each substance in the concentration dataset, together with the number of measurements, the percentage left-censored data, i.e. measurements below the level of detection (LOD) or level of quantification (LOQ) and mean concentrations per food and substance. Below some particularities for the different substances are provided.

2.12. Lead

Of all obtained samples, two aberrant samples (outliers) were removed from the FoodEx1 category 'wine'; one with 14 mg lead/kg and the other 21 mg lead/kg. Of all substances, lead concentration data were most abundantly available, 39,959 entries were obtained from 13 EU countries and for 358 different FoodEx1 codes, after clean-up of the data.

2.13. Methyl mercury

Data for methyl mercury were obtained for fish and sea food (60 foods) from 12 EU countries. Analytical results for both methyl mercury and total mercury were available. Fewer numbers of analytical values were available for methylmercury (n = 165) than for total mercury (n = 6,542). Therefore, we decided to include methyl mercury concentrations calculated out of total mercury concentrations using conversion factors established by EFSA (EFSA 2012a):

- 1 for fish meat, fish products, fish offal and unspecified fish and seafood;
- 0.8 for crustaceans, molluscs and amphibians, reptiles, snails and insects;
- 0 for all other food categories not containing fish or seafood.

For the samples with measured methyl mercury concentrations, total mercury concentrations were also available, allowing comparisons of measured and calculated methyl mercury concentrations. To do this, the mean of positive samples, i.e. samples with an analytical value of methyl mercury or total mercury above the LOQ value, was calculated. The mean calculated methyl mercury concentration was generally slightly higher than mean measured methyl mercury concentration (see Supplementary Material C). Given the smaller number of measured methyl mercury data and the slightly higher concentrations of calculated methyl mercury, it was decided to include only calculated methyl mercury data. After data cleaning, the dataset for methyl mercury contained 6,473 entries.

2.14. Inorganic arsenic

From the EFSA data warehouse, samples containing 'arsenic' between 2014 and 2018 were retrieved. Since we focused on the exposure to inorganic arsenic (iAs), 'organic arsenic' samples were omitted from the data and samples coded as 'arsenic and derivatives' and 'arsenic' were recoded to 'total arsenic' samples, following the approach taken by EFSA (2021b). Of samples for which both 'total arsenic' as well as 'inorganic arsenic' values were reported, the 'total arsenic' samples were omitted from the database. In addition, after closer examination of the original data, samphire ("*zeekraal*") samples analysed for 'arsenic' from the Netherlands were originally coded as 'leafy vegetables' and were consequently recoded as 'sea weeds'. The fraction of iAs was translated from the remaining 'total arsenic' samples according to the median ratios described in EFSA's Scientific Opinion (2021b). Similar to EFSA, total arsenic was not converted into iAs for fish. Supplemental material F lists the factors used for the conversion of total arsenic into iAs. Like EFSA, we used an additional LOQ-cut off of 100 μg/kg for iAS in cereal-based food for infants and young children.

The original dataset also contained 3104 entries for drinking water (tap and bottled). High concentrations of iAs in tap water (typically up to 7920 μg/litre), especially originating from one country, were present in the data set. In addition, the dataset contained non-detects with high LOQs (up to 900 μg/kg). A maximum level of 10 μg/L has been established for water intended for human consumption, without distinguishing among different arsenic forms (EU, 2020). In addition, a maximum level of 10 μg/L was established for total arsenic in natural mineral water (EC, 2003). In the most recent EFSA opinion on iAs, EFSA used concentration data over the years 2013–2018 and excluded values obtained from analytical methods with LOQs higher than 10 μg/L for that reason (EFSA, 2021b). To perform calculations using representative European iAs in drinking water, we did not use the received data from the Data Warehouse but used the mean values for the lower and upper bound as reported by EFSA in 2021. After data cleaning, 3,021 entries for iAs were obtained from 13 EU countries and for 117 different FoodEx1 codes.

2.15. Fluoride

Only fluoride concentrations in drinking water were available. Data were obtained from the EFSA data warehouse for bottled water, carbonated mineral water, still mineral water, well water and drinking water. Because of food conversions containing water, such as soft drinks and liquid infant formulae which are converted to water and other ingredients (see matching food consumption data and concentration data), all types of water were recoded into drinking water (A.15). Within the EFSA data warehouse information from limited countries was available. Therefore, additional fluoride concentration data obtained from the Dutch monitoring program for drinking water between 2014 and 2018 were included. It should be noted that those data were provided as mean values per pumping station. Mean values were calculated using a middle-bound scenario, in which samples below the limit of detection were substituted with a value equal to half the value of the level of detection. After data cleaning, 2011 entries for fluoride in drinking water were available.

2.16. NDL-PCBs

Concentration data were obtained for 6 NDL-PCBs, which are regarded as indicator congeners for the exposure to NDL-PCBs via food (EFSA 2005; JECFA 2016). Concentration data (n = 3,363 samples) for each of the 6 NDL PSBs were obtained from 9 countries. For many samples, the sampling type was not specified. To enlarge the number of observations, those samples were included. NDL-PCB concentrations in food were expressed on a whole weight- or on a percentage fat weight-basis. If for a sample data were available for both whole weight and percentage fat weight, the data expressed on whole weight were selected. If data were expressed based on percentage fat weight, the percentage fat in the original sample was provided in the SSD format. However, the original percentage fat in the sample was not always provided or higher than expected (up to 100%). To calculate the NDL-PCB concentration in those samples, the percentage fat weight according to the Dutch food composition database (NEVO; accessed November 2021)[2] was used. If NEVO provided two or more values for the percentage fat, the average fat weight was used for the calculations. After an initial run, high exposure estimates were obtained for NDL-PCBs in vegetable oil. This was mainly due to extreme NDL-PCBs concentrations analysed in one country. Average concentrations were approximately 250 times higher than those described for vegetable oil in the EFSA opinion (EFSA, 2012b). We therefore omitted the extreme NDL-PCBs concentrations analysed in one country. The mean concentration of the sum of 6 congeners now fell within the range published by EFSA (EFSA, 2012b).

As the sum of 6 indicator congeners comprises 50% of the total exposure to NDL-PCBs (EFSA 2012b), the 6 NDL-PCBs were summed per sample assuming equipotency (see paragraph scaling factors) and multiplied by 2 as a proxy for the total concentration of NDL-PCBs in food. In total, 20,103 data entries for the sum of NDL-PCBs in 60 food categories were used in the case study.

[2] Nederlands Voedingsstoffenbestand (NEVO) | RIVM. https://nevo-online.rivm.nl/Home/En

2.17. PBDEs

Concentration data were obtained from 4 countries and for 24 foods of animal origin (meat and meat products, fish and other seafood, eggs and milk). Like the NDL PCBs, PBDE concentrations in food were expressed based on whole weights or on percentage fat weight. Again, concentrations expressed on a whole weight basis were preferred over those expressed on percentage fat weight. In addition, the fat weight of the original sample was not always available, and the fat weights provided in the Dutch NEVO database was used to calculate PBDE concentrations expressed on whole weight. After data cleaning, the data set contained 1557 measurements for each of the four PBDEs.

Because some exposome studies or aggregated external exposure studies express the sum of PBDEs, concentration data for the four PBDEs −47, −99, −153 and −209 were summed per sample as lead-equivalents thus considering their SFs compared with lead (see paragraph scaling factors). Summing was performed following the lower bound and upper bound scenario (see Exposure scenarios).

2.18. Matching food consumption data and concentration data

As much as possible, food consumption data was linked to concentration data at the same level of detail. If that was not possible, food consumption was linked to a less detailed level FoodEx1 coding using the hierarchical FoodEx1 system. For example, consumption of turnips was linked to concentration data in root vegetables. As concentrations of substances are often available in raw agricultural products rather than processed products, a food translation table was used to link consumed processed food to substance concentrations in its raw agricultural commodity ingredients. For this we used the Dutch food translation table (Boon et al., 2015), which was based on Dutch recipes and contained conversion factors to convert foods classified according to FoodEx1 into their edible raw agricultural commodity ingredients (e.g. 167 g raw spinach is needed to produce 100 g cooked spinach). As this food translation table was developed for pesticide exposure calculations, it focused on fruit and vegetables. As such, the food translation table did not include animal-derived ingredients (fish, meat and milk) in composite food. Therefore, we updated the food consumption table with animal-derived ingredients as much as possible using Dutch recipes for composite foods.

2.19. Exposure scenarios

For each subpopulation the lower and upper bound scenarios following EFSA practice regarding handling concentrations below LOD LOQ EFSA, 2010c) were used for the exposure assessments. In the lower bound scenario, concentration values below the LOD or LOQ, as indicated accordingly in the SSD files, were assumed to equal 0. In the upper bound scenario, concentrations below the LOD or LOQ were assumed to equal the value of the respective limit.

2.20. Mixture risk assessment

Mixture risk assessment was performed using the MCRA tool version 9.1 (https://mcra.rivm.nl) for each country and subpopulation listed in Table 2, assuming dose additivity. Chronic (long-term) exposure was calculated using the Observed Individual Means (OIM) model. For each substance *s* in the assessment group and for each individual *i* in the food consumption data base, the consumed amount of a certain food *f* averaged over the total number of consumption days q_{if} was multiplied with the average concentration present in that food c_{ifs}. This was done for all consumed foods per individual. The subsequent obtained exposures per food were summed for each chemical *s* per individual over the *F* numbers of food consumed and divided by the bodyweight of the individual bw_i, which yielded the chronic exposure E_{is} to chemical *s* of the individual *i*.

$$E_{is} = \frac{\sum_{f=1}^{F} q_{if} c_{ifs}}{bw_i} \quad \text{(Equation 2)}$$

The chronic exposure of each chemical *s* in the assessment group E_{is} was then multiplied by the SF of the chemical (SF_s) and summed per individual to obtain the cumulative exposure per individual *Cum* E_i. As we used lead as the index chemical for deriving the SF, *Cum* E_j is the cumulative exposure of each individual expressed as lead equivalents:

$$Cum\ E_i = \sum_{s=1}^{S} E_{is} * SF_s \quad \text{(Equation 3)}$$

where *s* relates to the chemical considered. This yielded a distribution of the cumulative exposure, from which the median (P50) and the 95th exposure percentile were obtained.

Fold-exceedance of combined potency weighted tolerable exposures to the chemicals in the assessment group were characterised by dividing each individual's combined exposure in lead equivalents (*Cum* E_j) by the ESRV of the index compound lead ($ESRV_{Pb}$). We called the metric obtained in this way a *personalised modified reference point index* (mRPI).

$$personalised\ mRPI_i = \frac{Cum\ E_i}{ESRV_{Pb}} \quad \text{(Equation 4)}$$

This approach is similar to the mRPI introduced by Vejdovszky et al. (2019), as outlined in supplemental material D, and mathematically equivalent to the Hazard Index (Teuschler and Herzberg 1995). According to the EFSA guidance on harmonized methodologies, the hazard index is used in the context of health-based guidance values for the critical effect (such as the acceptable daily intake or the tolerable daily intake), whereas the reference point index (RPI), also known as the point of departure index, could be used for ESRVs that are not necessarily based on the critical effect (EFSA 2019). The RPI could typically use a single group UF (either a default or chemical-specific assessment factor) to assess the risk (EFSA 2019). Since UFs may vary depending on the derivation of the reference points, Vejdovszky et al. (2019) finetuned the RPI approach by applying chemical-specific uncertainty factors and named this the modified RPI (mRPI) approach. Because the reference points for IQ loss are not always based on the critical effect of a chemical and different UFs were applied, the mRPI approach was best suited to estimate the risk related to IQ loss.

Our approach yielded distributions of the personalised mRPI, of which the median and the 95th percentile of personalised mRPI were obtained. The personalised mRPI distributions obtained in this way were evaluated in terms of exceedances of combined "acceptable" exposures to lead equivalents relative to a value of 1. A personalised mRPI larger than 1 either means that a risk of the combined exposure cannot be excluded or that refinement is needed, depending on the direction of the uncertainties.

In addition to the calculation of percentiles, the contribution of substances to the personalised mRPI of the total population was assessed. For a particular substance *s,* the sum of the exposure *E* to that substance (expressed as lead-equivalents) of all individuals (n) in the food consumption database relative to the sum of the cumulative exposures of all individuals was calculated:

$$\%\ contribution\ s = \frac{\sum_{i=1}^{n} E_s}{\sum_{i=1}^{n} Cum\ E_i} * 100 \quad \text{(equation 5)}$$

Calculating the contributions for combinations of foods and substances is done in a similar way.

2.21. Uncertainty

The bootstrapping approach was used to quantify sampling uncertainty in food consumption and concentration data caused by a limited sampling size (Efron 1979; Efron and Tibshirani 1993). This approach

re-samples (with replacement) the original food consumption and concentration dataset to obtain a bootstrap of n observations. In the present calculation, we performed an uncertainty analysis using 100 re-sampling cycles with 10,000 iterations. This yielded 100 alternative exposure distributions, which might have been obtained during sampling from the population of interest and during sampling of foods. The mean and P95 were estimated for each of those 100 alternative exposure distributions, yielding 100 alternative exposure statistics. The median value (regarded as the best estimate) and its 95% uncertainty interval around the exposure estimates were obtained from those 100 alternative exposure statistics.

3. Results

3.1. Personalised modified reference point index

We calculated distributions of the potency weighted lead-equivalent exposures for substances relevant to IQ loss relative to the acceptable level of lead exposure, which we termed personalised modified reference point index (mRPI). Fig. 1 shows the personalised mRPI-distributions of women of child-bearing age from 8 European countries, calculated for the lower bound scenario, where analytical non-detects were set to zero. For the majority of the populations, personalised mRPIs were larger than 1. To make our findings comparable with risk assessments usually performed at the median (P50) or the 95th percentile (P95) of exposures, we additionally listed the P50 and P95 personalised mRPIs in Table 3. In the lower bound scenario, P50 personalised mRPI exceeded the ESRV of lead by between 1.3-fold for Slovenian women in their childbearing age and 7.6-fold for Italian toddlers. Approximately twofold higher P50 personalised mRPI were observed for the upper bound scenario in which we set analytical non-detects to the limit of detection. At P95, the personalised mRPI ranged from 4.4 for Austrian adolescents to 21 for Danish toddlers in the lower bound scenario. In the upper bound scenarios, 1.5-fold higher personalised mRPI were obtained. There was no exposure scenario, population subgroup or country, where the personalised mRPI stayed at or below the value of 1 for the entire population.

3.2. Main substances contributing to the personalised mRPI

Fig. 2 shows the contribution (expressed as percentages) of the different substances to the combined lead-equivalent exposures relative to the acceptable exposure to lead, personalised mRPI, of the various populations we examined in the selected countries.

Lead was an important contributor to the personalised mRPI in both the LB (non-detects set to zero) and UB scenarios (non-detects set at the level of quantification) in most of the countries and for several age groups. Lead alone made up between 15% of the personalised mRPI in Slovenian toddlers and 40% in Austrian adolescents in the LB scenario. In the UB scenario, this rose to between 35% for Danish toddlers and 52% for Austrian adolescents.

Non-dioxin-like PCBs also had a significant impact on the personalised mRPI, ranging from 17% for Austrian adolescents to 57% for Danish toddlers in the LB scenario and 14% for Austrian adolescents to 44% for Danish toddlers in the UB scenario.

For some countries, methyl mercury had a considerable influence on the personalised mRPI while for others its contribution was relatively small. It varied from 8% for Danish toddlers to 38% for Italian women in their childbearing age in the LB scenario. For the UB scenario it ranged from 6% in Austrian adolescents to 27% for Italian women in their childbearing age.

Fluoride showed differing impacts to the personalised mRPI, ranging from 4% for Slovenian toddlers in the LB scenario to 24% for Austrian women in their childbearing age. For the UB scenario, fluoride contributions varied between 4% (Italian toddlers) to 17% (Austrian women in their childbearing age).

Inorganic arsenic did not contribute strongly to the personalised mRPI, making up only 5–10% in the LB scenario and 8–11% in the UB scenario.

The sum of 4 PBDEs were of minor importance to the personalised mRPI in all countries. Their contribution amounted to only 2% or less in both the LB and UB scenarios.

3.3. Risk-driving food-substance combinations

Next, we analysed which food-substance combinations made up most of the intake of chemicals that considerably contributed to the personalised mRPI in the different countries (Table 4).

In all countries, and under both the lower and upper bound assessment scenarios, fluoride in drinking water contributed significantly to the personalised mRPI, varying from 6 to 24%. Methyl mercury in fish and seafood strongly impacted the personalised mRPI in all countries and age groups. This ranged from 8% (Denmark) to 38% (Italy). Lead derived from grains and grain products made up 5–11% of the personalised mRPI. Other notable sources of lead intake were vegetables and products thereof with a contribution to the personalised mRPI of up to 10% and fruit and fruit products (up to 12%). The most important source of NDL-PCB intake was from fish and seafood (8–38% of the personalised mRPI). Dairy products also significantly contributed to NDL-PCB intake (5–10% of personalised mRPI). In some countries, special foods were an important source of NDL-PCBs. This was due to fish oil supplements.

4. Discussion

Our case study shows that the component-based approach for performing MRA following EFSA guidance for grouping and exposure-based prioritisation of chemicals (EFSA 2021a) provides powerful information to risk managers on mixtures of different classes of dietary contaminants. Those mixtures have a high co-occurrence rate in biomonitoring studies. Apart from information on exceedances of the acceptable combined exposures, it provides information of sources of exposure, which could feed into re-evaluations of legal limits of substances in food. Considering chemicals relevant for IQ loss, the median and P95 personalised mRPIs exceeded the value of 1 in all populations. Lead and NDL-PCBs contributed strongly to the personalised mRPI, followed by methylmercury, fluoride and iAs. PBDEs only marginally influenced the combined risk. We also show that the food-substance combinations that contributed most to the combined risk are dairy, fish and seafood for NDL-PCBs, grains and fruits for lead, methyl mercury in fish and seafood, fluoride in drinking water and iAS in grains. There are some strengths and weaknesses in our case study which we discuss below.

4.1. Strengths and limitations of the study

4.1.1. Strengths

A major strength of our case study is in the use of combinations of food consumption data and data on the occurrence of our selected chemicals in food. This allowed us to establish country-specific distributions of personalised mRPIs. This level of detail was not achieved in MRA studies that relied on summary statistics of exposures at the median or the P95 (see for example Vejdovszky et al., 2019, EFSA 2019, Boberg et al., 2021, Sprong et al., 2020, Evans et al., 2016, Martin et al., 2017). The use of such summary statistics cannot deal with the fact that individuals highly exposed to one chemical will not necessarily experience high exposures to another substance. For example, in our study a vegetarian with high lead exposures due to large consumption of vegetables will not also be highly exposed to methyl mercury and NDL-PCBs in fish. The summing of lead exposure equivalents derived from high exposure percentiles is over-conservative. Distributions of personalised mRPIs for MRA provide rather realistic assessments and can therefore be regarded as a high tier MRA. Similar observations were made recently by

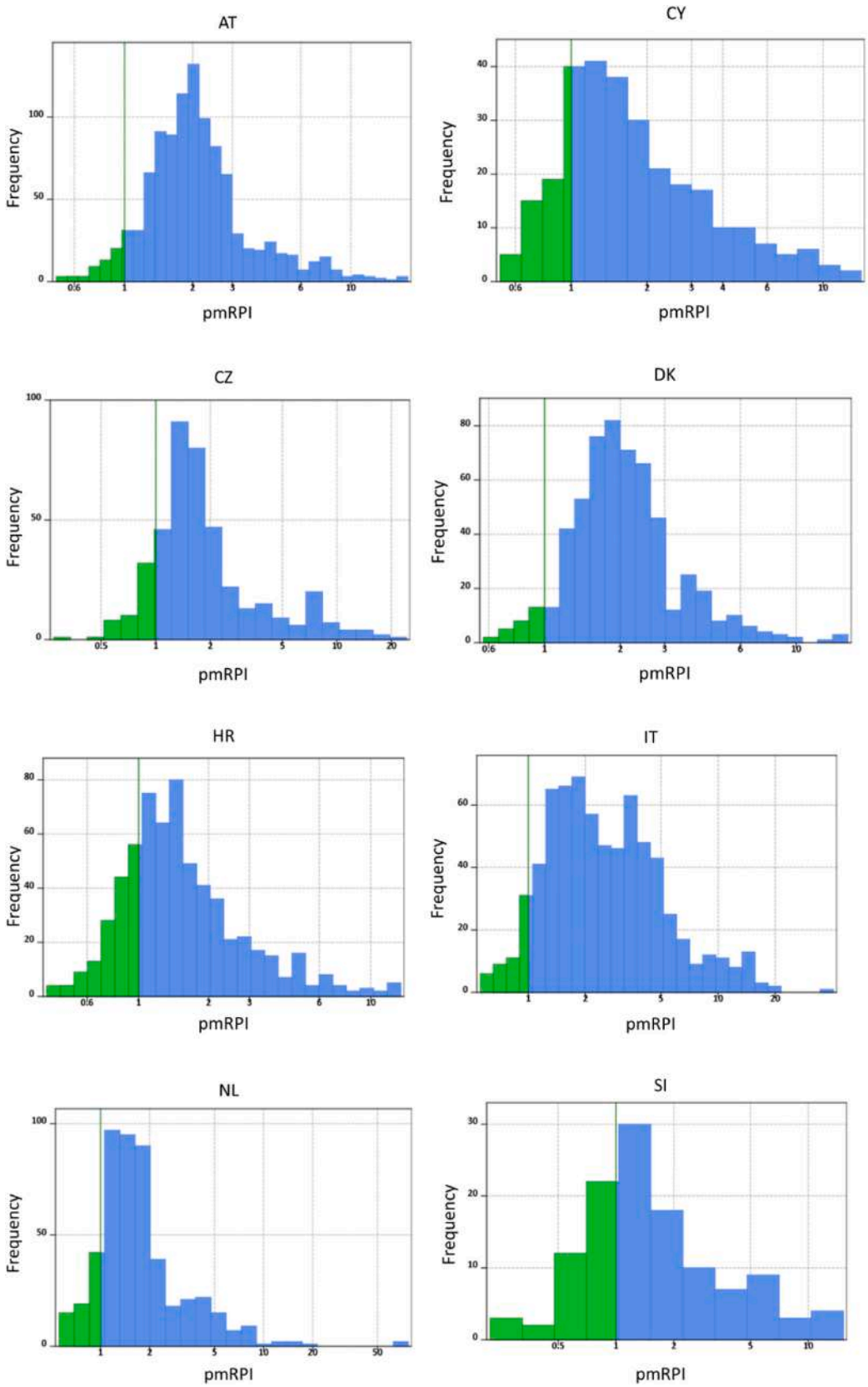
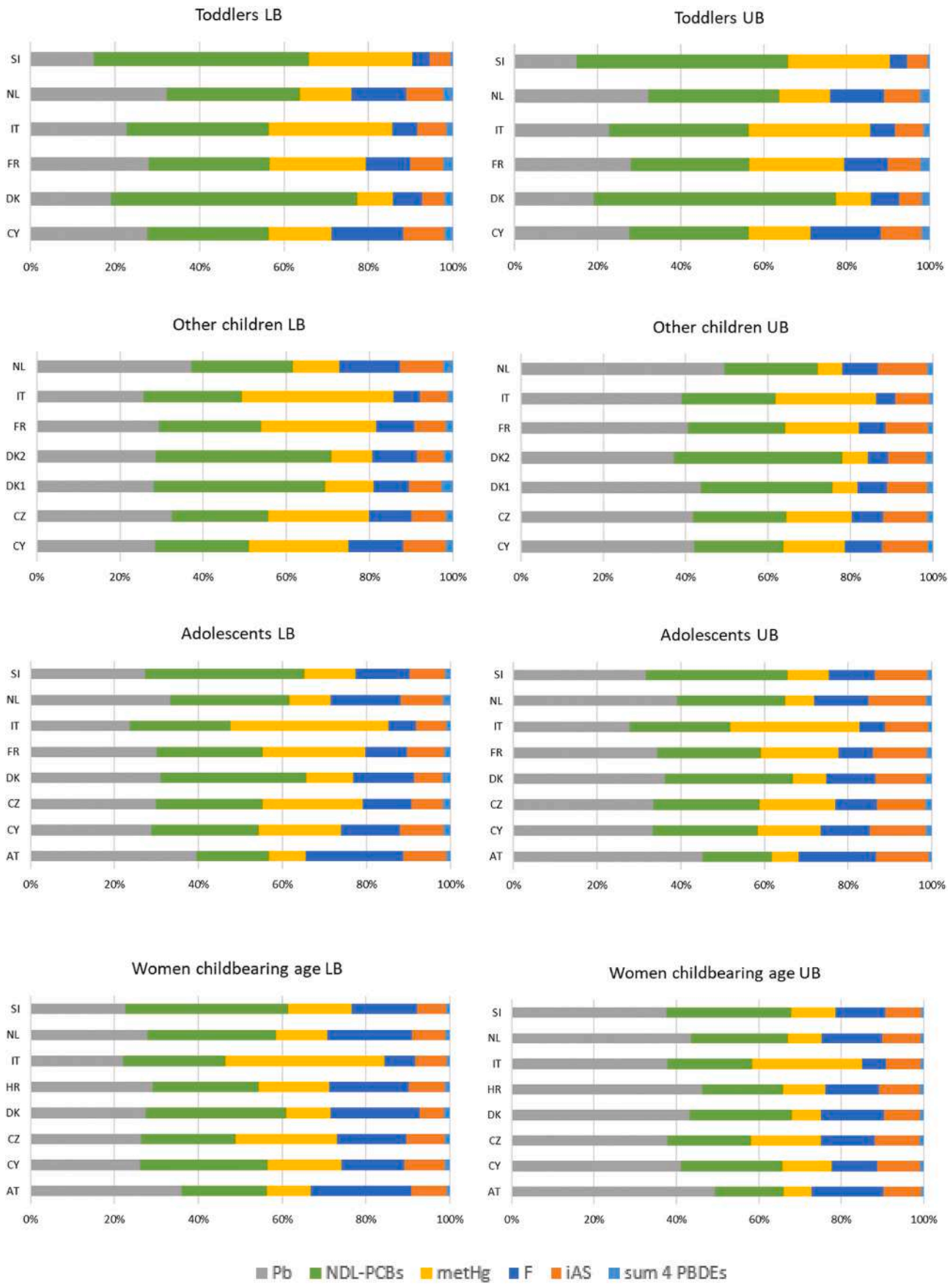


Fig. 1. Distribution of personalised modified reference point indices (pmRPI), which are potency weighted lead-equivalent exposures) for substances relevant to loss of intelligence scores (lead, methyl mercury, inorganic arsenic, fluoride, non-dioxin-like polychlorinated biphenyls and polybrominated diphenyl ethers) relative to the acceptable level of lead exposures (= 1), for women in their childbearing age (18–45 years) in 8 European countries. Results for the lower bound scenario, in which analytical values below the limit of detection or limit of quantification were assumed to equal 0, are shown. AT-Austria, CY-Cyprus, CZ-Czech Republic, DK-Denmark, HR-Croatia, IT-Italy, NL-Netherlands, SI-Slovenia. Values of pm RPI showing acceptable combined exposures (<1) are shaded green, those exceeding the index value of 1 are shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



(caption on next page)

Fig. 2. The percentage contribution of chemicals relevant to IQ loss to the personalised modified reference point index of the total population for toddlers (1–2 years old), other children (3–9 years old), adolescents (10–17 years old) and women in their childbearing ages (18–45 years) of 9 different European countries:Austria-AT, Cyprus-CY, Czech Republic-CZ, Denmark-DK (DK1 and DK 2 denote two different food consumption surveys for the particular subpopulation with DK1 providing the results of 3 years old children and DK2 results of children aged 4–9 years old), France-FR, Croatia-HR, Italy-IT, Netherlands-NL, Slovenia-SI, and for two different scenarios (lower bound-LB and upper bound-UB). For the LB scenario analytical values below the limit of detection or limit of quantification were assumed to equal 0, and for the UB scenario values below the limit of detection or quantification were assumed to equal the value of the particular limit. Pb: lead; NDL-PCBs: Non-dioxin-like PCBs; metHG: methyl mercury; F: fluoride; iAs: inorganic arsenic; PBDE: polybrominated diphenyl ethers.

Van den Brand et al. (2022) in their personalised mRPI distributions for mycotoxins and in the personalised MRA based on the HI approach for deteriorations of semen quality by Kortenkamp et al. (2022).

Another strength of our study is that we used reference values for specific effects, i.e. declines in cognitive ability as measured in terms of IQ loss. This is a rather refined way of performing MRA which avoids the mixing of toxicities as may be the case in low tier assessments based on HBGVs derived for different critical toxicities. Thus, in our case, we could rely on two HBGVs derived for IQ loss (lead, methyl mercury EFSA 2010a; US EPA 2001). In contrast, the HBGV for iAs is based on cancers of the lung, skin and bladder, as well as skin lesions; for NDL-PCBs it is based on liver and thyroid toxicity for NDL-PCBs (EFSA 2009; EFSA 2005). The use of these HBGVs would have biased our assessment. There is currently no HBGV for fluoride. We therefore estimated the corresponding ESRV for IQ loss for iAs, NDL-PCBs and fluoride based on epidemiological data. While there is evidence of associations of PBDE exposures with IQ loss (Eskenazi et al., 2013), there is no information on PBDE congener-specific associations which would have made it difficult to utilize the PBDE congener-specific food occurrence data. We therefore adopted the congener-specific hazard data derived by EFSA (2011a) for developmental neurotoxicity in rodents. Thus, assessments based on reference doses for specific endpoints, as we used in our approach and which shaped the personalised mRPI approach in Vejdovszky et al. (2019), the POD index (EFSA, 2019a), the chemical risk calculator (Boberg et al., 2021) and the normalized total margin of exposure approach (Sprong et al., 2020), provide a more realistic risk assessment.

However, the *de novo* derivation of reference values for specific endpoints can require extensive literature reviews and may be rather resource-intensive, while HBGVs or HBM-GVs are usually more readily available, e.g. in databases such as EFSA's OpenFoodTox database (Kovarich et al. 2016). Approaches based on such values also have merits in that they can provide lower tier MRAs which can be refined if the assessment indicates exceedance of combined acceptable levels (EFSA, 2019a).

4.1.2. Limitations

A major limitation of our study is that non-dietary routes of exposure, such as air, dust and soil, are not considered. Consequently, we very likely underestimated risks from combined exposures. However, for the general population in Europe there is good evidence that non-dietary exposures to lead, iAs, NDL-PCBs and PBDEs are of minor importance compared to dietary exposures. This may not always be the case for children, where uptake via dust and soil can be important routes of exposure to lead, iAs and PBDEs, particularly in highly contaminated areas (EFSA 2010a; EFSA 2011a; EFSA 2012a; EFSA 2005; EFSA 2009).

However, some studies revealed a larger role of non-dietary exposure to PBDE, since ingestion and dermal contact of dust were the major pathways of exposure to PBDE in an American study, accounting for 56–77% of the total exposure in toddlers, children, adolescents and adults, whereas diet only accounted for 20–40% (Johnson-Restepro and Kannan, 2009). In another recent American biomonitoring study, PBDEs exposure was the greatest contributor to IQ loss, followed by lead, organo-phosphates and methyl mercury (Gaylord et al., 2020), while our results show that the contribution of PBDEs to the combined exposure was only limited. This is likely explained by our inability to capture non-dietary exposures to PBDEs in our study; exposure from all routes is accounted for in human biomonitoring studies. Other factors can also explain the observed differences, among them the limited number of analytical data in our study, the differences in PBDE concentrations in dust and food between the US and Europe (Zota et al., 2008; EFSA 2009), the number of PBDEs included, i.e. PBDE- 47, -99, −153, −209 in our study, PBDE-47 in the study of Gaylord et al. (2020) and 20 PBDEs among which the congeners - 47, −99, −153, −209 in the study of Johnson-Restepro and Kannan (2009), and assuming equipotency of all PBDE isomers in other studies.

Some studies also pointed at a larger role for non-dietary sources of NDL-PCBs (Lehmann et al., 2015; Li et al. 2018)), Although banned in the United States and the European Union some decades ago (Lehmann et al., 2015; EFSA 2005), PCBs can be present in the indoor air and dust of many older buildings because of the use of NDL-PCB containing elastic sealants, caulking, paints, and flame retardant coatings (Lehmann et al., 2015). Large contributions of indoor air to the total exposure was shown for all age groups (Lehmann et al., 2015; Li et al. 2018), with contributions observed up to 60.8, 50.5, and 34.6% for children ages 2–3 years and 6–12 years and adults, respectively (Lehmann et al., 2015). Other dietary sources (e.g. tea) and routes of exposure are also relevant for fluoride, such as dental hygiene products, but the information accessible to us was too limited to draw conclusions on their contribution to total fluoride exposures (EFSA 2013). To obtain a more complete picture of the combined exposure to chemicals relevant for IQ loss, other routes of exposure can be included in external exposure assessment. Methodologies to aggregate the exposure from several routes are available (e.g. Husøy et al., 2020: aggregated exposure of di (2-ethylhexyl) phthalate from diet and personal care products) and have been implemented in MCRA (Van der Voet et al., 2020). However, chemical concentration data in consumer products and indoor air was not yet available for the substances included in our case study, and neither were levels in soil and outdoor air (IPCHEM database accessed 22 March 2022).

Another important limitation of our study that leads to an underestimation of the risk is that only certain contaminants were considered. Recently, an endpoint-specific reference value for IQ loss was estimated for cadmium (Chatterjee and Kortenkamp 2022). Cadmium is frequently detected in human biomonitoring samples (Haug et al., 2018; Buekers et al., 2021) at high occurrence rates (e.g. 99.6% and 96.5% quantifiable samples in mothers and child, respectively; Haug et al., 2018). Therefore, cadmium may significantly contribute to the risk of IQ loss.

In addition, human biomonitoring data showed co-exposure to substances from other regulatory domains, such as organophosphate pesticides (Haug et al., 2018), which are also relevant for IQ loss (Grandjean and Landrigan, 2006, 2014). As outlined in the method section, ESRVs of organophosphate pesticides for IQ loss were not readily available. Another issue with adding pesticide exposures to the combined exposure of contaminants is how to integrate different exposure scenarios deemed relevant for the particular regulatory silos. Where the LB and UB scenario is used by EFSA to estimate the risk of contaminants, such as the metals and persistent organic pollutants in our case study, for pesticides other refined scenarios with assumptions for agricultural use based on authorized uses are considered more realistic (EFSA 2020a; and b, EFSA et al., 2022; van Klaveren et al., 2019a and b). Currently, advanced exposure tools calculating exposure distributions are currently unable to deal with different exposure scenarios simultaneously and therefore, combined exposure of substances is often limited to summing percentiles (Sprong et al., 2020). The development of a tool that would be able to aggregate exposures from different regulatory frameworks by allowing simultaneous calculations using different exposure scenarios and

Table 4
Food-substance combinations contributing most to the combined dietary exposure to substances relevant for loss of intelligence presented for toddlers (1–2 years old), other children (3–9 years old), adolescents (10–17 years old) and women in their childbearing ages (18–45 years) of 9 different European countries (Austria-AT, Cyprus-CY, Czech Republic-CZ, Denmark-DK, France-FR, Croatia-HR, Italy-IT, Netherlands-NL, Slovenia-SI) and for two different scenarios (lower bound-LB and upper bound-UB)[a]. Percentages between brackets reflects the fraction of the personalised modified reference point index that can be attributed to the particular food-substance combinations.

Country	Toddlers	Other Children	Adolescents	Women child bearing age
Lower bound				
AT	–	–	F drinking water (23%) Pb grain (products) (11%) MetHg fish & seafood (9%) iAs grain(products) (6%) NDL-PCBs fish & seafood (6%)	F drinking water (24%) MetHg fish & seafood (10%) Pb grain (products) (7%) Pb vegetable (products) (7%) NDL-PCBs fish & seafood (7%)
CY	F drinking water (17%) MetHg fish & seafood (15%) NDL-PCBs fish & seafood (13%) NDL-PCBs dairy (8%) Pb Grain (products) (6%)	MetHg fish & seafood (23%) NDL-PCBs fish & seafood (13%) F drinking water (13%) Pb grain(products) (8%) NDL-PCBs dairy (6%)	MetHg fish & seafood (20%) F drinking water (14%) NDL-PCBs fish & seafood (14%) Pb grains (products) (7%) NDL-PCBs dairy (5%)	MetHg fish & seafood (18%) F drinking water (15%) NDL-PCBs fish & seafood (12%) NDL-PCBs special foods (9%) Pb grain (products) (6%)
CZ	-	MetHg fish & seafood (24%) NDL-PCBs fish & seafood (12%) F drinking water (10%) Pb grain(products) (7%) NDL-PCBs in fats and oils (6%)	MetHg fish & seafood (24%) NDL-PCBs fish & seafood (13%) F drinking water (12%) Pb grain(products) (7%) NDL- PCBs fats and oils (7%)	MetHg fish & seafood (24%) F drinking water (16%) NDL-PCBs fish & seafood (12%) NDL-PCBs fats and oils (5%) Pb grain (products) (5%)
DK	NDL- PCBs fish & seafood (47%) MetHg fish & seafood (8%) NDL-PCBs dairy (7%) F drinking water (7%) Pb grain(products) (6%)	DK1[b] NDL-PCBs fish & seafood (31%) F drinking water (10%) MetHg fish & seafood (9%) Pb grains (products) (8%) NDL-PCBs dairy (7%) DK2[b] NDL-PCbs fish & seafood (41%) MetHg fish & seafood (9%) NDL-PCBs dairy (8%) F drinking water (7%) Pb grains(products) (6%)	NDL-PCBs fish & seafood (22%) F drinking water (15%) MetHg fish & seafood (11%) Pb grain(products) (10%) NDL-PCBs in dairy (7%)	NDL-PCBs fish & seafood (24%) F drinking water (21%) MetHg fish & seafood (11%) Pb grain (products) (6%) Pb vegetable (products) (6%)
FR	MetHg fish & seafood (23%) NDL-PCBs fish & seafood (13%) F drinking water (11%) NDL-PCBs dairy (10%) Pb Grain (products) (7%)	MetHg fish & seafood (27%) NDL-PCBs fish & seafood (15%) Pb grain (products) (10%) F drinking water (9%) NDL-PCBs dairy (6%)	MetHg fish & seafood (24%) NDL-PCBs fish & seafood (14%) Pb grain(products) (11%) F drinking water (10%) iAs grain(products) (5%)	–
HR	-	–	–	F drinking water (19%) MetHg fish & seafood (17%) NDL-PCBs fish & seafood (15%) Pb grain(products) (7%) Pb vegetables (products) (6%)
IT	MetHg fish & seafood (29%) NDL-PCBs fish & seafood (22%) Pb grain (products) products (7%) NDL-PCBs in dairy (6%) F drinking water (6%)	MetHg fish & seafood (35%) NDL-PCBs fish & seafood (15%) Pb grain (products) (7%) F drinking water (6%) NDL-PCBs fats and oils (5%)	MetHg fish & seafood (38%) NDL-PCBs fish & seafood (14%) Pb grain(products) (7%) F drinking water (7%) Pb vegetable (products) (5%)	MetHg fish & seafood (38%) NDL-PCBs fish & seafood (15%) F drinking water (7%) Pb vegetable (products) (6%) Pb grain(products) (5%)
NL	F drinking water (13%) MetHg fish & seafood (12%) NDL-PCBs special foods (9%) NDL- PCBs dairy (9%) Pb grains (products) (8%)	F drinking water (14%) MetHg fish & seafood (10%) Pb grain(products) (9%) NDL-PCBs special foods (8%) NDL- PCBs dairy (8%)	F drinking water (17%) Pb grain (products) (10%) MetHg fish & seafood (10%) NDL-PCBs special foods (9%) iAs grain (products) (6%)	F drinking water (20%) NDL-PCBs special foods (16%) MetHg fish & seafood (12%) Pb vegetable (products) (7%) NDL-PCBs fish & seafood (6%)
SI	NDL- PCBs fish & seafood (13%) F drinking water (13%) MetHg fish & seafood (10%) Pb fruit and fruit products (9%) Pb grain(products) (9%)	–	NDL-PCBs fish & seafood (21%) F drinking water (13%) MetHg in fish & seafood (12%) Pb grain(products) p(8%) NDL-PCBs special foods (7%)	NDL-PCBs fish & seafood (17%) F drinking water (16%) MetHg fish & seafood (15%) NDL-PCBs special foods (15%) Pb grain (products) (5%)

(*continued on next page*)

Table 4 (*continued*)

Country	Toddlers	Other Children	Adolescents	Women child bearing age
Upper bound				
AT	-	-	F drinking water (16%)	F drinking water (17%)
			Pb grain (products) (12%)	Pb vegetable (products) (12%)
			Pb vegetable (products) (9%)	Pb grain (products) (8%)
			Pb fruit (products) (6%)	MetHg fish & seafood (7%)
			MetHg fish & seafood (6%)	Pb drinking water (6%)
CY	F drinking water (11%)	MetHg fish & seafood (15%)	MetHg fish & seafood (13%)	Pb vegetable (products) (12%)
	NDL-PCBs in dairy (9%)	F drinking water (9%)	F drinking water (10%)	MetHg fish & seafood (12%)
	MetHg fish & seafood (9%)	Pb vegetables (products) (9%)	NDL-PCBs fish & seafood (9%)	F drinking water (11%)
	NDL-PCBs fish & seafood (8%)	Pb grain (products) (9%)	Pb vegetable (products) (8%)	NDL- PCBs fish & seafood (8%)
	Pb vegetables (products) ((7%)	NDL-PCBs fish & seafood (8%)	Pb grain (products) (8%)	Pb grain (products) (6%)
CZ	-	MetHg fish & seafood (16%)	MetHg fish & seafood (16%)	MetHg fish & seafood (17%)
		NDL-PCBs fish & seafood (8%)	F drinking water (9%)	F drinking water (13%)
		F drinking water (8%)	NDL-PCBs fish & seafood (9%)	NDL-PCBs fish & seafood (9%)
		Pb grains (products) (8%)	Pb grain (products) (8%)	Pb vegetable (products) (7%)
		Pb vegetable (products) (6%)	Pb vegetable (products) (6%)	Pb grain (products) (6%)
DK	NDL-PCBs fish & seafood (31%)	DK1	NDL-PCBs fish & seafood (13%)	NDL-PCBs fish & seafood (15%)
	NDL-PCBs dairy (9%)	NDL-PCBs fish & seafood (19%)	F drinking water (10%)	F drinking water (15%)
	Pb dairy (7%)	Pb vegetables (products) (10%)	Pb grain (products) (9%)	Pb vegetable (products) (10%)
	Pb grain (products) (6%)	Pb grain (products) (8%)	Pb vegetable (products) (9%)	MetHg fish & seafood (7%)
	MetHg fish & seafood (6%)	NDL-PCBs dairy (8%)	NDL-PCBs dairy (7%)	Pb grain (products) (6%)
		F drinking water (7%)		
		DK2		
		NDL-PCBs fish & seafood (27%)		
		NDL-PCBs dairy (9%)		
		Pb grain (products) (7%)		
		Pb vegetables (products) (7%)		
		Pb dairy (6%)		
FR	MetHg fish & seafood (14%)	MetHg fish & seafood (18%)	MetHg fish & seafood (16%)	-
	NDL-PCBs in dairy (11%)	NDL-PCBs fish & seafood (10%)	Pb grain (products) (12%)	
	Pb foods for infants and small children (9%)	Pb grain (products) (10%)	NDL-PCBs fish & seafood (9%)	
	NDL-PCBs fish & seafood (8%)	Pb vegetable (products) (8%)	Pb vegetable (products) (8%)	
	F drinking water (7%)	NDL-PCBs dairy (7%)	F drinking water (7%)	
HR	-	-	-	F drinking water (13%)
				Pb vegetable (products) ((13%)
				MetHg fish & seafood (10%)
				NDL-PCBs fish & seafood (9%)
				Pb grain (products) (8%)
IT	MetHg fish & seafood (20%)	MetHg fish & seafood (24%)	MetHg fish & seafood (26%)	MetHg fish & seafood (27%)
	NDL- PCBs fish & seafood (15%)	NDL-PCBs fish & seafood (11%)	NDL-PCBs fish & seafood (10%)	Pb vegetable (products) (13%)
	Pb grains (products) (8%)	Pb vegetable (products) (9%)	Pb vegetable (products) (10%)	NDL-PCBs fish & seafood (11%)
	NDL-PCBs dairy (7%)	Pb grain (products) (9%)	Pb grains (products) (9%)	Pb grain (products) (7%)
	Pb vegetables (products) (6%)	Pb fruit (products) (5%)	F drinking water (5%)	F drinking water (6%)
NL	Pb fruit (products) (10%)	Pb grain (products) (9%)	F drinking water (11%)	F drinking water (15%)
	NDL-PCBs dairy (9%)	F drinking water (9%)	Pb grain (products) (10%)	Pb vegetable (products) (11%)
	F drinking water (8%)	Pb vegetable (products) (8%)	Pb vegetable (products) (8%)	NDL-PCBs special foods (10%)
	Pb grain (products) (8%)	Pb fruit (products) (8%)	MetHg fish & seafood (6%)	MetHg in fish & seafood (8%)
	Pb dairy (7%)	NDL-PCBs dairy (8%)	iAs grain (products) (6%)	Pb grain (products) (6%)
SI	Pb fruit (products) (12%)	-	NDL-PCBs fish & seafood (14%)	F drinking water (12%)
	Pb grain (products) (9%)		Pb grain (products) (10%)	NDL-PCBs fish & seafood (12%)
	F drinking water (8%)		F drinking water (10%)	MetHg fish &seafood (11%)
	NDL-PCBs fish & seafood (7%)		MetHg fish & seafood (8%)	Pb vegetable (products) (10%)
	Pb vegetable (products) (7%)		Pb vegetable (products) (7%)	NDL-PCBs special foods (10%)

Pb: lead; NDL-PCBs: Non-dioxin-like PCBs; metHG: methyl mercury; F: fluoride; iAs: inorganic arsenic

[a] For the LB scenario analytical values below the limit of detection or limit of quantification were assumed to equal 0, and for the UB scenario values below the limit of detection or quantification were assumed to equal the value of the particular limit.

[b] Two Danish food consumption sources were available. DK1 reflects the results obtained for the DANSDA 2005–08 food consumption survey (children aged 4–9 years old) and DK2 those of IAT 2006–07 food consumption survey (children aged 3-years old).

results in the generation of exposure distribution would allow for more realistic exposure assessments (Sprong et al., 2020). For the purpose of this case study, only different classes of contaminants were considered.

In our study we summed the indicator NDL-PCBs in food. To obtain the total exposure to NDL-PCBs we multiplied the resulting values by 2 (EFSA, 2005). In doing so, information on the risk driving congener(s) is lost. If such information is needed, additional calculations for NDL-PCBs and PBDEs using the individual congeners could be performed.

In our study, we focussed on MRA of dietary chemicals. However, non-chemical stressors may also affect cognitive development of children. A cumulative impact assessment includes such non-chemical stressors. A recent meta-analysis showed that alongside toxic chemicals, several non-chemical stressors such as maternal health, the mother's ability to access information relevant to a healthy pregnancy, dietary nutrients and quality of social interaction, had a significant impact on the child's cognitive development (Nilsen et al., 2020). It was beyond the scope of our case study to include those non-chemical stressors.

4.1.3. Uncertainties

In common with all risk assessments, the assessment performed in our case study is affected by uncertainties. Their identification is needed to assess whether the assessment represents an over- or an underestimation of risks. This is challenging in the case of a MRA, as the uncertainties behind every single chemical assessment may multiply leading to rather complex patterns of under- or over-estimations of combined risks. For a meaningful interpretation of the risks, an integration of those uncertainties into a final conclusion of the magnitude of over- or underestimation is needed. For pesticide MRAs, EFSA used a probabilistic approach for the integration of uncertainties into an overall conclusion (EFSA 2020a; and b, 2022). They identified 31, 34 and 41 sources of uncertainty (in food consumption, concentration data, hazard data and MRA methodology) for combined exposures to pesticides relevant for chronic effects on the thyroid and for pesticides relevant for acute effects on the nervous system, and craniofacial malformation respectively (EFSA 2020a; and b, 2022). Uncertainties were quantified at the high level of exposures using expert knowledge elicitation following principles of the EFSA guidance (EFSA 2014). This methodology resulted in a factor 3–5 lower combined risks to pesticides relevant for acute effects on the nervous system and a factor 2–4 lower for chronic effects on the thyroid (EFSA, 2020a and b). It was not the purpose of our study to perform such a sophisticated uncertainty analysis as this is resource intensive, and requires several experts with different backgrounds. Instead, we identified the major uncertainties, which future in-depth uncertainty analysis could build upon. Those are described below. Where possible, the direction of the uncertainty (over- or underestimation was given).

4.1.4. Endpoint IQ loss

We selected the loss of 1 IQ point as a measure of cognitive deficits in the developing child, since this degree of cognitive decline at the population level can have an economic impact on societies (Gould, 2009; Grandjean et al., 2012; Bellanger et al., 2013; Trasande and Liu, 2011; Pichery et al., 2011; Gaylord et al., 2020). IQ tests usually consists of several subtests, each measuring a different aspect of cognitive development, such as memory, verbal and spatial reasoning, planning, learning and the comprehension and use of language. Developmental neurotoxicants could affect a certain aspect of cognitive functioning rather than all aspects (EFSA 2010a). The PODs in our study were obtained from heterogenous endpoints, varying from general cognitive indexes (methyl mercury and fluoride), full scale IQ scores (lead, NDL-PCBs) or raw verbal IQ scores (iAs; Table 1). The use of such heterogenous endpoints in a combined assessment could result in uncertainty. This is probably limited for the general cognitive index, which showed concurrent validity with intelligence tests, including the Stanford–Binet IQ (r0.81) and full-scale IQ score (r0.71) from the Wechsler Preschool and Primary Scale of Intelligence (WPPSI) (Grandjean et al., 2022; Kaplan and Sacuzzo, 2010). The uncertainty caused by only including a particular subset (e.g. only verbal IQ or performance IQ) could be larger as exemplified for iAs. Here, the endpoint was based on raw verbal IQ scores. While iAs also affected full scale IQ score, the association between iAs intake and adverse effect in children was by a factor of 3 lower compared with verbal IQ scores as performance and processing speed were not affected (Tsuji et al., 2015). Thus, the use of heterogenous endpoints (full IQ scores or particular IQ scores) may have biased our calculation of personalised mRPI. One could therefore question whether a MRA should be performed based on mixing ESRV for full IQ scores and more specific cognitive functions.

The chemicals in our cumulative assessment group showed sex-specific sensitivities on cognitive development. For example, the ESRV of iAs was based on IQ loss observed in girls, which showed larger IQ losses than boys. In contrast, for fluoride larger effects on IQ loss were observed in boys. Yet, both boys and girls were included for the derivation of the ESRV. As generally the ESRV is based on the most sensitive gender, inclusion of both boys and girls in the derivation of the ESRV would have led to an underestimation of the personalised mRPI.

4.1.5. Model assumptions

Implicit in our adding up of lead-equivalent risk quotients in the personalised mRPI is the assumption of dose addition. The possibility of synergisms or (partial) antagonisms was not considered and this is a potential source of under- or overestimations of risks. However, a recent systematic review of the frequency of synergisms has shown that dose addition provides a good approximation of expected mixture effects (Martin et al., 2022).

4.1.6. Likelihood of co-exposure

In our case study, the likelihood of co-exposure to several pollutants was addressed by reviewing human biomonitoring detection rates (i.e. percentage of measurements above the LOD or LOQ, whatever applicable). For example, in the study described by Haug et al. (2018) occurrence rates varied from 54% for PBDE-153 in children aged 6–12 years to 100% for lead in maternal and children's blood. Occurrence rates of 90% of higher were observed for several PCBs, PBDE-47, mercury and lead, which indicates a high chance of co-occurrence. Besides PBDE-153, a lower occurrence rate was observed for arsenic (59% in maternal blood and 67% in children aged 6–12 years). Hence the chance of co-exposure to PBDE-153 and iAs is smaller, but still present. Assuming 100% co-exposure as we did in our study may have overestimated the personalised mRPI. More sophisticated methods to assess co-exposure patterns based on biomonitoring data are available, such as network analysis (Ottenbros et al., 2021), or external exposures such as the Sparse non-negative matrix under-approximation which has been applied to mothers' milk (EFSA 2021a; Crépet et al., 2022). Application of these methodologies may refine our analysis.

Human biomonitoring studies usually only analyse total mercury and arsenic (e.g. Haug et al., 2018; Julvez et al., 2021). As only methyl mercury and iAs are relevant for IQ loss, establishing co-exposures based on inspecting occurrence rates of total mercury and arsenic may introduce an element of uncertainty regarding co-exposures to methyl mercury and iAs. Measuring different forms of the metals may improve determination of co-exposures. Inclusion based on occurrence rates of total mercury and arsenic may have resulted in overestimations of exposure in the assessment, since occurrence rates of methylmercury and iAs may differ.

We also included fluoride in our case group, because fluoridation of drinking water is common practice in some European regions. Fluoride is not always considered in human biomonitoring studies. As fluoride may be obtained from other (dietary) sources (see 4.2.1 limitations) inclusion of fluoride in biomonitoring programs would be helpful to establish real-life mixtures.

4.1.7. Assessment group membership

With respect to the grouping of substances based on an effect on IQ loss, it should be noted that adverse effects of iAs and fluoride on IQ loss are still under debate. Based on the available evidence, the overall association between low-dose iAs exposure and IQ loss was considered as weak and therefore Tsuji et al. (2015) included only the study of Hamadani et al. (2011), a well-controlled study from the Bangladesh cohort with the most pronounced effect of iAs on IQ loss for the establishment of an ESRV. Some may question whether a substance can be considered as a member of the assessment group for IQ loss, based on overall weak associations with cognitive declines. According to the EFSA guidance on grouping, a higher degree of certainty in grouping efforts can be achieved when knowledge of an adverse outcome pathway (AOP) or the mode of actions is available (EFSA 2021a). Comprehensive AOPs for IQ loss have not yet been constructed and there is limited information on the mode of actions for IQ loss for the substances in our assessment group. Only the metals play a role in the AOP for deficits in learning and cognition (Von Stackelberg et al., 2015), but for the other substances included in our study the available information is limited. A putative AOP for developmental neurotoxicity as part of an integrated approach to testing and assessment was proposed recently by the EFSA PPR panel (EFSA 2021c). The use of AOPs in the classification of substances that have an effect on IQ loss can be evaluated in future studies. As iAs was included in the AOP of von Stackelberg et al. (2015), we included iAs in the assessment group IQ loss.

The detrimental effect of fluoride on cognitive function at low dose exposures in community fluoridation areas has been doubted by Guth et al. (2020, 2021). According to those authors, effects are predominantly observed in highly contaminated areas and from studies with shortcomings in design, such as small sample size and no adjustment for important cofounders, such as maternal IQ and co-exposure to other neurotoxicants. Some well-designed prospective studies from community water fluoridation areas which allowed for controlling well-known confounding factors showed contradictory results.

With respect to PBDE, two recent systematic reviews showed an adverse association between PBDE exposure and cognitive development in children (Lam et al., 2017; Gibson et al., 2018). Lam et al. (2017) concluded that sufficient evidence existed to support and association between developmental PBDE exposure in humans and IQ loss in children, Gibson et al. (2018) were more precautious in their conclusion because several uncontrolled confounders, such as co-exposure to known neurotoxicants, lack of statistical power due to small sample sizes and no statistical correction for multiple comparison, might have affected the outcome and impaired comparison across studies. Therefore, Gibson et al. (2018) advocated standardization of outcome assessment in future work.

4.1.8. ESRVs

Benchmark dose modelling is the preferred approach to establish ESRVs, since it makes a more extended use of dose-response data and it allows for quantification of the uncertainties in the dose-response data, in contrast to more simple approaches such as the NOAEL (EFSA 2017). To take the uncertainty of the benchmark dose into account, the lower bound of the confidence interval BMDL around the bench mark dose is used to derive the POD. In our case study, ESRVs based on BMDLs were obtained for lead, methyl mercury, fluoride, NDL-PCBs (Table 1 main text; all based on epidemiological data) and PBDEs (animal data). For iAs, only a LOAEL was available, which indicates that the ESRV of iAs is less robust.

In our study, we predominantly used ESRVs that were already published. As the aim of our case study was a proof of principle rather than a comprehensive risk assessment, we did not update established ESRVs as this was beyond the scope of our case study. Future research could update and/or refine ESRVs by using data from well-equipped mother/child cohorts addressing cognitive development, such as the HOME cohort; Kalloo et al., 2020, Braun et al., 2017 or the HELIX cohorts (Maitre et al., 2018). In our paper, we describe the uncertainties and indicate, where possible, whether this led to an under- or overestimation of the risk and the subsequent identification of the risk drivers. Table 5 summarizes those uncertainties, together with the direction of the effect on the risk. A detailed explanation on the uncertainties around the ESRVs is provided in Supplemental material E. A general uncertainty was the extrapolation of ESRVs derived for a certain age group to another age group, as was done for several substances (Tables 1 and 5). When extrapolating a reference point in urine or blood into an external dose, differences in kinetics between children and adults should be taken into account. Frequently noted uncertainties leading to overestimations of the personalised mRPI were: uncontrolled confounding (lead and iAs), cumulation of conservative assumptions for kinetic modelling (lead, iAs and PBDEs), and the choice of UFs (methyl mercury and PBDEs). An underestimation of the personalised mRPI was considered due to extrapolation of BMDLs (lead: extrapolation of the BMDL of women in their child-bearing age to toddlers and other children), assumptions for kinetic modelling (for fluoride), uncontrolled confounding for positive effects of fish consumption (methylmercury), inclusion of all dioxin-like PCBs in the ESRV of NDL-PCBS, and ignoring other relevant PBDE congeners.

4.1.9. Exposure data

Uncertainties in exposure data are related to food consumption data, occurrence data and matching food consumption data to concentration data. Sampling uncertainty in food and consumption data due to limited sampling size was quantified by bootstrapping (Efron 1979; Efron and Tibshirani 1993). This yielded the boundaries of the uncertainty interval around the personalised mRPI listed in Table 3, which indicate what the personalised mRPI could have been if other samples from the population and foods were used, assuming that representative sampling was applied. Generally, the upper boundary was about a factor 1.2 higher than the lower boundary at median personalised mRPI estimates, for the P95 the upper boundary was about a factor 1.5 higher. Only for some subpopulations was the ratio between the upper and lower boundary larger. This was predominantly applicable for subpopulations with a smaller size of less than 200 (Table 2). Exposure percentiles obtained for small subpopulations are statistically less robust. EFSA indicated that percentiles calculated over a number of subjects/days lower than 60 for the 95th percentile requires a cautious interpretation of the results since they may not be statistically robust (EFSA 2011b). As none of the lower boundaries of the uncertainty interval around the personalised mRPI is smaller than 1, the impact of sampling uncertainty on MRA is small.

Uncertainty around samples below the LOQ was addressed by the lower and upper bound scenario where those samples were substituted by zero or the value of the LOQ, respectively. Those scenarios were selected as they are frequently performed in risk assessment of contaminants. Other more realistic scenarios are available, such as the median bound (in which samples below the LOQ are assumed to equal half the value of the LOQ) and more sophisticated scenarios considering the distribution of samples below and above the LOQ.

Several other sources of uncertainties could not be quantified in our assessment. Those are listed in Table 6. A detailed description of the uncertainties is provided in Supplemental material F. Uncertainties included the use of the food coding system and assumptions made to handle data gaps. Table 6 also indicates the direction of the uncertainty: over- or underestimation of the personalised mRPI. In many cases, the direction of uncertainty was indeterminate. An exception was the use of conversion factors for methylmercury which resulted in an overestimation of the personalised mRPI and the contribution of methylmercury to the personalised mRPI. In addition, aggregation of foods in higher hierarchical FoodEx groups if less than 50 measurements per food group resulted in an overestimation of the iAs exposure and thus the personalised mRPI. Due to aggregating of foods (e.g. pasta, which could consist of rice-based pasta, such as rice noodles, and wheat-based pasta) and the oversampling of rice-based products compared with products

Table 5
Summary of sources of uncertainty around the endpoint-specific references values for IQ loss for lead, methyl mercury, inorganic arsenic (iAs), fluoride, non-dioxin-like perchlorinated biphenyls (NDL-PCBs) and polybrominated diphenyl ethers (PBDEs) and their effect on the personalised modified reference point index (personalised mRPI).

Substance	Type of uncertainty	Description	Direction effect on personalised mRPI	Reference
Lead	Uncontrolled confounding	Uncontrolled confounding, measurement error and other potential causal factors as common weaknesses were identified in the study of Lanphear, particularly for lead concentrations in blood below 50 or 100 μg/L. Whether this also affected piecewise linear function with breakpoint at 100 μg/L used by EFSA for the derivation of the $BMDL_{01}$ is not known to us.	+/−	Wilson and Wilson (2016), Van Landingham et al. (2021)
	Kinetic modelling	Conservative assumptions used for modelling dietary exposure out of blood concentrations.	+	EFSA 2010a
	Extrapolation $BMDL_{01}$ women childbearing age to other age groups	EFSA derived two $BMDL_{01}$s for IQ loss, one of 0.5 μg/kg bw per day for children aged 0–7 years and another one of 0.54 μg/kg bw/day for women in their child bearing age. Only 0.54 μg/kg bw/day was used in our study.	- (toddlers, other children)	EFSA 2010a
	Choice of UF	EFSA was in their opinion on the risk of lead not very clear which margin of exposure would be adequate, it could be interpreted as both 1 or 10. We used an UF of 1, while 10 could have been more appropriate.	–	EFSA 2010a
Methyl mercury	Point of departure	Considerable study uncertainty in the quantification of IQ loss upon prenatal methyl mercury exposure, with regression coefficients varying from 0 (no effect) to 1.5 (i.e. increase in the maternal hair concentration with 1 μg/g resulted in a loss of 1.5 IQ point). Differences could be explained by distinct exposure patterns, population genetic variability and nutrition (e.g. n-3 fatty acid intake).	+/−	Cohen et al. (2005)
	Uncontrolled confounding	While Rice et al. (2003) investigated the confounding effect of PCBs, they did not consider confounding beneficial effects of n-3 fatty acids in fish. When those were taken into account the ESRV was close to 0.1 μg/kg bw. Appropriate UFs were not provided.	- (if an UF is to be taken into account)	Groth (2017)
	Linear extrapolation $BMDL_{05}$ to $BMDL_{01}$	Not clear whether the UF of 10 covers the uncertainty caused by linear extrapolation of a $BMDL_{05}$ to a $BMDL_{01}$ as we did in our study	+/−	
	Choice of UF	Choice of UF varied from 10 in studies for IQ loss to 6.4 for other DNT effects derived from the same population(s). Difference is based on whether inter-species differences in toxicodynamics would require an additional UF. Rice et al., adapted the UF of US EPA (10), which based their study on 1 population. Rice et al. showed that the ESRV would not change when other (more sensitive) populations were included. EFSA and JECFA concluded that an UF for inter-species differences in toxicodynamics was not needed as a sensitive population was included. It should be noted that JECFA concluded that the UF could be further refined and reduced.	+	Rice et al. (2003) US EPA (2001) JECFA (2004) EFSA (2012)
iAs	Point of departure	Study uncertainty in the quantification of IQ loss due to large variability in studies caused by different study designs. The LOAEL was based on one well-designed study in a possible sensitive population due to malnutrition. Other well-designed studies were performed after the study of Hamadani. BMD modelling from all eligible studies would reduce uncertainty.	+/−	Tsuji et al. (2015)
	Uncontrolled confounding	Hamadani incompletely assessed maternal IQ, which is well-known confounder. Adjustment for study IQ in another study attenuated the association between iAs exposure and IQ loss. Studies performed after the study of Hamadani showed modest declines of IQ scores, with effects being more pronounced in girls than in boys. Residual confounding, such as exposure to other neurotoxicants could not be excluded.	+	Wasserman et al. (2011) Vahter et al. (2020)
	Kinetic model	Choice of parameter values and assumptions: Conservative assumption urinary excretion rates	+	Tsuji et al. (2011)
		Fraction of oral dose excreted in urine based on monkeys	+/−	
Fluoride	Point of departure	Boys more sensitive to the effect than girls. BMCL for pooled data (boys and girls used).	–	Grandjean et al. (2022)
	Kinetic model	The fractional retention of fluoride is only constant (i.e. 36% for adults) at a daily dietary intake of 2 mg/kg or higher (Villa et al., 2010). Below a total daily intake of 0.8 mg/day, fluoride excretion exceeds the intake, resulting in a negative fluoride balance. This means that at a daily urinary excretion of 0.3 mg, the consequent intake would be smaller than 0.3 mg/day instead of 0.6 mg/kg. Taken together, a urinary excretion of 0.3 mg/day leading to 1 IQ point loss is highly uncertain.	–	Rugg-Gunn et al. (2011), Villa et al. (2010)
		Extrapolation of maternal kinetics to children. Kinetics differ for different age groups	–	
		Use of upper boundary of interval around intake levels (highest intake), selection of midpoint would have led to a lower ESRV	–	
NDL-PCBs	Point of departure	ESRV based on total PCBs, which included dioxin-like PCBs, which are not associated with developmental neurotoxicity.	–	Jacobson et al. (2002), JECFA 2016 EFSA 2018

(*continued on next page*)

Table 5 (*continued*)

Substance	Type of uncertainty	Description	Direction effect on personalised mRPI	Reference
	Congener specific toxicity	Toxic potency could also differ between congeners. Preliminary neurotoxicity equivalency factors have been proposed, but not included in our study. The use of well-established neurotoxicity equivalency factors would result in a more precise estimation of the contribution of (individual) NDL-PCB to the combined exposure.	+/−	Simon et al. (2007), Rayne & Forest (2010), Pradeep et al., 2019).
	Kinetic model	Use of half-life of 10 years for all NDL-PCBs. NDL-PCB half-lives differ from 2.6 years for PCB 52 to 14.1 years for PCB 153	+/−	Ritter et al. (2011)
	Choice of UF	UF of 2 was applied for the extrapolation $BMDL_{05}$ to $BMDL_{01}$	+/−	
PBDEs	Point of departure	$BMDL_{10}$ for developmental neurotoxicity in animals rather than humans. It is not clear how the findings in the developmental neurotoxicity study in animals actually relate to IQ loss in children. Ideally data from epidemiological studies should be used. A recent meta-analysis of four prospective cohort studies pointed at a dose-dependent relationship between PBDE exposure and IQ loss. However, it was not possible to derive ESRVs for the different congeners. More human data on individual congeners are needed.	+/−	Lam et al. (2017), EFSA 2011a
		EFSA summarized the uncertainties in the animal studies affecting the ESRVs for developmental neurotoxicity, which included use of technological mixtures instead of pure congeners for toxicity studies, unknown levels of impurities, single dose administration during the pre- and postnatal period, no stratification of litter mates.	+	EFSA 2011a
	Congeners not considered	8 congeners, i.e. −28, −47, −99, −100, −153, −154, −183 and 209 were considered of primary importance by EFSA because of the composition of the technical PBDE mixtures and concentration in the environment and in food. Only PBDE -47, −99, −153, and −209 were included in the case study, because only ESRV were available for those congeners.	–	EFSA 2011a
	Kinetic model	Limited data on half-lives are available for PBDEs in human, and available data pointed at large variability. The largest half-lives of the individual congeners was used.	+	EFSA (2011a)
	Choice of UF	UF of 100 for PBDE-209, based on the study of Martin et al. According to EFSA, the animal $BMDL_{10}$ of 1.7 mg/kw bw expressed as an external dose can be compared with the estimated human dietary exposure, and EFSA related the exposure of PBDE-209 to the minimal margin of exposure of 2.5.	+	Martin et al. (2017) EFSA (2011a)

based on other grains, the personalised mRPI was overestimated. Exclusion of data for which no occurrence data were available (e.g. methylmercury in foods other than fish and seafood, NDL-PCBS in vegetable foods) resulted in an underestimation of the personalised mRPI.

The issue of limited concentration data used for certain substances can be addressed by including the entire EFSA data set which comprises 27 countries, rather than the 4–13 countries included in our study. However, this would not resolve the issue for NDL-PCBs and PBDEs congeners. For NDL-PCBs, concentration data for congeners other than the 6 indicator congeners were very limited. As those 6 indicator NDL-PCBs comprise approximately 50% of the total PCBs (EFSA 2005, 2012b; JECFA 2016), we calculated the exposure by multiplying the sum of the 6 congeners of NDL-PCB with a factor two. Particular if congeners would differ in potency, as described under uncertainties in ESRVs, the sum of 6 indicator congeners multiplied by two could have led to an under- or overestimation of the exposure. Once better information on potencies of the different congeners is available, the NDL-PCB congeners to be analysed in food can be reconsidered. Regarding PBDEs, concentration data (life stock meat, cow milk and eggs) for the four other congeners deemed relevant for dietary exposure by EFSA (2011a) were available in the concentration database.

5. Conclusions

Our case study shows that specific and targeted MRA using a component-based personalised mRPI approach can be performed for mixtures of dietary chemicals. The mixtures were selected in 1) having a high co-occurrence rate in human biomonitoring studies and 2) sharing a common adverse effect. By using this approach to estimate external dietary exposure, we performed MRA for the deleterious effect of combined exposure of lead, methyl mercury, iAs, fluoride, NDL-PCBs and PNDEs on cognitive development, determined by IQ loss.

All included populations exceeded the acceptable level of combined exposure. NDL-PCBs in fish, other seafood and dairy, lead in grains and fruits, methylmercury in fish and other seafoods, and fluoride in water contributed most to exposure and the subsequent risk. PBDEs hardly contributed to the combined exposure.

Uncertainties were identified for the likelihood of co-exposure, assessment group membership, values of ESRVs based on epidemiological (lead, methylmercury, iAS, fluoride and NDL-PCBs) and animal data (PBDE), and exposure data. Those uncertainties lead to a complex pattern of under- and overestimations, which would require probabilistic modelling based on expert knowledge elicitation for integration of the identified uncertainties into an overall uncertainty estimate. In addition, the listed uncertainties could be used to refine future MRA for cognitive decline.

CRediT author statement

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Table 6
Non-quantified uncertainties in exposure data.

Input data	Source	Description	Direction	Comment
Food consumption data	Food coding	Recoding FoodEx2 into FoodEx1	+/−	Not all food consumptions surveys were available in FoodEx1, the food coding used to match food consumption data to occurrence data. FoodEx2 is more refined, but currently the Dutch recipes database is not available in FoodEx2
		Food coding not always discriminate different food products (e.g. crackers which could be rice-based or wheat based).	+/−	FoodEx2 is more refined and could have prevented this issue
	Representativeness	Under sampling of specific consumption patterns	+/−	E.g. vegetarians, vegans
		Infrequently consumed foods	+/−	E.g. fish consumption in the Netherlands
	Number of reporting days	Extrapolation of few days of consumption to long-term exposure	+/−	Higher number of consumption days included (e.g. 7 days for Denmark) resulted in less uncertainty.
	Reporting foods	Underreporting of non-healthy foods and overreporting of health foods, frequency of consumption	+/−	
Occurrence data	Reported concentrations	Errors in reported concentrations or units	+/−	
	Measurement uncertainty	Analytical method not provided, measurement error	+/−	
	Limited data	Use of conversion factors:		Not applicable for other substances
		- iAs, mean of large range ratio iAs to total As (see Annex G)	+/−	
		- Methyl mercury (see Annex C)	+	
		- NDL-PCBs (Sum 6 indicator congeners times 2)	+/−	
		Aggregation of foods if less than 50 measurements were available:		
		- iAs	+	iAs in rice vs lower concentrations in other grains
		- Other substances	+/−	
		Exclusion of foods for which no data was obtained and for which above mentioned assumptions could not be used	-	
	Concentration data expressed per fat weight	Inaccurate description of the percentage fat weight in a sample. Assuming mean fat content of Dutch food composition database (NEVO)	+/−	Accounts only for NDL-PCBs and PBDEs
	Regional variability	Concentration in food and drinking water may vary between regions	+/−	Particularly for lead, iAs and fluoride in drinking water. Sensitivity analysis showed up to 14% lower pmRPIs when Dutch drinking water concentrations were used
	Measurements below LOQ	Assumed to be 0	–	Particularly when non-sensitive analytical methods (high LOQ) are used.
		Assumed to be value of LOQ	+	
	Processed foods	Processing (e.g. washing, cooking) may affect the concentration in food. Concentrations are often provided in raw agricultural commodities (e.g. wheat) or ingredients (wheat flour) but not in processed foods (e.g. wheat bread).	+/−	Processing factors were not used.
Matching food consumption data to occurrence data	Regional variability	Use of (mean of) Dutch food recipes data may not be representative to other countries	+/−	

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Appendix A. Supplementary data

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Air pollution and stroke; effect modification by sociodemographic and environmental factors. A cohort study from Denmark

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ABSTRACT

Objectives: Air pollution increases the risk of stroke, but the literature on identifying susceptible subgroups of populations is scarce and inconsistent. The aim of this study was to investigate if the association between air pollution and risk of stroke differed by sociodemographic factors, financial stress, comorbid conditions, and residential road traffic noise, population density and green space.

Methods: We assessed long-term exposure to air pollution with ultrafine particles, $PM_{2.5}$, elemental carbon and NO_2 for a cohort of 1,971,246 Danes aged 50–85 years. During follow-up from 2005 to 2017, we identified 83,211 incident stroke cases. We used Cox proportional hazards model (relative risk) and Aalen additive hazards models (absolute risk) to estimate associations and confidence intervals (CI) between 5-year running means of air pollution at the residence and risk of stroke in population strata.

Results: All four pollutants were associated with higher risk of stroke. The association between air pollution and stroke was strongest among individuals with comorbidities, with shorter education, lower income and being retired. The results also indicated stronger associations among individuals living in less populated areas, and with low noise levels and more green space around the residence. Estimates of absolute risk seemed better suited to detect such interactions than estimates of relative risk. For example for $PM_{2.5}$ the hazard ratio for stroke was 1.28 (95%CI: 1.22–1.34) and 1.26 (95%CI: 1.16–1.37) among those with mandatory and medium/long education respectively. The corresponding rate difference estimates per 100,000 person years were 568 (95%CI: 543–594) and 423(95%CI: 390–456)

Conclusion: The associations between air pollution and risk of stroke was stronger among individuals of lower socioeconomic status or with pre-existing comorbid conditions. Absolute risk estimates were better suited to identify such effect modification.

1. Introduction

Stroke is a leading global cause of death and morbidity, ranking third among causes of years of life lost (GBD 2017 Causes of Death Collaborators, 2018) and first among causes of disability-adjusted life years (GBD 2016 Stroke Collaborators, 2019). Air pollution affects hypertension, oxidative stress, systemic inflammation, imbalance of the nervous system and atherosclerosis, which are all pathophysiological

Abbreviations: $PM_{2.5}$, Particulate matter <2.5μm diameter; UFP, Ultrafine particles <0.1μm diameter; EC, elemental carbon; DEHM, Danish Eulerian Hemispheric Model; UBM, Urban background model; OSPM, Operational street pollution model; TWA, time weighted average.

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mechanisms associated with stroke (Lederer et al., 2021). Air pollutants with the strongest public health concern include particulate matter with diameter <2.5 μm ($PM_{2.5}$) and nitrogen dioxide (NO_2), and several other pollutants including elemental carbon (EC) and ultrafine particles (UFP, <0.1 μm diameter) indicate risk, but currently with insufficient data (WHO, 2021). A recent meta-analysis found that a 5null$\mu g/m^3$ increase in long-term exposure to air pollution with $PM_{2.5}$ is associated with a 6.3% increased risk of stroke (Alexeeff et al., 2021). Studies on long-term exposure to air pollution with NO_2 are less conclusive but generally indicate a positive association (Olaniyan et al., 2021). Only few studies have investigated the potentially more potent UFP, indicating an association with cardio- and cerebrovascular disease (Downward et al., 2018; Li et al., 2017; Poulsen et al., 2023a,b). Studies on elemental carbon (EC) (or black carbon or $PM_{2.5}$ absorbance) generally indicate no or a weak association with risk of stroke (Beelen et al., 2014; Downward et al., 2018; Ljungman et al., 2019; Poulsen et al., 2023a,b; Stafoggia et al., 2014; Stockfelt et al., 2017; Wolf et al., 2021).

The association between air pollution and risk of stroke may be modified by other factors. In a Danish study, NO_2 was associated with ischemic stroke only at residences with high noise levels (Sorensen et al., 2014). Some studies have linked access to green areas near the residence or living in rural areas with lower incidence and mortality from stroke or cardiovascular disease (Crouse et al., 2019; Hystad et al., 2020; Kim et al., 2019; Klompmaker et al., 2021; Seo et al., 2019; Stafoggia et al., 2014) possibly due to reduced psychological stress, increased physical activity, or by lower levels of air pollution (Nieuwenhuijsen et al., 2017).

Poor socioeconomic conditions are associated with risk of stroke, likely due to an unhealthy lifestyle, including smoking, alcohol consumption, stress, depression, poor diet, obesity, sedentary lifestyle and hypertension, which are all established risk factors for stroke (Boehme et al., 2017). Some studies have indicated stronger associations between air pollution and stroke among people with lower socioeconomic status (Rodins et al., 2020; Stafoggia et al., 2014; Yang et al., 2021), whereas this was not found in other studies (Hystad et al., 2020; Klompmaker et al., 2021).

Elderly people, children, women,and persons with pre-existing comorbidities including stress have been suggested to be more susceptible to the effects of air pollution (Clougherty, 2010; Schwartz et al., 2011). A recent meta-analysis of 11 cohort studies, found evidence of excess risk of ischemic heart disease in women compared to men in relation to long-term $PM_{2.5}$ exposure, but no difference in risk between sexes in relation to stroke (Zhang et al., 2022). Hypertension and diabetes are established risk factors for stroke (Boehme et al., 2017), but studies investigating effect modification by comorbidity have produced conflicting results (Amini et al., 2020; Hart et al., 2015; Hystad et al., 2020; Olaniyan et al., 2021; Shin et al., 2019; Stafoggia et al., 2014). A study found that self-reported stress could modify the short-term association between $PM_{2.5}$ and blood pressure (Hicken et al., 2014), indicating that stress conditions might also modify the association between air pollution and stroke.

At present, there is insufficient evidence to conclusively identify susceptible sub-populations for which the association between long-term exposure to air pollution and stroke is strongest, and where targeted interventions could be merited. When comparing associations between air pollution and risk of stroke in population groups, relative risk (i.e. how many times greater the hazard in one group compared to another, for example hazard ratios (HR)) and absolute risk (additional cases per 100,000 person-years, rate differences) might provide different results. The difference between these two measures arise when the basic disease rates between the groups of interest differ, for example when the incidence of disease is higher in persons of low versus high educational level, which is often the case. In such situations, the hazard ratio for a given exposure between these two groups may be similar, but the absolute rate difference will be higher in the group of low educational level compared to high – provided that the exposure is positively associated with the outcome. From a public health perspective, the knowledge on whether the absolute health effects of exposure to air pollution differ across subpopulations is important. We are only aware of one previous study of air pollution and risk of stroke, which has applied an additive model for estimation of absolute risk (Danesh Yazdi et al., 2021).

The aim of this nationwide study was to investigate if the association between long-term exposure to air pollution and risk of stroke differed by sociodemographic factors, financial stress, comorbid conditions, and road traffic noise, population density and green space at the residence.

2. Methods

The study was set in Denmark, where a unique personal identification number applied since 1968 allows all citizens to be followed in administrative and health registers (Schmidt et al., 2014). From the Danish Civil Registration System (Pedersen, 2011), we retrieved residential address histories until emigration for all persons living in Denmark (excluding Greenland and Faeroe Islands) in 1979 or born in Denmark any time hereafter. Eligible for the present study were 2,048,282 Danes born after 1920 (data on educational level was not available for those born earlier), living in Denmark on 1 January 2005, and who were at least 50 years old any time between this date and 31 December 2017.

2.1. Outcome

From the Danish national patient register (Lynge et al., 2011a,b) and the register of cause of death (Helweg-Larsen, 2011), we identified all stroke cases (ICD8: 431–434, 436, ICD10 I61-I64) recorded as primary cause of death or admission in the period 1977–2017. We excluded prevalent cases at baseline and only counted incident events.

2.2. Air pollution

The identified addresses were geocoded by means of the Building and Housing Registry (Christensen, 2011). Address-specific air pollution concentrations were quantified by adding air pollution contributions modelled at 3 scales using the Danish DEHM/UBM/AirGIS system (Brandt et al., 2003; Jensen et al., 2017; Khan et al., 2019). The system combined 1) the Danish Eulerian Hemispheric Model (DEHM), covering the northern hemisphere, for the long-range transported regional background concentrations, set up with four domains with two-way nesting capabilities for higher resolution over Denmark (Brandt et al., 2012), 2) the Urban Background Model (UBM) (Brandt et al., 2003; Frohn et al., 2022), covering local background at 1 × 1 km resolution for all of Denmark, calculated from Danish emissions of air pollution at the same resolution (Plejdrup et al., 2021) and 3) the Operational Street Pollution Model (OSPM®), modelling air pollution from traffic in the address street if the traffic density was above 500 vehicles per day (Kakosimos et al., 2010; Ketzel et al., 2012). The OSPM calculations took into account traffic density and composition, emission factors, street and building configuration, and meteorology (Jensen et al., 2017; Khan et al., 2019). We modelled $PM_{2.5}$, EC and NO_2 concentrations. Additionally, we modelled particle number concentration, which in the present paper is denoted UFP, as these quantities are highly correlated. This is a new addition to the modelling system, validated and detailed elsewhere (Frohn et al., 2021; Ketzel et al., 2021). The modelling system performs well when compared to measurements. For example for yearly concentrations of UFP at street level, the correlation coefficient was 0.95 (Ketzel et al., 2021). For all pollutants, monthly mean air pollutant levels were aggregated from modelled hourly concentrations. Combining these data with individual address histories, we calculated running 5-year time-weighted average (TWA) exposures for all cohort members.

2.3. Individual socioeconomic covariates

Potential confounders were selected *a priori*. Statistics Denmark provided annually updated data on Civil status (married/cohabiting, other), highest attained educational level (mandatory, secondary/vocational, medium/long), highest occupational level since 1990 (white collar, blue collar, retired), country of origin ("Danish origin": having Danish citizenship or having at least one parent who has; "Other country of origin": all other), personal income and household income. Both income variables were categorized in sex- and calendar-year specific quintiles.

2.4. Financial stress events and comorbidity

From the registers of Statistics Denmark, we identified potentially stressful financial events:

Loss of job, having household or personal income drop more than 50% between two consecutive years or having a household income of less than half the Danish median household income. For each participant, we created a time-dependent variable indicating if they experienced any of these events in the past five years.

We established a running Charlson comorbidity index from National Patient Registry data (Lynge et al., 2011a,b) on diseases in the five previous years. We incorporated a 1-year lag period to ensure that the index did not reflect the stroke outcome under study. The index was categorized as 0, 1 or ≥2.

2.5. Neighborhood characteristics

In the year 2017, Denmark comprised 2160 parishes with a mean area of 16.2 km^2 and a median population of 1032 persons. Using annually updated data from Statistics Denmark, we calculated population density and proportion of parish inhabitants with only mandatory education for each parish. Population density was categorized into <100, 100–2000, and >2000 persons per km^2 and the proportion with only mandatory education was dichotomized by the highest quintile (<11.6% vs. ≥11.6% or more).

2.6. Green space

We used BASEMAP02, a high resolution (10 × 10 m) land-use map of Denmark (Levin et al., 2017) to quantify green space estimated as proportion of greenness within 150 m of the residence including recreational areas, forests and open nature areas, private gardens and agricultural areas. Green space was categorized into tertiles: <55.1%, 55.1–63.5%, ≥63.6%.

2.7. Road traffic noise

Estimation of road traffic noise has been described in detail previously (Thacher et al., 2020). In brief, noise at the most exposed facade of the residence was modelled by the Nordic prediction method (Bendtsen, 1999) with input data on address-specific geocodes, building height, road type, light/heavy vehicle distributions, travel speed, and annual average daily traffic for all Danish road links (Jensen et al., 2019). The model included screening and reflection effects from buildings, terrain and noise barriers. A-weighted sound pressure estimates were aggregated as L_{den} for the years 2000, 2005, 2010, and 2015. Linear interpolation was used to quantify exposures in the intervening years. Noise was categorized into three levels: <55, 55–59, ≥60 dB.

2.8. Statistical methods

We calculated Spearman rank correlations between air pollutants and covariates. The association of air pollutants with risk of stroke was evaluated in two different models: A Cox proportional hazards model, to calculate relative risk estimates (HR) and an Aalen additive hazards model to calculate absolute risk estimates (rate differences per 100,000 person-years). In both models, age was the time-scale, and both models were adjusted for the same covariates: sex, calendar year (categorical, in two-year categories), educational level, occupational status, civil status, country of origin, personal and household income and proportion of parish inhabitants with only mandatory education (above or below highest quintile). All variables were modelled time-dependently, allowing subjects to change levels of exposure and covariates (except sex and country of origin).

Cohort members were followed up from 50 years of age or 1 January 2005, whichever came last, until stroke, age 86 years, more than 14 consecutive days of unknown address, emigration, death, or 31 December 2017, whichever came first.

To facilitate comparison with other studies risk of stroke was investigated per fixed increment ($PM_{2.5}$: 5null$\mu g/m^3$, NO_2: 10null$\mu g/m^3$, EC: 1null$\mu g/m^3$ and UFP: 10,000 particles/cm^3). We stratified analysis by sex, income, educational level, occupation, financial stress event, comorbidity, proportion of parish inhabitants with only mandatory education, population density, road traffic noise and green space within 150 m.

R (version 3.6.3) was used for Aalen analyses; all other analysis was performed in SAS 9.4 (SAS Institute Inc., NC, USA). By Danish law, entirely register based studies do not require ethical approval.

3. Results

From the 2,048,282 eligible Danes we excluded 54,416 persons diagnosed with stroke before baseline and 22,620 with missing covariate information. This left 1,964,702 persons representing 17,790,121 years of follow-up, during which 83,211 cases of incident stroke occurred. The cohort is described in Table 1. Residential exposure to UFP above the median was associated with higher age, being female, non-Danish origin, longer education, higher personal and household income, being retired, high population density, lower proportion of inhabitants with low education in the neighborhood, low level of green space within 150 m of the residence, and higher levels of road traffic noise. The interquartile ranges were 1.85null$\mu g/m^3$ for $PM_{2.5}$, 4248 particles/cm^3 for UFP, 0.28null$\mu g/m^3$ for EC and 7.15null$\mu g/m^3$ for NO_2. The Spearman rank correlations between pollutants ranged from 0.71 ($PM_{2.5}$ vs EC) to 0.86 (UFP vs NO_2) (Table 2).

Overall, we observed a HR for stroke of 1.28 (95% CI: 1.24–1.33) for 5null$\mu g/m^3$ higher $PM_{2.5}$, 1.12 (95% CI: 1.09–1.15) per 10,000 particles/cm^3, 1.06 (95% CI: 1.04–1.09) per 1null$\mu g/m^3$ of EC and 1.06 (95% CI: 1.04–1.07) per 10null$\mu g/m^3$ NO_2 in models adjusted for age, sex, calendar-year, education, occupational status, civil status, personal income, household income country of origin, and area-level deprivation (percentage of parish population with only mandatory education). Similarly, stroke rate (per 100,000 person-years) differences for the same exposure contrasts were 509 (95% CI: 490–529) for $PM_{2.5}$, 282 (95% CI: 266–298) for UFP, 229 (95% CI: 209–250) for EC, and 120 (95% CI: 112–128) for NO_2.

In general, HRs for stroke were similar, and with overlapping CIs, for different levels of the individual sociodemographic characteristics shown in Table 3, whereas rate differences were consistently higher in association with shorter education, being retired and with low income. Table 4 shows a consistent pattern of higher HRs and higher rate differences with higher level of comorbidity. With some exceptions, more green space within 150 m of the residence, low levels of traffic noise, low population density, and low level of education in the neighborhood were associated with higher HRs and higher rate differences.

4. Discussion

This large nationwide cohort study showed that: 1) air pollution with UFP, $PM_{2.5}$, BC and NO_2 was associated with higher risk of stroke, 2)

Table 1
Sociodemographic and exposure characteristics of the study population at baseline; total and by 5-year exposure to ultrafine particles (below and above the median).

Baseline Characteristics	Total (N = 1,964,702)	UFP <11,064 particles/cm^3 (N = 980,586)	UFP ≥11,064 particles/cm^3 (N = 984,116)
Individual-level			
Female (%)	53	51	54
Age, years (median, 5–95 pctl)	58 (50–79)	55 (50–78)	60 (50–80)
Civil status (%)			
Married or cohabiting	73	77	69
Widow(er) or divorced or single	27	23	31
Individual income (%)			
Low (quintile 1)	25	25	25
Medium (quintile 2, 3 and 4)	55	57	53
High (quintile 5)	20	18	23
Household income (%)			
Low	21	20	21
Medium	55	58	52
High	25	22	27
Highest attained education (%)			
Mandatory	36	38	34
Secondary/ vocational	45	46	45
Medium/long	19	16	21
Country of origin (%)			
Danish	98	99	97
Other	2	1	3
Occupational status (%)			
White collar	46	47	46
Blue collar	37	39	35
Retired or unemployed	16	14	19
Financial stress event[a], past 5 years (%)			
Yes	82	81	84
No	18	19	16
Charlson comorbidity index[b] (%)			
0	85	87	83
1	8	7	9
≥2	7	6	8
Address-level			
Road traffic noise, 5-year (%)			
<55 dB	50	57	43
55–60 dB	22	20	23
>60 dB	28	22	34
Green space[c] within 150 m (%)			
<55.1%	38	26	50
55.1–63.5%	27	25	29
≥63.6%	35	48	21
Air pollution, 5-year (median, 5–95 pctl)			
$PM_{2.5}$ (μg/m^3)	11.2 (8.7–12.6)	10.2 (8.2–11.7)	11.7 (10.0–13.2)
UFP (particles/cm^3)	11,106 (7,212–17,239)	9,301 (6,622–10,002)	13,721 (11,304–18,831)
EC (μg/m^3)	0.7 (0.4–1.1)	0.5 (0.4–0.7)	0.8 (0.6–1.4)
NO_2 (μg/m^3)	15.3 (9.3–27.3)	12.5 (8.3–16.4)	19.5 (14.3–31.7)
Area-level			
Parish inhabitants with only mandatory education (%)			
<11.6%	62	52	72
≥11.6%	38	48	28
Population density (%)			
<100 per km^2	26	47	6
100–2000 per km^2	55	49	61
>2000 per km^2	19	4	33

[a] Financial stress events were defined as ≥1 of the following events during the last 5 years: family income below Danish relative poverty limit, personal income drop of ≥50% between two consecutive years, family income drop of ≥50% between two consecutive years, and/or loss of job.

[b] Comorbidity defined as a Charlson comorbidity index of 0, 1 or ≥2 during the last 5 years, with a lag-period of 1 year.

[c] Green space defined as the proportion of gardens, fields, recreational areas, forest, and wet/dry open nature areas within a 150 m of the residence.

Table 2
Spearman correlations between 5-year exposure to $PM_{2.5}$, ultrafine particles, elemental carbon and NO_2 for the study population, 2005–2017.

	$PM_{2.5}$	UFP	EC	NO_2
$PM_{2.5}$	1	0.80	0.71	0.75
UFP	0.80	1	0.84	0.86
EC	0.71	0.84	1	0.82
NO_2	0.75	0.86	0.82	1

both relative and absolute risk of stroke in association with air pollution were higher among individuals with comorbidity, 3) the association between air pollution and absolute risk for stroke was strongest among individuals with shorter education, lower income and being retired; no such patterns was detected for relative risk, and 4) mostly, both relative and absolute risk for stroke in association with air pollution were higher among individuals with more green space, less noise, low population density and low level of education in the neighborhood.

4.1. Individual factors

We found a consistent pattern of stronger associations between air pollution and the risk of stroke among people with higher levels of comorbidity. This was also seen in a large Canadian study where $PM_{2.5}$ was associated with higher relative risk of stroke, particularly among participants with Charlson comorbidity scores ≥5 (Olaniyan et al., 2021). In the US Nurses' Health Study, the association between $PM_{2.5}$ and stroke was strongest among women with diabetes (Hart et al., 2015). However, in one study, the association between$PM_{2.5}$ and stroke was strongest among subjects without pre-existing cardiovascular disease (Hystad et al., 2020) and other studies have not found effect modification by diabetes or cardiovascular diseases (Amini et al., 2020; Shin et al., 2019; Stafoggia et al., 2014). Our consistent pattern of effect modification by comorbidity might relate to the size of our study and that we identified cases both from hospital and mortality registers and that we accounted for multiple medical conditions. Comorbidity might be associated with biological changes potentially leading to higher susceptibility to air pollution effects. In addition, if comorbidity increases the likelihood of having a stroke detected by the health care system, such surveillance effect might have contributed to our result.

We observed stronger associations between air pollution and absolute risk of stroke among individuals with short education, low income and being retired, In line with our study, the absolute risk of stroke from $PM_{2.5}$ and NO_2 was higher among subjects of lower socioeconomic status (SES) in a US study of 63 million Medicare members (Danesh Yazdi et al., 2021). We are not aware of other studies estimating the absolute risk of stroke in relation to long-term air pollution. In terms of relative risk we found no effect modification by SES whereas the Medicare study found that the relative risk of cerebrovascular disease was lower among subjects of low SES (Klompmaker et al., 2021). Two Canadian cohorts have reported stronger associations between $PM_{2.5}$ and NO_2, and relative risk of stroke among less affluent individuals (Olaniyan et al., 2021; Shin et al., 2019), but a multinational cohort did not find income to modify the association with $PM_{2.5}$ (Hystad et al., 2020). With one exception (Andersen et al., 2012) previous studies have, in line with our results, reported no difference in the association between air pollution and relative risk of stroke by educational level (Beelen et al., 2014; Hystad et al., 2020; Ljungman et al., 2019; Stafoggia et al., 2014; Yang et al., 2021).

Table 3
Association between air pollution and risk for stroke by sociodemographic factor.

	cases	$PM_{2.5}$ (5null$\mu g/m^3$)		UFP (10000#/cm^3)		EC (1null$\mu g/m^3$)		NO_2 (10null$\mu g/m^3$)	
		Cox model HR (95% CI)[a]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a and b]	Cox model HR (95% CI)[a]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a and b]	Cox model HR (95% CI)[a]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a and b]	Cox model HR (95% CI)[a]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a and b]
Sex									
Women	38,061	1.29 (1.23–1.35)	490 (467–512)	1.11 (1.07–1.15)	277 (259–296)	1.05 (1.01–1.09)	230 (206–255)	1.05 (1.03–1.07)	116 (107–125)
Men	45,150	1.28 (1.22–1.34)	538 (512–564)	1.13 (1.09–1.17)	287 (267–307)	1.07 (1.04–1.11)	228 (200–256)	1.06 (1.05–1.08)	124 (114–135)
Highest education level									
Short	39,738	1.28 (1.22–1.34)	568 (543–594)	1.13 (1.09–1.17)	366 (341–390)	1.07 (1.03–1.11)	320 (285–354)	1.06 (1.04–1.08)	170 (157–183)
Medium	33,156	1.29 (1.23–1.36)	456 (431–480)	1.12 (1.08–1.16)	233 (214–252)	1.05 (1.01–1.09)	172 (148–196)	1.06 (1.04–1.08)	98 (88–108)
Long	10,317	1.26 (1.16–1.37)	423 (390–456)	1.10 (1.03–1.17)	199 (175–222)	1.07 (1.00–1.15)	164 (130–198)	1.03 (0.99–1.06)	69 (57–81)
Occupation									
White collar	29,446	1.26 (1.20–1.33)	352 (327–377)	1.09 (1.05–1.14)	151 (134–167)	1.05 (1.01–1.10)	113 (92–135)	1.05 (1.03–1.07)	62 (53–71)
Blue collar	29,900	1.31 (1.25–1.38)	355 (328–382)	1.16 (1.12–1.21)	173 (154–192)	1.09 (1.04–1.13)	126 (104–149)	1.08 (1.06–1.10)	77 (67–88)
Retired/ unemployed	23,865	1.27 (1.21–1.34)	681 (651–711)	1.11 (1.07–1.15)	633 (596–671)	1.05 (1.00–1.09)	616 (552–680)	1.04 (1.02–1.06)	304 (283–325)
Personal income, quintiles									
1st (low)	34,401	1.28 (1.22–1.34)	547 (519–574)	1.11 (1.07–1.15)	322 (297–347)	1.06 (1.02–1.10)	257 (224–291)	1.06 (1.04–1.08)	145 (131–159)
2nd–4th	41,938	1.29 (1.23–1.36)	505 (481–529)	1.14 (1.10–1.18)	287 (267–306)	1.07 (1.04–1.11)	243 (218–268)	1.06 (1.04–1.08)	122 (113–132)
5th (high)	6,872	1.21 (1.10–1.34)	367 (334–401)	1.08 (1.00–1.16)	159 (136–182)	1.00 (0.91–1.10)	103 (75–131)	1.02 (0.98–1.07)	53 (41–65)
Household income, quintiles									
1st (low)	31,611	1.27 (1.21–1.33)	606 (578–635)	1.10 (1.06–1.14)	415 (386–444)	1.04 (1.00–1.08)	358 (318–397)	1.04 (1.02–1.06)	179 (163–194)
2nd–4th	42,707	1.30 (1.24–1.37)	461 (438–485)	1.15 (1.11–1.18)	252 (234–270)	1.08 (1.05–1.12)	203 (180–226)	1.07 (1.05–1.09)	110 (101–119)
5th (high)	8,893	1.25 (1.15–1.37)	338 (309–366)	1.11 (1.04–1.19)	145 (125–164)	1.03 (0.95–1.12)	94 (70–117)	1.05 (1.01–1.09)	52 (41–62)

HR = hazard ratio.

[a] Analyses were adjusted for age, sex, calendar-year, education, occupational status, civil status, personal income, household income country of origin, and area-level deprivation (percentage of parish population with only mandatory education).

[b] Risk estimates calculated in Aalen additive hazard model are given as a rate difference per 100,000 person-years (pyrs).

Table 4
Association between air pollution and risk for stroke by financial stress, comorbidity, population density, road traffic noise and green space.

	Cases	$PM_{2.5}$ (5null$\mu g/m^3$)		UFP (10000#/cm^3)		EC (1null$\mu g/m^3$)		NO_2 (10null$\mu g/m^3$)	
		Cox model HR (95% CI)[aa]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a,ba and b]	Cox model HR (95% CI)[aa]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a,ba and b]	Cox model HR (95% CI)[aa]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a,ba and b]	Cox model HR (95% CI)[aa]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a,ba and b]
Any financial stress event in past 5 years[cc]									
No	71,834	1.29 (1.24–1.34)	520 (499–540)	1.12 (1.09–1.16)	294 (277–311)	1.06 (1.04–1.09)	244 (222–266)	1.05 (1.04–1.07)	125 (117–134)
Yes	11,377	1.27 (1.17–1.36)	434 (398–470)	1.12 (1.06–1.19)	214 (187–241)	1.07 (1.01–1.14)	155 (123–188)	1.08 (1.04–1.11)	91 (77–106)
Charlson comorbidity index[d]									
0	46,470	1.11 (1.06–1.16)	342 (324–361)	1.02 (0.98–1.05)	165 (151–180)	0.97 (0.93–1.01)	115 (99–132)	1.00 (0.98–1.02)	62 (55–69)
1	16,187	1.26 (1.18–1.35)	798 (745–852)	1.08 (1.03–1.13)	505 (455–555)	1.04 (0.99–1.10)	468 (397–540)	1.02 (1.00–1.05)	214 (187–242)
≥2	20,554	1.31 (1.24–1.39)	841 (793–890)	1.14 (1.10–1.20)	594 (546–643)	1.09 (1.04–1.13)	545 (471–620)	1.07 (1.05–1.1)	279 (252–306)
Proportion of parish inhabitants with only mandatory education									
<11.6%	64,979	1.25 (1.20–1.30)	489 (469–509)	1.12 (1.09–1.15)	256 (240–272)	1.06 (1.03–1.08)	198 (178–217)	1.05 (1.04–1.07)	103 (95–111)
≥11.6%	18,232	1.40 (1.32–1.49)	525 (490–560)	1.13 (1.08–1.19)	253 (224–282)	1.12 (1.05–1.20)	290 (249–332)	1.08 (1.05–1.11)	137 (119–154)
Road traffic noise									
<55 dB	40,275	1.36 (1.29–1.43)	541 (516–566)	1.15 (1.11–1.20)	312 (290–334)	1.12 (1.07–1.17)	384 (347–421)	1.13 (1.09–1.16)	219 (203–234)
55–60 dB	18,270	1.29 (1.21–1.38)	559 (525–594)	1.13 (1.08–1.19)	317 (288–345)	1.04 (0.99–1.10)	240 (196–284)	1.07 (1.04–1.11)	172 (154–189)
>60 dB	24,666	1.16 (1.11–1.22)	460 (433–488)	1.05 (1.01–1.09)	239 (217–261)	1.00 (0.96–1.04)	163 (138–188)	1.01 (0.99–1.03)	85 (75–94)
Green space within 150 m[e]									
<55.1%	34,936	1.20 (1.15–1.26)	503 (477–529)	1.06 (1.02–1.09)	269 (247–290)	1.01 (0.97–1.04)	179 (154–203)	1.02 (1.00–1.03)	94 (84–103)
55.1–63.5%	22,155	1.27 (1.20–1.35)	543 (513–572)	1.10 (1.05–1.15)	307 (283–331)	1.07 (1.01–1.14)	307 (269–346)	1.08 (1.05–1.11)	165 (150–180)
≥63.6%	26,120	1.36 (1.28–1.44)	503 (474–532)	1.22 (1.16–1.28)	327 (301–353)	1.13 (1.08–1.19)	315 (270–359)	1.15 (1.11–1.19)	187 (170–204)
Population density									
<100 per km^2	20,446	1.38 (1.29–1.47)	540 (508–572)	1.21 (1.15–1.29)	455 (413–496)	1.05 (1.01–1.10)	189 (143–235)	1.22 (1.16–1.28)	293 (267–319)
100–2000 per km^2	47,360	1.34 (1.28–1.41)	564 (539–590)	1.14 (1.10–1.19)	353 (330–375)	1.10 (1.06–1.14)	331 (299–363)	1.11 (1.08–1.13)	191 (178–204)
>2000 per km^2	15,405	1.13 (1.05–1.20)	485 (451–518)	1.05 (0.99–1.10)	324 (294–354)	0.96 (0.90–1.02)	239 (205–272)	1.00 (0.98–1.02)	99 (86–112)

HR = hazard ratio.
[a] Analyses were adjusted for age, sex, calendar-year, education, occupational status, civil status, personal income, household income, country of origin, and area-level deprivation (percentage of parish population with only mandatory education).
[b] Risk estimates calculated in Aalen additive hazard model are given as a rate difference per 100,000 person-years (pyrs).
[c] Financial stress events were defined as ≥1 of the following events during the last 5 years: family income below Danish relative poverty limit, personal income drop of ≥50% between two consecutive years, family income drop of ≥50% between two consecutive years, and/or loss of job.
[d] Comorbidity defined as a Charlson comorbidity index of 0, 1 or ≥2 during the last 5 years, with a lag-period of 1 year.
[e] Green space defined as the proportion of gardens, fields, recreational areas, forest, and wet/dry open nature areas within a 150 m of the residence.

We found slightly higher relative risk estimates among blue collar workers compared to white collar workers. A German cohort study reported associations between $PM_{2.5}$ and NO_2 and relative risk of stroke to be stronger among those being unemployed or retired (Rodins et al., 2020), which we did not observe. However, we found much higher absolute risk estimates among those being retired; we are not aware of other studies estimating absolute effect of air pollution on risk of stroke by occupational status.

We found a stronger absolute risk among those without financial stress events in past 5 years, which might seem counterintuitive. A possible explanation could be diagnostic bias, if people under stress are less likely to prioritize seeking medical attention for symptoms of milder strokes.

We observed no convincing effect modification by sex, which is in accordance with two recent meta-analyses on $PM_{2.5}$ (Yuan et al., 2019; Zhang et al., 2022). We could not corroborate the results of Danesh Yazdi et al.(Danesh Yazdi et al., 2021) showing stronger associations between $PM_{2.5}$ and stroke in females.

In summary, both the present and previous studies have provided some evidence of stronger associations between air pollution and risk of stroke among the less privileged. Our study showed that an additive model, providing absolute risk estimates, was able to detect interactions, which remained undetected when using the (traditional) multiplicative Cox models and relative risk estimates. We are only aware of one previous study that has applied additive models, and would recommend future studies to do so when investigating risk associations by population groups with different underlying disease/mortality rates. This applies for example to different socioeconomic groups since many aspects of low SES are established or putative risk factors for cardiovascular disease (Powell-Wiley et al., 2022).

4.2. Residential and area factors

Our results showed higher relative and absolute risks of stroke in association with air pollution among individuals living in areas with low educational level. In a US cohort, the association of $PM_{2.5}$ with mortality

from stroke and ischaemic heart disease combined did not differ by area level, income or education; stroke, however, constituted only 20% of cases (Hayes et al., 2020). One explanation for our result could be that the area level indicator acts as proxy for some unaccounted for individual factor, although our model included a range of individual socioeconomic indicators.

The associations of air pollution with stroke, both absolute and relative, were generally stronger in less populated areas and areas with less traffic noise, both indicating rural conditions. In a multinational study, including countries of all income levels, the association between $PM_{2.5}$ and stroke was strongest in rural populations (Hystad et al., 2020), whereas the association between $PM_{2.5}$ and stroke hospitalization was not modified by level of urbanicity in an Italian cohort (Gandini et al., 2018). The Danish nurse cohort study found no clear effect modification by noise level or urbanicity (Amini et al., 2020).Important sources of non-traffic air pollution in Denmark include shipping and non-industrial combustion plants, and we have previously demonstrated that in Denmark, air pollution from non-traffic sources is a stronger risk factor for stroke than air pollution from traffic with $PM_{2.5}$ estimates of 1.004 (95% CI: 0.998–1.011) for traffic sources and 1.091 (95% CI: 1.074–1.108) for non-traffic per IQR (Poulsen et al., 2023a,b). A possible explanation for the weaker association between air pollution and risk of stroke in more urban populations in the present study could thus be that a higher proportion of urban exposure hails from traffic.

Our study showed stronger associations between air pollution and risk of stroke among individuals with much green space around the residence. Previous studies on stroke have found the same (Klompmaker et al., 2021), the opposite (Crouse et al., 2019) or no effect modification (Avellaneda-Gomez et al., 2022). It has been suggested that green space might positively influence cardiovascular health by promoting physical activity and decreasing stress (Nieuwenhuijsen et al., 2017), but it could also be an indicator of differential susceptibility as a consequence of differences in lifestyle or different composition of air pollution (for example a smaller proportion of traffic-related air pollution). Finally, it could also reflect the urban-rural differences discussed above. Further studies will be needed to elucidate the potential effect modifying properties of green space (Poulsen et al., 2023).

4.3. Strengths and limitations

Our study was nationwide and register-based, minimizing the potential for selection bias. Outcome data as well as information on individual and area level SES-covariates were obtained from updated public registers of high quality (Helweg-Larsen, 2011; Lynge et al., 2011a, 2011b; Schmidt et al., 2014, 2015). Furthermore, we had data on financial stress, comorbidity and green space at the residence. Also, we supplemented the standard use of Cox models with additive Aalen models, which accounted for differences in base rates between subpopulations. Such differences are not accounted for by Cox models producing relative risk estimates. Another strength of our study was the detailed address-level assessment of air pollution and noise with high spatiotemporal resolution (Khan et al., 2019).

Some exposure misclassification is, however, inevitable when modelling air pollution, and we did not have information about non-residential exposures. Such misclassification is likely to be independent of case status and may, thus, have attenuated risk estimates. If our 5-year exposure time-window does not optimally capture relevant exposure, this could also attenuate risk estimates. We have, however, previously demonstrated that in this cohort, shorter (1-year) and longer (10-year) exposure windows produce similar risk estimates for the association between air pollution and stroke (Poulsen et al., 2023a,b).

It was a limitation that we did not have data on individual lifestyle factors, such as overweight, diet or smoking, which are considered risk factors of stroke and also potentially linked to choice of residence and thus the individual level of air pollution exposure. However, we have previously shown that if analyses of associations between air pollution and stroke is adjusted for sociodemographic factors from registers, similar to the present study, there was little additional impact of adjusting for individual lifestyle factors (smoking, diet, physical activity and body mass index) (Sorensen et al., 2022).

Even though the validity of our stroke definition has been shown to be high (83.5%) (Luhdorf et al., 2017), our data did not allow a reliable subdivision of stroke by subtype; previous studies suggested that the association with air pollution may differ by stroke subtype (Andersen et al., 2012; Shin et al., 2019).

Our cohort is a predominantly white, middle-aged or older, Western population. Air pollution composition and correlations between environmental and individual risk factors may differ in other settings. These factors should be taken into consideration before generalizing our results to other populations. Another thing to consider in future studies is that all though all four air pollutants are highly correlated and have large contributions from combustion processes and from non-traffic sources (Poulsen et al., 2023a,b) they may represent different aspects of air pollution and we have previously found indications that $PM_{2.5}$ from non-traffic sources, may be closest associated with risk of stroke (Poulsen et al., 2023a,b). It may therefore be worthwhile to apply multipollutant models including multiple sources or types of air pollutants to better identify the true risk factors for stroke. In such analysis one could also consider effect modification by environmental factors such as urbanicity and green space.

5. Conclusion

The associations between air pollution and risk of stroke were stronger among individuals of lower socioeconomic status or with pre-existing comorbid conditions. Absolute risk estimates were better suited to identify such effect modification. Our results substantiate the need for preventive strategies targeted towards reducing air pollution exposure among these specific susceptible groups, with the potential to reduce more cases of stroke.

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Ethics

By Danish law informed consent and ethical approval are not required for entirely register based studies.

Declaration of competing interest

The authors declare they have nothing to disclose.

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Air pollution exposure and social responsiveness in childhood: The cincinnati combined childhood cohorts

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ABSTRACT

Autism Spectrum Disorder (ASD) affects about 1 in 44 children and environmental exposures may contribute to disease onset. Air pollution has been associated with adverse neurobehavioral outcomes, yet little research has examined its association with autistic-like behaviors. Therefore, our objective was to examine the association between exposure to air pollution, including NO_2 and $PM_{2.5}$, during pregnancy and the first year of life to ASD-like behaviors during childhood. Participants (n = 435) enrolled in the Cincinnati Childhood Allergy and Air Pollution Study and the Health Outcomes and Measures of the Environment Study were included in the analysis. Daily exposures to NO_2 and $PM_{2.5}$ at the residential addresses of participants were estimated using validated spatiotemporal models and averaged to obtain prenatal and first year exposure estimates. ASD-like behaviors were assessed via the Social Responsiveness Scale (SRS) questionnaire at age 12. Linear regression models adjusting for confounders were applied to estimate the association between pollutants and SRS scores. After adjusting for covariates, the association between NO_2 and $PM_{2.5}$ and SRS scores remained positive but were no longer statistically significant. Prenatal and first year exposure to NO_2 were associated with total SRS T-scores with an estimated 0.4 point increase (95% CI: −0.7, 1.6) per 5.2 ppb increase in NO_2 exposure and 0.7 point (95% CI: −0.3, 1.6) per 4.2 ppb increase in NO_2 exposure, respectively. For $PM_{2.5}$, a 2.6 $\mu g/m^3$ increase in prenatal exposure was associated with a 0.1 point increase (95% CI: −1.1, 1.4) in SRS Total T-scores and a 1.3 $\mu g/m^3$ increase first year of life was associated with a 1 point increase (95% CI: −0.2, 2.3). In summary, exposure to NO_2 and $PM_{2.5}$ during pregnancy and the first year of life were not significantly associated with higher autistic-like behaviors measured with SRS scores after adjustment of covariates. Additional research is warranted given prior studies suggesting air pollution contributes to ASD.

1. Introduction

Autism spectrum disorder (ASD) is a behavioral condition characterized by deficits in communication and social interaction, and an increase in restricted and repetitive patterns in behaviors, interests, and activities (Diagnostic and Statistical Manual of Mental Disorders, 2013). ASD is associated with lifelong consequences including functional deficits, difficulty maintaining relationships, and challenges with living and working independently (Lord et al., 2018). ASD is among the most common neurodevelopmental disorders of childhood, and its prevalence is increasing worldwide (Zeidan et al., 2022) and within the United States (Christensen et al., 2019). The prevalence of ASD is 4 times higher

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among boys than girls, and about 1 in 44 (2.3%) children were identified with ASD (Werling and Geschwind, 2013) in 2018. According to estimates from the Autism and Developmental Disabilities Monitoring Network, 2.3% represents a 23 percent increase from 2016 (1 in 54), and more than double that of 2000 (1 in 150) (National Center on Birth Defects, 2022). The rise in ASD cases is likely partially attributed to increased monitoring and a broadening of diagnostic criteria (Rice et al., 2012); however, these factors do not fully explain the rising prevalence of ASD (Hertz-Picciotto and Delwiche, 2009).

While genetics and family history play a role in ASD, a growing body of evidence suggests that *in utero* exposure to air pollution, a complex mixture of particles, gases, trace metals and adsorbed organic contaminants, may also be neurotoxic and could contribute to the development of ASD (Ortega et al., 2014; Peters et al., 2006; Muhlfeld et al., 2008; Bekkar et al., 2020; Shang et al., 2021; Costa et al., 2017, 2020). Exposure to air pollution has been linked to adverse physical and developmental effects on the fetus including low birth weight, preterm birth, and high infant mortality (Currie et al., 2009; Stillerman et al., 2008). In children, air pollutants including NO_2 and fine particulate matter ($PM_{2.5}$) have been associated with numerous neurobehavioral outcomes: impaired cognitive abilities (Harris et al., 2015; Suglia et al., 2008), deficits in attention-related behaviors (Chiu et al., 2013; Newman et al., 2013; Siddique et al., 2011), symptoms of anxiety/depression (Ali and Khoja, 2019; Bakolis et al., 2021; Yolton et al., 2019), as well as ASD (Raz et al., 2018; Volk et al., 2013; Roberts et al., 2013; Windham et al., 2013). Toxicological studies have explored plausible biological pathways linked to autism, noting that airborne pollutant particles cause systemic inflammation, alter the neonatal immune system, contribute to neuronal injury, induce oxidative stress, and affect the development of the central nervous system (Peters et al., 2006; Muhlfeld et al., 2008; Li et al., 2003; Pangrazzi et al., 2020). The brain is already vulnerable to oxidative stress due to its high metabolic activity and low levels of antioxidants, and children with ASD may be at greater risk for oxidative stress (Pangrazzi et al., 2020; MohanKumar et al., 2008). Investigating postnatal air pollution exposure is also indicated because brain development continues, doubling in size through a child's first year of life, and environmental toxicant insults can perturb neurodevelopment (Rice and Barone, 2000). Exposure to fine particulate matter in early infancy has been found to influence patterns of structural brain development in childhood, specifically hemispheric differences in gray matter across cortical regions (Cserbik et al., 2020).

Previous studies have found children born to mothers who live close to freeways have twice the risk of ASD (Volk et al., 2011). Furthermore, epidemiological studies have reported associations of ASD diagnosis and prenatal exposure to various air pollutants, including nitrogen dioxide (NO_2) (Volk et al., 2013; Ritz et al., 2018) and fine particulate matter ($PM_{2.5}$) (Volk et al., 2013; Raz et al., 2015; Becerra et al., 2013). The role of postnatal air pollution exposure is uncertain as studies in California (Volk et al., 2013), Pennsylvania (Talbott et al., 2015), Israel (Raz et al., 2018), and Denmark (Ritz et al., 2018) have found that exposure during early childhood is associated with increased odds of ASD, while other studies found the association to be very weak or not significant (Raz et al., 2018; Gong et al., 2014, 2017; Guxens et al., 2016; Pagalan et al., 2019; Kaufman et al., 2019). The objective of this study was to examine the association between NO_2 and $PM_{2.5}$ during early brain development and ASD-related traits and behaviors in two well-characterized longitudinal cohorts with detailed residential histories.

2. Methods

2.1. Study population

We analyzed pooled and harmonized data collected from participants enrolled in two cohorts from the Cincinnati, Ohio region: the Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS) and the Health Outcomes and Measures of the Environment (HOME) Study. The combined study population, referred to as the Cincinnati Combined Childhood Cohort (C4), leverages the two cohorts' compatibility regarding geographic proximity, demographics, available exposure data, neurobehavioral outcomes, and harmonized data for analysis.

A complete description of both cohorts' eligibility, enrollment, and methods is available elsewhere (Yolton et al., 2019; LeMasters et al., 2006; Ryan et al., 2005; Brunst et al., 2015; Braun et al., 2017, 2020). Briefly, CCAAPS is a longitudinal cohort study of children examining the associations between traffic-related air pollutants and respiratory and neurobehavior health (LeMasters et al., 2006; Ryan et al., 2005). Study eligibility required a birth address either <400 or >1500 meters (m) from a major roadway and at least one biological parent with atopy confirmed by skin prick testing (LeMasters et al., 2006). Children were enrolled at approximately age 6 months from 2001 to 2003 and completed study visits at ages 1, 2, 3, 4, 7, and 12. At the 12-year study visit, a comprehensive neurobehavioral assessment battery was completed (Yolton et al., 2019). The HOME Study is a longitudinal pregnancy and birth cohort study that enrolled pregnant women to examine the association between common low-level environmental toxicants and child health and neurobehavioral outcomes (Braun et al., 2017). Study eligibility required living in the study region, <19 weeks pregnant, >18 years old, residing in a home built in or before 1978, not living in a mobile or trailer home, HIV-negative, not taking medications for seizures or thyroid disorders, planning to continue prenatal care and deliver at the collaborating clinics and hospitals, planning to live in the greater Cincinnati area for the next year, fluent in English, and no diagnosis of diabetes, bipolar disorder, schizophrenia or cancer that resulted in radiation treatment or chemotherapy (Braun et al., 2017, 2020). Pregnant women were enrolled from 2003 to 2006 and their children completed study visits at birth, 4 weeks, annually 1–5 years, 8 years, and 12 years. The study visit at age 12 included a comprehensive assessment of mental health and brain development. Both studies were approved by the Institutional Review Boards of the University of Cincinnati and/or Cincinnati Children's Hospital Medical Center. Participants and parents provided informed assent and consent, respectively prior to completing study procedures.

2.2. Air pollutants exposure assessment

We used validated models (Di et al., 2019, 2020) to estimate daily exposure to $PM_{2.5}$ and NO_2 at the homes of C4 participants prenatally through age one year. At each study visit, beginning at enrollment and through the most recently completed 12-year visit, caregivers reported all residential addresses and corresponding dates when the child lived at each location. Daily estimates of air pollutants were aggregated to derive average exposures for each pollutant during two time periods: 1) prenatal exposures during pregnancy (date of conception to date of birth) and 2) exposure during the participant's first year of life (date of birth to first birthday). When more than one address fell into a time interval, we created a weighted average to account for changes in residence.

We estimated NO_2 exposure based on validated spatiotemporal models covering the entire contiguous U.S. with daily predictions on 1-km-level grid cells from 2000 to 2016 (Di et al., 2020). This ensemble model integrated multiple machine learning algorithms, including neural network, random forest, and gradient boosting, with multiple predictor variables, including chemical transport models, to provide estimates with high spatiotemporal resolution (Di et al., 2020). The relationship between daily monitored and predicted NO_2 is almost linear; the ensemble produced a cross-validated R^2 of 0.788 overall, a spatial R^2 of 0.844, and a temporal R^2 of 0.729. Daily estimates of NO_2 concentrations were derived at each participant's home based on the residential history provided. Daily estimates were aggregated and weighted (if residence changed) to calculate the daily average prenatal and first year exposure to NO_2.

We estimated $PM_{2.5}$ exposures using a validated ensemble model that

integrated multiple machine learning algorithms, including neural network, random forest, and gradient boosting (Di et al., 2019). The three machine learning algorithms used more than 100 predictor variables, ranging from satellite data, land-use data, meteorological data, and CTM predictions, with cross-validation controlling for overfitting. A generalized additive model combined $PM_{2.5}$ estimates from the machine learning algorithm to account for geographic differences. The trained model predicts daily $PM_{2.5}$ for the entire contiguous United States from 2000 to 2016 at every 1 km × 1 km grid cell, with excellent performance, a spatial R^2 of 0.89 and temporal R^2 of 0.85. Similar to NO_2 exposure, daily estimates of $PM_{2.5}$ concentrations at the home of each participant were averaged to obtain the average daily exposure prenatally and in the first year of life.

2.3. ASD-related traits and behavior assessment

At the age 12 study visit for both the CCAAPS and HOME cohorts, the Social Responsiveness Scale-2 (SRS-2) was completed by the child's caregiver. The SRS-2 is a 65-item questionnaire that assesses behavioral and social-communicative traits used to identify the presence and severity of social impairment for both the general (non-clinical) population and in clinical settings (Bolte et al., 2008; Constantino and Gruber, 2012; Constantino and Todd, 2005; Frazier et al., 2014). This measure has been used widely in studies with children because of its ease of administration, strong psychometric properties, high internal validity, reliability, and reproducibility (Constantino et al., 2003). It is routinely administered as part of the comprehensive diagnostic assessment for ASD and has been validated against widely implemented clinical diagnostic tools, the Autism Diagnostic Interview-Revised (Duku et al., 2013; Chan et al., 2017) and the Autism Diagnostic Observation Schedule (Bolte et al., 2008, 2011). In addition to a continuous Total score reflecting severity of social deficits associated with the autism spectrum, the SRS-2 generates scores for five treatment subscales: Social Awareness, Social Cognition, Social Communication, Social Motivation, and Restricted Interests and Repetitive Behavior. The SRS-2 also offers two DSM-5 Compatible Subscales, Social Communication and Interaction (SCI) and Restricted Interests and Repetitive Behavior (RRB). Parents of C4 participants completed the SRS-2 during the 12-year visit, and SRS raw scores were created utilizing publisher-supplied software. Sex-specific T scores, with a mean = 50 and standard deviation = 10, were determined based on the publisher's instructions, with higher scores representing more severe impairment and behaviors consistent with ASD.

2.4. Covariates

We collected maternal and child characteristics at enrollment and subsequent follow-up visits using self-report interviews and surveys. Covariates considered for inclusion in adjusted models were identified using a directed acyclic graph and selected from prior literature on their potential roles as confounders of the relationship between air pollution and ASD risk (Fig. S1). These included maternal education (some college education or less/obtained a college degree or graduate/doctoral degree) and a measure of community-level socioeconomic status. We calculated community deprivation, an indicator of community socioeconomic disadvantage, based on residential birth address utilizing a previously developed index that combines several census tract level socioeconomic data to provide a score ranging from 0 to 1, with values indicating greater deprivation (Brokamp et al., 2019).

2.5. Statistical analysis

Descriptive statistics and graphical plots were used to examine the distribution of all variables, examine potential outliers, and describe the cohort, exposures, and outcome measures. Means and standard deviations are reported for continuous variables; number of individuals and frequencies are reported for categorical variables. Missing maternal baseline education levels (n = 21) were replaced with reports obtained in later follow up visits.

We developed separate unadjusted linear regression models to initially evaluate associations between prenatal and first-year of life exposures to air pollutants (NO_2 and $PM_{2.5}$) and the six SRS-2 component scores. Adjusted linear regression models were subsequently developed with covariates. All effect estimates are presented as a interquartile range (IQR) unit increase in exposure concentration. We assessed potential effect modification of NO_2 and $PM_{2.5}$ exposure by child sex by including an interaction term in the adjusted models. We then conducted a stratified analysis by child sex for models that contained an interaction term of significance ($p < 0.1$). We conducted a sensitivity analysis utilizing a logistic regression model with dichotomized outcomes to asses diagnostically-relevant SRS cut points at 60 and 75. Additionally, we explored average exposures during each trimester of pregnancy. All statistical analyses were conducted using R Studio (version 4.1.1; R Development Core Team) and figures were created with GraphPad Prism (version 9.3.1).

3. Results

3.1. Participant characteristics

A total of 435 children (CCAAPS: n = 180, HOME: n = 255) and their caregivers attended the age 12 study visits and completed the SRS questionnaire (Table 1). Children were, on average, 12.4 years at the time of the visit, with slightly fewer males than females (48% vs. 52%). Most participants (64.6%) self-reported their race as White, reflecting the racial distribution of the greater Cincinnati region. On average, mothers were 29.1 years of age at delivery; most of whom had college degree (60.8%), were married or living with a partner (80.9%)) and did not smoke during pregnancy (88.7%).

For the CCAAPS cohort, the SRS questionnaire was added to the study protocol after the start of the age 12 study visits and not all individuals who completed the age-12 visit completed this assessment. No significant differences were identified for participants who completed the age 12 study visit and those who did not (n = 806) with respect to sex, maternal education level, and household income but participants who did not complete the age 12 visit were more likely to be white and be married (Table S1). No significant differences in sex, birthweight, maternal age at delivery, maternal education level, smoking, household income, NO_2 exposure level, and all SRS scores were found between participants of the two cohorts (Table 1). There were significant differences between the cohorts in racial composition, marital status, and community deprivation status with CCAAPS participants more likely to be White, have greater daily exposure to $PM_{2.5,}$ have parents who were married or living together, and live in a census tract with higher deprivation index as compared to HOME Study participants. The mean total SRS score of children in this cohort was 50.8 [standard deviation (SD) = 9.4]. Seventy-four (17.0%) children had total SRS scores ($T \geq 60$) indicating deficiencies in reciprocal social behavior that may interfere with daily social interactions. Ten children (2.3%) had scores considered to be severe ($T \geq 75$) and strongly associated with a clinical diagnosis of ASD (Constantino and Gruber, 2012).

3.2. Exposure to air pollution

Average [SD] exposure to NO_2 among the participants during the prenatal period (32.3 [4.4] ppb) was similar to average exposure during the first year of life (32.0 [4.0] ppb) (Fig. 1A). However, individual participants' exposures to NO_2 differed from prenatal to the first year of life due to changes in both residential locations and temporal variation; the correlation between prenatal and first year of life NO_2 exposure was 0.57. Additionally, exposure to $PM_{2.5}$ among the participants during the prenatal period, 15.7 [1.8] $\mu g/m^3$, was similar to average exposure

Table 1
Child, Maternal, and Household Characteristics of C4, CCAAPS, and HOME cohorts.

Characteristics	C4		CCAAPS		HOME		p-value
	N (%) or Mean (SD)						
Child Characteristics							
N	435		180		255		
Sex							0.06
Male	209	(48.0)	96	(53.3)	113	(44.3)	
Female	226	(52.0)	84	(46.7)	142	(55.7)	
Race							<.01
White	281	(64.6)	131	(72.8)	150	(58.8)	
Black or African American	122	(28.0)	36	(20.0)	86	(33.7)	
Other	32	(7.4)	13	(7.2)	19	(7.5)	
Birthweight (kg)	3.36	(0.6)	3.42	(0.6)	3.31	(0.6)	0.06
Maternal Characteristics (at time of birth)							
Age at Delivery	29.07	(5.9)	29.48	(5.9)	28.78	(5.9)	0.2
Education at Time of Birth							0.7
HS graduate or less	98	(22.6)	41	(22.8)	57	(22.4)	
Some college or technical school	264	(61.1)	45	(25.0)	73	(28.7)	
College graduate or advanced	72	(16.6)	94	(52.2)	124	(48.8.)	
Marital Status							<.01
Married or living with partner	336	(80.1)	155	(88.1)	181	(75.7)	
Not married and living alone	79	(19.0)	21	(11.9)	58	(24.3)	
Smoking	49	(11.3)	21	(11.7)	28	(11.0)	1.0
Household Characteristics (at time of birth)							
Household Income							0.1
<$40,000	157	(37.1)	57	(32.6)	100	(40.3)	
$40,000 - $89,999	185	(43.7)	78	(44.6)	107	(43.1)	
>$90,000	81	(19.1)	40	(22.9)	41	(16.5)	
Deprivation Index	0.36	(0.2)	0.33	(0.1)	0.38	(0.2)	<.01
Air Pollutant Exposure							
NO_2							
Prenatal (ppb)	32.3	4.4	32.3	3.9	32.4	4.7	0.9
First Year of Life (ppb)	32.0	4.0	31.7	4.1	32.3	4.0	0.2
Fine Particulate Matter ($PM_{2.5}$)							
Prenatal ($\mu g/m^3$)	15.7	1.8	16.2	1.4	15.4	2.0	<.01
First Year of Life ($\mu g/m^3$)	15.5	0.9	15.9	0.8	15.3	0.9	<.01
SRS Score							
Total	50.8	(9.4)	51.2	(9.5)	50.5	(9.4)	0.5
Social Awareness	52.8	(9.7)	53.5	(9.7)	52.3	(9.8)	0.2
Social Cognition	50.5	(9.4)	50.5	(9.6)	50.4	(9.4)	0.9
Social Communication	50.6	(9.5)	50.9	(9.5)	50.3	(9.5)	0.5
Social Motivation	50.6	(10.0)	50.9	(10.4)	50.3	(9.8)	0.5
DSM Social Communication and Interaction	51.0	(9.5)	51.4	(9.5)	50.7	(9.5)	0.4
DSM Restricted Interests and Repetitive Behavior	49.8	(9.4)	50.1	(9.7)	49.7	(9.1)	0.6

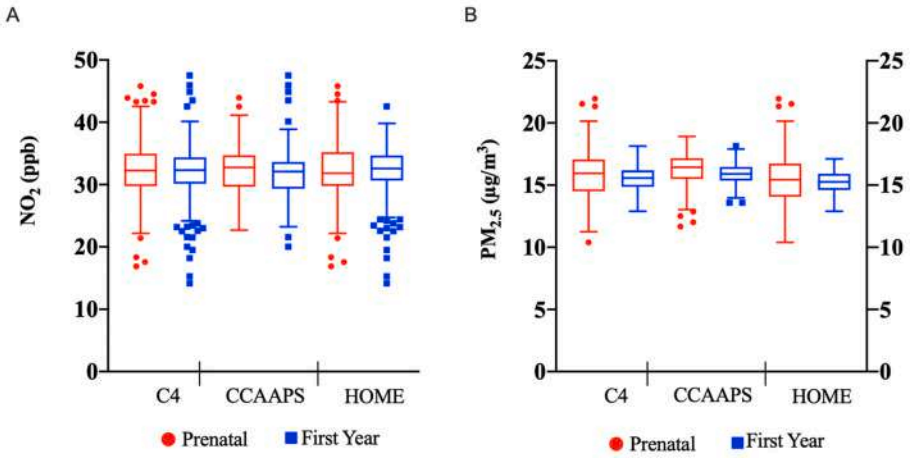

Fig. 1. Prenatal and First Year of Life Air Pollution Exposure
Distribution of prenatal (red, circle) and first year (blue, square) of life NO_2 and $PM_{2.5}$ exposure for C4, CCAAPS, and HOME Study cohorts. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

during the first year of life, 15.5 [0.9] μg/m^3 (Fig. 1B), but the correlation among individual exposure between the two time points was 0.14.

3.3. Air pollution and Social Responsiveness Scale scores

Results are presented in IQR increase of each exposure: prenatal NO_2 (5.2 ppb), first year NO_2 (4.2 ppb), prenatal $PM_{2.5}$ (2.6 μg/m^3), first year $PM_{2.5}$ (1.3 μg/m^3). In unadjusted models, prenatal and first year exposure to NO_2 were significantly associated with total SRS T-scores with an estimated 1.2 point increase (95% CI: 0.17, 2.3) per 5.2 ppb increase in NO_2 exposure (Fig. 2A) and 1.3 point (95% CI: 0.4, 2.2) per 4.2 ppb increase in NO_2 exposure (Fig. 2B), respectively. Prenatal NO_2 exposure was also positively associated with Social Cognition (β = 1.1, 95% CI: 0.009, 2.1), Social Motivation (β = 1.3, 95% CI: 0.2, 2.4), DSM SCI (β = 1.2, 95% CI: 0.1, 2.2), and DSM RRB (β = 1.1, 95% CI: 0.1, 2.2) component scores. First year NO_2 exposure was positively associated Social Awareness (β = 1.1, 95% CI: 0.2, 2.1), Social Cognition (β = 1.5, 95% CI: 0.6, 2.5), Social Motivation (β = 1.3, 95% CI: 0.3, 2.2), DSM SCI (β = 1.3, 95% CI: 0.3, 2.2), and DSM RRB (β = 1.2, 95% CI: 0.3, 2.1). After adjusting for maternal education and census tract deprivation index, positive but nonsignificant associations were observed with prenatal (β = 0.4, 95% CI: −0.7, 1.6) and first year of life (β = 0.7, 95% CI: −0.3, 1.6) NO_2 exposure and SRS Total T-scores. Increased maternal education was significantly associated with decreased SRS Total T-scores in both prenatal and first year of life in the fully adjusted models (Table S2).

For first year $PM_{2.5}$ exposure, in the unadjusted models, a 1.3 μg/m^3 increase in exposure during the first year was significantly associated with a 1.3 point increase (95% CI: 0.02, 2.7) in SRS Total T-scores (Fig. 3B). Additionally, the Social Cognition (β = 1.4, 95% CI: 0.1, 2.8) and DSM SCI (β = 1.4, 95% CI: 0.04, 2.7) component T-scores were significantly associated with the exposure. No significant associations were observed with prenatal $PM_{2.5}$ exposure in the unadjusted model (Fig. 3A). Similar to NO_2 models, increased maternal education was associated with decreased SRS T-scores in the fully adjusted models (Table S2).

When examining potential effect medication by sex, there was a significant interaction between sex and prenatal NO_2 for SRS Total, Social Cognition, Social Communication, and DSM RRB scores (Fig. S2), with female children having increased association (Fig. S2) though the effect of prenatal NO_2 exposure on SRS scores remained not significant. Sensitivity analysis with dichotomized outcomes to asses diagnostically-relevant SRS cut points had no significant findings. In addition, there were no significant associations between trimester specific windows of exposure during pregnancy and SRS scores.

4. Discussion

In this longitudinal study, we found elevated SRS scores associated with exposure to NO_2 and $PM_{2.5}$ during pregnancy and the first year, but these were not statistically significant after adjusting for maternal education and community deprivation. Maternal exposure to NO_2 during pregnancy and child's NO_2 exposure during first year of life seemed to indicate greater ASD risk with elevated SRS total T-scores and component scores for Social Cognition, Social Motivation, DSM RRB, and DSM SCI categories. However, after adjustment for socioeconomic and community confounders, all associations were no longer statistically significant. Similar nonsignificant trends were observed for $PM_{2.5}$ exposure during the first year.

Although prior studies have examined the association between air pollutants and ASD diagnosis, to our knowledge this is the first epidemiologic study to utilize the SRS to study air pollution and ASD-like behaviors in typically developing children. Our findings are consistent with a meta-analysis by Chun et al. which suggested there is weak evidence for a positive association between maternal exposure to NO_2 and ASD in children (Chun et al., 2020). Pagalan et al. conducted a study using a Canadian population-based birth cohort and found that the adjusted odds ratios for NO_2 exposure per interquartile range were not significantly associated with ASD diagnosis, assessed with the Autism Diagnostic Interview–Revised and Autism Diagnostic Observation Schedule (Pagalan et al., 2019). Other studies utilizing ASD diagnosis for case ascertainment have also reached similar conclusions26,38,40. Moreover, studies that studied NO_2 exposure and childhood autistic traits, assessed using the Autism Spectrum Disorder module of the Autism—Tics, Attention Deficit and Hyperactivity Disorders, and Other Comorbidities (A-TAC) inventory, have not found significant associations (Gong et al., 2014; Guxens et al., 2016). However, there is no consensus regarding NO_2 exposure as a risk factor for ASD because some studies reported contradictory findings. Positive association between ASD diagnosis and prenatal and first year NO_2 exposure were observed in the United States (Volk et al., 2013). In Taiwan (Jung et al., 2013), one year preceding diagnosis was found to be a significant exposure window. In a nationwide study of Danish children, Ritz et al. found that pollution exposures in early infancy but not in pregnancy contributed to an increased risk of ASD (Ritz et al., 2018).

Studies analyzing $PM_{2.5}$ exposure and ASD have also reported inconsistent findings. In contrast to our findings, two California based studies reported significant associations between prenatal $PM_{2.5}$ exposure and ASD diagnosis, assessed with the Autism Diagnostic Interview–Revised and Autism Diagnostic Observation Schedule (Volk et al., 2013; Becerra et al., 2013). However, another study of children in California, found no association (Goodrich et al., 2018; Kerin et al., 2018). Consistent with our findings, additional studies have found effect estimates for $PM_{2.5}$ to be elevated but not reaching a significance level of association with ASD diagnosis (Talbott et al., 2015; Pagalan et al., 2019; Kaufman et al., 2019) or ASD-like traits (Guxens et al., 2016). Raz et al. found that while the exposure during the entire pregnancy may not be relevant, exposure during certain time points, specifically the third

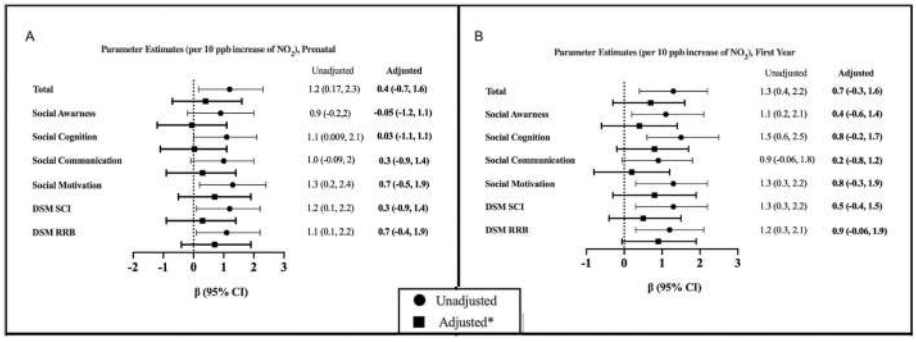

Fig. 2. Linear Regression Parameter Estimates for NO_2 Exposure and SRS Scores
Unadjusted (circle) and adjusted* (square) parameter estimates (per prenatal and first year IQR ppb increase, 5.2 and 4.2, respectivel) for the association between prenatal (A) and first year NO_2 (B) and SRS Total and component scores. *Adjusted for maternal education and community deprivation index.

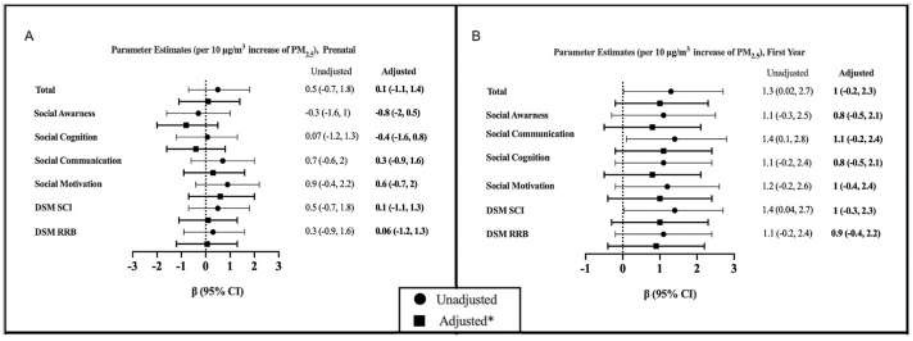

Fig. 3. Linear Regression Parameter Estimates for $PM_{2.5}$ exposure and SRS Scores
Unadjusted (circle) and adjusted* (square) parameter estimates (per prenatal and first year IQR μg/m^3 increase, 2.6 and 1.3 μg/m^3, respectively) for the association between prenatal (A) and first year (B) and $PM_{2.5}$ and SRS Total and component scores. *Adjusted for maternal education and community deprivation index.

trimester, was significant (Raz et al., 2015). Of those studies that specifically looked at first year of life exposure and ASD diagnosis, Volk et al. found this time period to be of significance (Volk et al., 2013) while Talbott et al. and Kaufman et al. did not observe any significant associations (Talbott et al., 2015; Kaufman et al., 2019). Other studies examining postnatal time points, such as Chen et al. examined exposure in the 2nd and 3rd year of life and Ritz et al. at 9 months of age, found statistically significant associations (Ritz et al., 2018; Chen et al., 2018).

Our study did have some limitations that should be considered in the context of our findings and comparisons to previous studies. First, our study population consists of typically developing children and does not represent the phenotypic extreme present in the case–control studies. Thus, only a few participants met the threshold for clinically relevant levels of autistic behaviors. Unmeasured confounders may also be present in our study and affected our observations. Studies have shown that higher gestational concentrations of some phthalate metabolites are associated with higher scores of autistic traits (Oulhote et al., 2020). Similarly, we do not have data regarding maternal folic acid intake which has been shown to modify the effects of air pollution on risk for ASD (Goodrich et al., 2018). In addition, our power to detect statistically significant associations was limited by our sample size. In particular, in models of prenatal and first year of life NO_2 and first year of life $PM_{2.5}$ the parameter estimates were consistently positive and similar between adjusted and unadjusted models.

Our study had multiple strengths including the availability of residential locations for all study participants, allowing us to assign modeled pollutant exposures for developmentally relevant time points while accounting for changes in home address prenatally and during early childhood. In contrast, the majority of previous studies on air pollution and adverse child health outcomes rely on addresses obtained from the birth certificate to classify exposure which may result in misclassification due to maternal residential mobility during pregnancy. A study conducted by Chen. et al. utilizing a New York birth cohort, found that 13% of mothers moved once during pregnancy and 3.5% moved at least twice (Chen et al., 2010). Our ability to characterize exposures throughout changes in residence during pregnancy and the first year of life was advantageous. In addition, we used air pollution estimates from validated models that have been utilized frequently in other studies (Qian et al., 2021; Xi et al., 2022; Qiu et al., 2022; Wyatt et al., 2022; Liao et al., 2021; Rahman et al., 2022; Lu et al., 2021; Shi et al., 2021; Yazdi et al., 2021). Furthermore, our study population is a combination of two well-established longitudinal cohorts, CCAAPS and HOME Study, which both used similar data collection measures allowing them to be merged for increased sample size and power. Also, to our knowledge, this was the first study to utilize the Social Responsiveness Scale as the outcome measure, allowing us to explore the effect of air pollution exposures on an entire range of ASD-associated traits and behaviors that vary in severity. Our study was also the first to analyze this association in this unique geographic region as three major interstates intersect in the Cincinnati, Ohio region to create one of the busiest commercial truck routes in the US (Institute ATR, 2019). The confluence of nearby coal-fired power plants in the Ohio River Valley, industrial emissions, and other sources of $PM_{2.5}$ in the region also result in annual $PM_{2.5}$ concentrations that place the region as the 13th worst metropolitan region in the US for air quality (Association, 2021). Thus, C4 participants reside in a region greatly impacted by NO_2 and $PM_{2.5}$.

5. Conclusions

ASD has lifelong social and behavioral implications with limited treatment options, emphasizing the need to identify modifiable risk factors for these disorders. Air pollution represents an important exposure to consider in the etiology of ASD since brain development and function are susceptible to insult from environmental toxicants during the prenatal and postnatal windows. While our results were not conclusive, we did observe a consistent elevated relationship between air pollution exposure and SRS scores. Collectively, the biological plausibility of air pollution affecting brain development and our results suggest the need for further study, particularly given the limited and inconclusive results from longitudinal cohort studies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114172.

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Ambient ozone exposure and depression among middle-aged and older adults: Nationwide longitudinal evidence in China

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ABSTRACT

Background: Epidemiological studies have linked long-term ozone (O_3) exposure with depression in developed countries. However, available literature is sparse and exists great heterogeneities. We aimed to investigate the association of long-term O_3 exposure with depression among Chinese middle-aged and older adults.
Methods: We designed a repeated measurement study based on longitudinal data from four waves (2011, 2013, 2015, and 2018) of the China Health and Retirement Longitudinal Study (CHARLS). Annual mean O_3 concentrations assessed through machine learning–based spatiotemporal models were assigned to each participant at city level. Depression score was measured using the 10-item Center for Epidemiologic Studies Depression scale (CES-D-10), with scores above the cut-off point of ten defined as depressive symptom. Mixed-effects models were used to evaluate the impact of O_3 on depression score and depressive symptom, and quantify the concentration-response (C-R) relationships. Subgroup analyses were performed to examine the potential effect modifications.
Results: A total of 19,582 participants with 60,125 visits were included in our analysis, with mean depression score of 8.1 (standard deviation: 6.3). Multivariable-adjusted mixed-effects model estimated a 6.34% (95% confidence interval [CI]: 3.34%, 9.43%) increase in depression score and an odds ratio (OR) of 1.29 (95% CI: 1.16, 1.45) for depressive symptom associated with per 10-μg/m^3 rise in annual mean O_3 exposure. Significantly elevated risks were identified only at high concentrations (approximately ≥90 μg/m^3). Participants who suffered from chronic diseases had a significant increased risk of depression (% Change in depression score: 8.42% [95% CI: 4.79%, 12.17%], and OR: 1.42 [95% CI: 1.24, 1.62]), and an evident effect modification was identified for depressive symptom ($P = 0.01$).
Findings: Our study provided novel evidence that long-term O_3 exposure could be a risk factor for depression among Chinese middle-aged and older adults. Our findings may have significant implications for formulating policies in reducing disease burden of depression by controlling air pollution.

1. Introduction

Depression gives rise to a large proportion of the health burden from mental disorder and substantially diminishes quality of life with an increased prevalence across the globe (Moreno-Agostino et al., 2021; Vos et al., 2020). Given the social and health care burden of this disorder, it is imperative to identify modifiable risk factors for prevention of depression. In addition to the identified social and behavioral factors (e.g., low socioeconomic status and smoking) (Li et al., 2021; Ribeiro et al., 2017), ambient air pollution has increasingly been recognized as

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an emerging risk factor for depression (Chen et al., 2018; Pun et al., 2017; Xue et al., 2021). Fine particulate matter ($PM_{2.5}$) has been widely identified as an important environmental determinant of depression onset and aggravation in several recent meta-analysis (Borroni et al., 2022; Braithwaite et al., 2019), while other major air pollutants such as ozone (O_3) has not been adequately investigated.

Associations of O_3 exposure with mental health deserve more epidemiological investigations. Biological mechanism study indicated that exposure to O_3 may be a potential risk factor for depression due to the high neurotoxicity and powerful oxidizing properties (Zhang et al., 2019). Emerging epidemiological studies had reported a positive association between short-term O_3 exposure and depression across the globe (Lu et al., 2020; Nguyen et al., 2021; Tsai et al., 2020), while limited researches investigated the long-term impact of O_3 exposure on depression (Borroni et al., 2022). Available longitudinal O_3-depression evidence was mainly reported in North America (Kioumourtzoglou et al., 2017) and Europe (Bakolis et al., 2021; Pelgrims et al., 2021), wherein great heterogeneities still existed between studies. For instance, a large American cohort study reported increased risk of depression associated with long-term O_3 exposure (Kioumourtzoglou et al., 2017), while non-significant association was observed in a recent regional study in Britain (Bakolis et al., 2021). Besides, the effect of O_3 exposure on depression may vary among subpopulations, which might be due to differential susceptibility of the subgroups to health effects of air pollution (Simoni et al., 2015) and confounding effect of comorbidities (Loop et al., 2013). Related evidence was largely sparse in developing countries such as China, where most locations have been experiencing serious O_3 air pollution (Lu et al., 2018) and rapid increase in depression prevalence during recent decades (Ferrari et al., 2022).

To fill this research gap, we designed a repeated measurement study based on a Chinese nationwide cohort of middle-aged and older men and women during 2011–2018. We primarily aimed to quantify the long-term association between O_3 exposure and depression in Chinese adults, and to depict the concentration-response (C-R) relationship across a wide range of exposure levels.

2. Materials and methods

2.1. Study population

The participants of this study were recruited from the China Health and Retirement Longitudinal Study (CHARLS), an ongoing nationwide longitudinal survey on the health and socioeconomic status of Chinese middle-aged and older adults. A representative sample of ~18,000 Chinese adults from 28 provinces was involved in the baseline survey conducted in 2011–2012, and was generally followed up every 2–3 years through a face-to-face computer-assisted interview (Zhao et al., 2014). Details of the study design and the purpose of the CHARLS are provided elsewhere (Chen et al., 2015; Zhao et al., 2014). The CHARLS study had been approved by the Ethics Committee of Peking University Health Science Center, and all participants gave written informed consent before participation (No. IRB00001052-11015).

Based on publicly available data from CHARLS surveys in 2011, 2013, 2015, and 2018 waves, we involved a sample of 25,458 individual adults with 76,768 observations. To conduct a longitudinal analysis, we focused on participants who had valid CES-D-10 records (n = 23,937). Each visit represented each face-to-face interview with the participant, and could generate a single observation for the participant. We further excluded the adults <40 years old at survey time and participants visited only once. Finally, this study involved 60,125 observations from 19,582 individual adults (Fig. 1) distributed across 125 cities spanning 28 provinces (Fig. 2). Details for description of sample inclusion and exclusion were presented in supplementary materials.

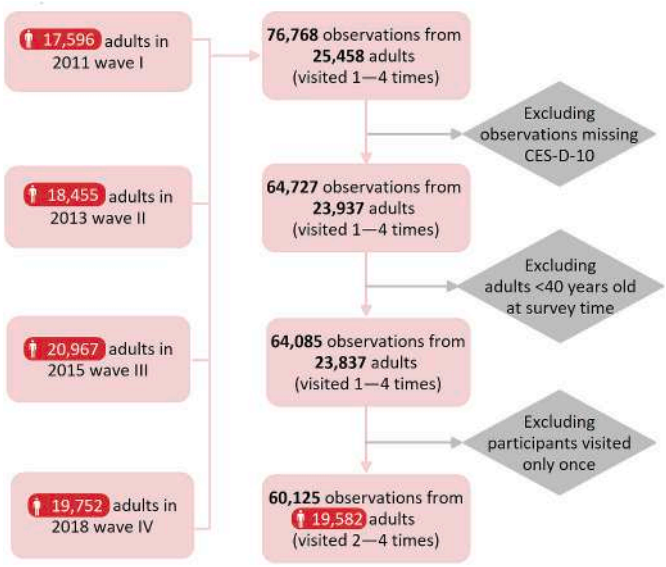

Fig. 1. Flowchart of sample inclusion and exclusion.

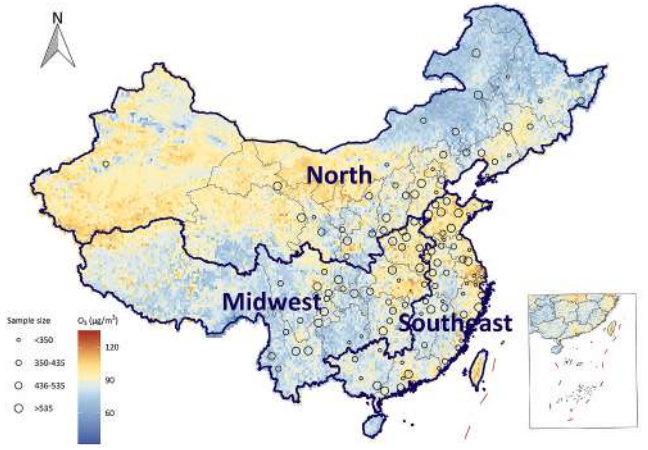

Fig. 2. The map of participants' geographic distribution with long-term averages of O_3 concentrations (2010–2018).

2.2. Environmental exposure

The concrete residential addresses of participants were unable to obtain due to limited access to privacy data in CHARLS, we thus assessed O_3 exposure at the city-level in the longitudinal analysis. By aggregating gridded O_3 estimates at the resolution of 0.1° × 0.1° into city-level averages, we first derived monthly ozone concentrations of 125 prefectural cities from 2010 to 2018, and then calculated average O_3 concentrations over the 12 months preceding the surveyed month for each individual as the exposure. The same method was used to assign annual $PM_{2.5}$ exposure to participants. For privacy consideration, the survey date of CHARLS available for public only included the year and month, which limited our ability to assess O_3 exposure directly based on specific survey date. However, for long-term exposure assessments (e.g., annual mean exposure), calculation using monthly averages instead of daily estimates would not greatly affect the assessments.

Monthly O_3 concentrations across Chinese mainland during 2005–2018 at a resolution of 0.1° were estimated through a data fusion of observations and models. In brief, the daily maximum 8-h O_3 monitoring data (MDA8) of 1713 stations in China from 2013 to 2017 were used to establish a national MDA8 prediction model based on eXtreme

Gradient Boosting (XGBoost). Regional monitors data during 2005–2012 and nationwide measurements in 2018 were applied to test the prediction accuracy with high R^2 values (range: 0.60–0.87) and low root mean square error (RMSE, 12.94–18.41 μg/m^3) in different years (Liu et al., 2020a). The daily ground-level O_3 monitoring data were obtained from the China National Environmental Monitoring Center. More modeling details for O_3 estimates could be found in prior publication (Liu et al., 2020a).

Gridded $PM_{2.5}$ concentrations at a 0.1° × 0.1° resolution from 2010 to 2018 on a monthly scale were derived from Tracking Air Pollution (TAP, http://tapdata.org.cn/en, accessed on April 14th, 2023), which was known as a near real-time air pollutant database in China. Daily $PM_{2.5}$ concentrations were estimated based on a two-stage machine learning model combined with a tree-based gap-filling method and the synthetic minority oversampling technique (Geng et al., 2021). The predicted concentrations of $PM_{2.5}$ showed good consistency comparing against the ground observations, with out-of-bag cross-validation R^2 of 0.80–0.88 and RMSE of 13.9–22.1 μg/m^3 for different years between 2013 and 2020 (Xiao et al., 2021).

2.3. Measurement of depressive symptom

The CHARLS used the 10-item Center for Epidemiologic Studies Depression scale (CES-D-10) to measure the mental health status of the participants, which were collected during a computer-assisted interview by fieldworkers. Cronbach's alpha coefficient was calculated to evaluate the reliability of the CES-D-10 scale used in our analysis. This coefficient ranged between 0.76 and 0.81 in selected waves (2011–2018) of the CHARLS survey (Table S1), which displayed considerably high internal consistencies across the total sample. Prior studies had examined the validity of the CES-D-10 among Chinese adults (Boey, 1999). The CES-D-10 questionnaire included 10 questions and each question measured the frequency of negative mood using a score of 0 (rarely or none of the time), 1 (some or a little of the time), 2 (occasionally or a moderate amount of the time), or 3 (most or all of the time). Two questions were scored in a negative direction. The sum of scores for 10 specific questions were calculated as the depression score to indicate the general status of depressive symptom for each participant. The depression score ranged from 0 to 30, and a higher score represented a higher severity of depression. The depression score greater than or equal to 10 was used as a cut-off for categorizing the status of depressive symptom (Liu et al., 2020b; Yao et al., 2022). Intraclass correlation coefficients (ICC) for depression score during different waves ranged from 0.67 to 0.80 (Table S2), which suggested a relatively high reproducibility of measurements.

2.4. Covariates

We considered a set of covariates in accordance with prior epidemiological studies (Shi et al., 2022; Xue et al., 2021), enabling us to separate the effect of ambient O_3 from potential confounders. The covariates included demographic characteristics (i.e., sex, age, marital status, educational level, employment status, residence, and region), behavioral factors (i.e., cigarette smoking and alcohol drinking), health factors (i.e., chronic diseases, disability in activities of daily living and health status), and environmental factors (i.e., household cooking fuel and outdoor temperature).

To be concrete, marital status was defined as a dichotomous variable, with "yes" representing the participant has been married and lived with a spouse or cohabitation, and "no" otherwise; educational level was divided into below primary school (0 y), primary and middle school (1–9 y), or above middle school (>9 y); employment status was divided into "yes" or "no" according to whether participant was employed; residence was classified as either rural or urban; region was divided into Midwest, Southeast, and North area; cigarette smoking was classified as "yes" or "no"; alcohol drinking were categorized into never, rarely, and frequently; chronic disease was classified as "yes" for participants suffering one or more physician-diagnosed chronic diseases (e.g., stroke, diabetes, coronary heart disease, and hypertension, etc.); health status was divided into "good", "fair" or "poor" according to the self-report judgement; disability in activities of daily living was classified as "yes" or "no"; household cooking fuel was categorized as clean fuel (i.e., electricity, liquefied and natural gas) and solid fuel (i.e., wood, coal, biomass charcoal, and straw). Monthly average temperatures at the city-level were aggregated from daily estimates of the European Center for Medium-Range Weather Forecasts (ECMWF) atmospheric reanalysis data set of the global climate at a resolution of 0.1° × 0.1°.

2.5. Statistical analysis

Continuous variables were described using mean and standard deviation (SD) and category variables were presented by frequency and percentage. Given the identified impact of ambient $PM_{2.5}$ on depression onset and aggravation (Borroni et al., 2022; Braithwaite et al., 2019; Xue et al., 2021), we applied a bi-pollutant model to estimate the associations between O_3 exposure and depression. Considering the longitudinal repeated measurement study design, mixed-effects linear regression model was used to analyze the impact of O_3 on depression score. The model was specified as follows:

$$Log\left(Score_{i,j}+1\right)=\beta_0+\beta x_{i,j}+\gamma z_{i,j}+city_i+\eta(community_i)+\mu(i),$$

where i denotes the subject index; j refers to visit index; β_0 represents the intercept; β is the regression coefficients for air pollutants; $z_{i,j}$ denotes a set of adjusted covariates and γ represents the corresponding regression coefficients; $city_i$ denotes a fixed effect to control the unmeasured city-specific risk factors of depression (Ng, 1997); η and μ denotes two random intercepts to model the correlations between records from the same community or the same subject, respectively. The change in depression score was calculated as $100\% * [exp\ (10 * \beta) - 1]$ to quantify the impact of O_3 on depression score. We adopted a sequential adjustment approach to define the models with different levels of adjustment. The crude model (model 1) was developed to incorporate study cities and the concentrations of air pollutants (O_3 and $PM_{2.5}$) as the fixed-effect term and set individual ID and community as the random-effect terms. Model 2 controlled for demographic characteristics based on the crude model. Model 3 further adjusted behavioral and health factors. Multivariable-adjusted model (model 4) further accounted for environmental factors. Variance inflation factor (VIF) was used to test collinearity in the multivariable-adjusted model, and the VIF-based assessments did not imply appreciable multicollinearity (Table S3). The associations between O_3 and depressive symptom was assessed through mixed-effects logistic regression models with the same adjustments of covariates and parameters as the linear regression models.

To investigate the C-R relationship between O_3 with depression score and depressive symptom, O_3 exposure was fitted as a smoothing term using a restricted cubic spline (RCS) function in the multivariable-adjusted model (Zhang et al., 2022). We adopted three knots to fit the curve according to Akaike information criterion and Bayesian information criterion (Table S4). Likelihood-ratio test was applied to examine the potential nonlinearity of the relationship (Liu et al., 2019). We performed subgroup analyses to examine potential effect modifications, stratified by sex (male or female), age group (<65 or ≥65 years), educational levels (0 y, 1–9 y, or >9 y), employment status (yes or no), cigarette smoking (yes or no), alcohol drinking (never, rarely or frequently), residence (urban or rural), region (Midwest, North, or Southeast) and chronic diseases (yes or no). Meta-regression methods were used to examine whether the differences between subgroups were statistically significant (Liu et al., 2021; Yang et al., 2015).

Sensitivity analyses were performed to assess the robustness of our results. First, we compared the effects of alternative exposure time windows using 2-year average O_3 and $PM_{2.5}$ concentrations. Second, to

Table 1
Constant variables of all studied subjects.

Variable	Subgroup	No. (%)
Total number of Subjects		19582
Educational levels	Below elementary school	7615 (38.9)
	Elementary and middle school	7976 (40.7)
	Above middle school	2377 (12.1)
	Unknown	1614 (8.2)
Sex	Female	10171 (51.9)
	Male	9398 (48.1)
	Unknown	13 (<0.1)
Place of residence	Rural	11698 (59.7)
	Urban	7884 (40.3)
Region	Midwest	6445 (32.9)
	North	5531 (28.2)
	Southeast	7606 (38.8)

Because of using rounding-off method, it may occur that the sum of the percentages does not equal 100%.

comprehensively assess O_3 effects, we re-estimated O_3-depression association using a single-pollutant model without the adjustment of $PM_{2.5}$. Third, considering that air pollutants may have an interactive effect with age on depression (Xue et al., 2021), we adjusted the interaction term of age and O_3 in the multivariable-adjusted model. Fourth, the directed acyclic graph (DAG) was used to identify a minimal sufficient adjustment set of variables (Fig. S1), which was widely used to select covariates for minimizing confounding bias in epidemiological studies (Textor et al., 2016). Fifth, to better capture the long-term effect of O_3 exposure on depression, we excluded participants who only had twice consecutive visits (n = 3238), and re-estimated the associations based on the multivariable-adjusted analysis (model 4). Sixth, we further performed an additional subgroup analysis to explore the potential modification effect of type-specific chronic diseases on O_3-depression associations. Additionally, we used multiple imputation to fill in the missing values and rerun the multivariable-adjusted model (model 4) to verify the stability of our main results.

All analyses were conducted by R software (Version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria), with the "lme 4" package for analyses of mixed-effects models, the "mice" package for multiple imputation, the "rms" package for nonlinear smoothing using restricted cubic spline, and the "effects" package for prediction of C-R relationship. Two-sided test with *P*-value <0.05 was considered statistically significant.

3. Results

Table 1 summarizes sociodemographic and behavioral characteristics of 19,582 middle-aged and older adults included in this study. During a total of 60,125 visits from 2011 through 2018, each participant

Table 2
Longitudinal variables of the studied subjects and environmental exposures at baseline and three consequent waves.

Variable	Total, n (%)	2011 CHARLS	2013 CHARLS	2015 CHARLS	2018 CHARLS
Number of visits	60125	13530	14739	16842	15014
Age (yrs), mean (SD)	59.5 (9.5)	58.3 (9.2)	59.3 (9.4)	59.1 (9.8)	61.1 (9.3)
Depressive symptom	20565 (34.2)	4945 (36.5)	4574 (31.0)	5490 (32.6)	5556 (37.0)
Depression score	8.1 (6.3)	8.3 (6.3)	7.8 (5.8)	7.9 (6.4)	8.5 (6.5)
Married and lived together					
No	10098 (16.8)	2097 (15.5)	2317 (15.7)	2857 (17.0)	2827 (18.8)
Yes	50020 (83.2)	11433 (84.5)	12416 (84.2)	13984 (83.0)	12187 (81.2)
Unknown	7 (0.1)	0	6 (0.1)	1 (0.1)	0
Cigarette smoking					
No	34776 (57.8)	8269 (61.1)	8503 (57.7)	9447 (56.1)	8557 (57.0)
Yes	25321 (42.1)	5260 (38.9)	6235 (42.3)	7380 (43.8)	6446 (42.9)
Unknown	28 (0.1)	1 (0.1)	0	15 (0.1)	11 (0.1)
Alcohol drinking					
Frequent	16253 (27)	3413 (25.2)	4050 (27.5)	4660 (27.7)	4130 (27.5)
Rare	4868 (8.1)	1049 (7.8)	1179 (8.0)	1477 (8.8)	1163 (7.7)
Never	39000 (64.9)	9068 (67.0)	9507 (64.5)	10704 (63.6)	9721 (64.7)
Unknown	4 (0.1)	0	3 (0.1)	1 (0.1)	0
Employment status					
No	18474 (30.7)	3894 (28.8)	4427 (30.0)	5121 (30.4)	5032 (33.5)
Yes	41580 (69.2)	9610 (71.0)	10291 (69.8)	11701 (69.5)	9978 (66.5)
Unknown	71 (0.1)	26 (0.2)	21 (0.1)	20 (0.1)	4 (<0.1)
Health status					
Fair	22027 (36.6)	4426 (32.7)	4786 (32.5)	5312 (31.5)	7503 (50.0)
Good	11629 (19.3)	2145 (15.9)	2506 (17.0)	3167 (18.8)	3811 (25.4)
Poor	26447 (44.0)	6957 (51.4)	7434 (50.4)	8360 (49.6)	3696 (24.6)
Unknown	22 (<0.1)	2 (<0.1)	13 (0.1)	3 (<0.1)	4 (<0.1)
Chronic diseases					
No	17784 (29.6)	4358 (32.2)	4706 (31.9)	4738 (28.1)	3982 (26.5)
Yes	38511 (64.1)	9063 (67.0)	9555 (64.8)	9476 (56.3)	10417 (69.4)
Unknown	3830 (6.4)	109 (0.8)	478 (3.2)	2628 (15.6)	615 (4.1)
ADL disability					
No	44664 (74.3)	11409 (84.3)	11359 (77.1)	11931 (70.8)	9965 (66.4)
Yes	15433 (25.7)	2113 (15.6)	3380 (22.9)	4898 (29.1)	5042 (33.6)
Unknown	28 (0.1)	8 (0.1)	0	13 (0.1)	7 (0.1)
Household cooking fuel					
Clean	34147 (56.8)	6004 (44.4)	7934 (53.8)	10015 (59.5)	10194 (67.9)
Solid	25802 (42.9)	7476 (55.3)	6742 (45.7)	6791 (40.3)	4793 (31.9)
Unknown	176 (0.3)	50 (0.4)	63 (0.4)	36 (0.2)	27 (0.2)
Environmental exposure, mean (SD)					
O_3 concentration($\mu g/m^3$)	88.7 (7.5)	89.9 (7.2)	88.4 (7.4)	88.1 (7.5)	88.4 (7.7)
$PM_{2.5}$ concentration ($\mu g/m^3$)	50.8 (21.9)	58.2 (23.9)	57.3 (24.5)	48.8 (19.2)	40 (14.0)
Temperature (°C)	13.6 (5.0)	13.1 (5.0)	13.6 (5.2)	13.7 (4.9)	13.7 (5.0)

Notes: Because of using rounding-off method, it may occur that the sum of the percentages does not equal 100%. Abbreviations: O_3, ozone; $PM_{2.5}$, fine particulate matter; SD, standard deviation; ADL disability, disability in activities of daily living.

Table 3
Estimated associations of O_3 exposure with depression score and depressive symptom.

	Depression score (% Change, 95% CI)	Depressive symptom (OR, 95% CI)
Model 1	3.72 (0.96, 6.56)	1.18 (1.06, 1.31)
Model 2	3.84 (1.05, 6.69)	1.19 (1.07, 1.32)
Model 3	5.35 (2.50, 8.28)	1.26 (1.13, 1.40)
Model 4	6.34 (3.34, 9.43)	1.29 (1.16, 1.45)

Model 1 was crude model.
Model 2: Adjusted for covariates in model 1 plus demographic characteristics including age, sex, educational level, marital status, employment status, residence and region.
Model 3: Adjusted for covariates in model 2 plus behavioral and health factors including alcohol drinking, cigarette smoking, ADL disability, chronic diseases and health status.
Model 4: Adjusted for covariates in model 3 plus environmental factors including household cooking fuel and outdoor temperature. Model 4 was multivariable-adjusted model.
Abbreviations: O_3, ozone; OR, odds ratio; CI, confidence interval.

was visited 3.1 times on average. Participants were aged 59.5 (SD: 9.5) years (range: 40–108 years), and nearly half of them were males (48.0%). The summary of longitudinal variables for participants in each wave of CHARLS (2011, 2013, 2015, and 2018 waves) were presented in Table 2. Among all visits, the mean depression score was 8.1 (SD: 6.3), and 34.2% were measured as depressive symptom. The characteristics for depressed and non-depressed participants were presented in Table S5. Annual mean concentrations of $PM_{2.5}$ for 125 cities showed a substantial decline from 2011 (58.2 μg/m^3) to 2018 (40.0 μg/m^3), while the average concentrations of O_3 fluctuated around 88.7 μg/m^3 (SD: 7.5 μg/m^3) during same period.

Table 3 estimates associations of ambient O_3 exposure with depression score and depressive symptom. Consistently positive effects of O_3 were observed in different models (model 1–4). According to the multivariable-adjusted model (model 4), a 10-μg/m^3 increase in annual mean O_3 exposures was associated with an excess risk of 6.34% (95% confidence interval [CI]: 3.34%, 9.43%) for elevated depression score. In the secondary analysis based on depressive symptom (depression score ≥10), positive associations were also identified using different models, with comparable odds ratio (OR) estimates ranging from 1.18 (95% CI: 1.06, 1.31) in model 1 to 1.29 (95% CI: 1.16, 1.45) in model 4.

Fig. 3 outlines the shape of C-R curves for long-term associations between annual average O_3 exposure and percentage change in depression score and OR for depressive symptom. We identified a nonlinear relationship between O_3 exposure and depression score (P = 0.02 for nonlinearity), with significantly elevated risks only at high concentrations (approximately ≥90 μg/m^3). In terms of depressive symptom, no evident violation of linear C-R association (P = 0.06) was identified, but we also detected steeper slopes at high concentrations.

Fig. 4 illustrates results from subgroup analysis on O_3-depression relationship stratified by demographic, behavioral and health factors. Males suffered from higher risk of depression (% Change in depression score: 8.33% [95% CI: 3.84%, 13.02%], and OR: 1.44 [95% CI: 1.21, 1.71]) associated with a 10-μg/m^3 rise in O_3 exposure. Participants under 65 years old were at higher risk when exposure to outdoor O_3 pollution, with corresponding excess risk of 6.58% (95% CI: 2.89%, 10.41%), and OR of 1.33 (95% CI: 1.16, 1.52) respectively. O_3-depression associations were more evident among individuals who were smoking or drinking frequently, although effect modifications were non-significant between stratum. Only participants who suffered from chronic disease had a significantly risk of depression (% Change in depression score: 8.42% [95% CI: 4.79%, 12.17%], and OR: 1.42 [95% CI: 1.24, 1.63]), and an evident effect modification were identified for depressive symptom (P = 0.01). The sub-region analyses presented positive associations in the Midwest and Southeast, while non-significant relationship was observed in North. Although participants in Midwest suffered from higher increased risk of depression associated with O_3 exposure than those in North, the difference was only significant for the depression score.

Sensitivity analyses largely supported the findings from our main

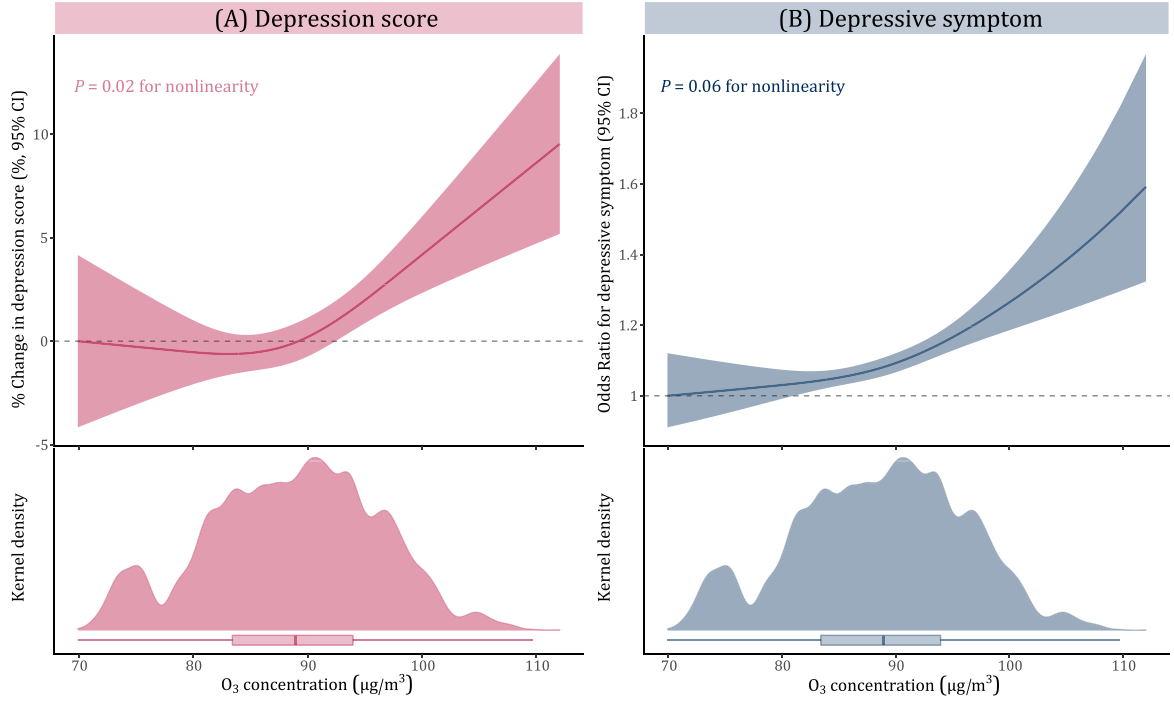

Fig. 3. Shapes of concentration-response relationships of O_3 exposure with depression. Dash area represents the 95% confidence interval (CI). Notes: the kernel density plot and box plot were used to describe the distribution of O_3 exposure for participants during study period, with 83.4 μg/m^3, 88.9 μg/m^3, 94.0 μg/m^3 for 25th, 50th, and 75th percentile O_3 concentrations, respectively.

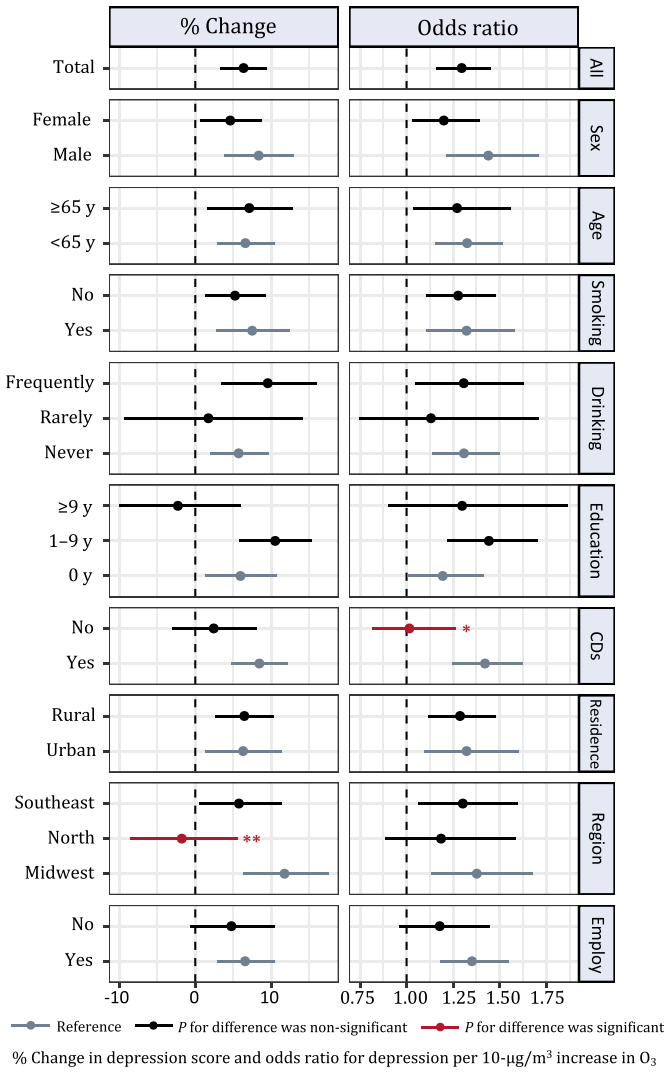

Fig. 4. Subgroup-specific associations of O_3 exposure with depression score and depressive symptom. Notes: † represents the reference group. Error bars, 95% confidence intervals. *P*-values were shown for tests of the null hypothesis that the point-estimate of the association was identical between subgroups; *$P < 0.05$, **$P < 0.01$. The associations were estimated using model 4. Abbreviations: CDs, chronic diseases; O_3, ozone.

analysis (Table S6). The association of O_3 exposure with depression was insensitive to use single-pollutant model (percentage change in depression score: 4.85% [95% CI: 1.91%, 7.86%], and OR: 1.13 [95% CI: 1.02, 1.27]). When using exposure concentrations at the 2-year scale, we observed comparable estimates of increase in depressive score (4.56% [95% CI: 0.41%, 8.87%]) and risk of depressive symptom (1.37 [95% CI: 1.17, 1.60]). The associations were also robust by excluding participants who only had twice consecutive visits, adjusting the minimum set of variables based on directed acyclic graph, and adding the interaction term of age and O_3 in the multivariable-adjusted model. Compared with participants without asthma, significantly higher percentage increase in depression score (29.95, 95% CI: 11.89, 50.94) associated with O_3 exposure was observed among asthma participants, while there was non-significant difference between the stratums when considering the depressive symptom as the outcome (Table S7).

4. Discussion

To the best of our knowledge, this is the first national study to investigate long-term effect of O_3 on depression in China. In this longitudinal study, we found a significantly positive association between long-term O_3 exposure and depression among Chinese middle-aged and older adults. O_3-depression associations were robustness by performing sensitivity analyses and adjusting different categorized covariates, while some evidence for effect modification by chronic diseases was observed. Our findings provided essential longitudinal evidence for O_3-depression associations and may contribute to the primary prevention of depressive disorders.

We observed a positive association between long-term O_3 exposure and depression, which is generally consistent with several prior longitudinal studies (Kioumourtzoglou et al., 2017; Zhao et al., 2020). However, great heterogeneity in epidemiologic evidence existed across investigations. A prospective cohort study suggested an increased risk of depression associated with exposure to O_3 among 41,844 older women from 11 US states (Kioumourtzoglou et al., 2017). Meanwhile, insignificant associations were reported by a prospective longitudinal survey in London enrolling 1698 adults (Bakolis et al., 2021) and a cross-sectional study in Brussels enrolling 1325 inhabitants aged ≥15 years (Pelgrims et al., 2021). These discrepancies may be attributed to differences in study population and design, the magnitude of sample size, as well as demographic characteristics. Risk of depression relating to O_3 exposure is biologically plausible (Martinez-Lazcano et al., 2013; Thomson, 2019) and could be supported by an animal experimental evidence (Li et al., 2019). For instance, inhalation of ambient O_3 could strengthen the release of some hormones (i.e., glucocorticoids and stress hormones) by activating the hypothalamic-pituitary-adrenal (HPA) axis (Henriquez et al., 2019), which was recognized as a pivotal mechanism in the development of mental disorders (Thomson, 2019).

Few prior studies investigated the C-R curve between O_3 exposure and depression. In this study, our analysis highlighted a nonlinear C-R relationship between long-term exposure to O_3 and depression scores. We observed significant increases of depression scores only at high exposure concentrations (approximately ≥90 μg/m^3), suggesting a potential threshold in O_3-related risk of depression. A recent repeated measurement survey (Shi et al., 2022) had reported similar effect of mid-term O_3 exposure on depression scores among 3445 middle-aged and older people in China. This association also could be interpreted by previous studies of neural mechanisms (Martinez-Lazcano et al., 2013; Thomson, 2019). At low levels of O_3 exposure, given the short half-life, it is difficult for O_3 to accumulate in sufficient concentrations to enter the brain to affect the nervous system (Martinez-Lazcano et al., 2013). Despite non-significant evidence ($P = 0.06$) for nonlinear C-R association with depressive symptom, we detected a similar pattern with steeper slopes at high O_3 concentrations. Since depressive symptom was defined by depression scores (depression score ≥10) rather than clinical diagnoses, there may exist possible misclassifications in depression, which would have an inevitable impact on the analysis of O_3-depression relationship (Shi et al., 2022).

Most existing epidemiological studies failed to investigate effect modifications by demographic and behavioral characteristics when assessing relationships of depression with long-term O_3 exposure (Bakolis et al., 2021; Kioumourtzoglou et al., 2017; Pelgrims et al., 2021; Zhao et al., 2020). When performing subgroup analyses stratified by demographic and behavioral characteristics, we did not observe significant differences except for the covariate of region. Comparing with participants living in North, a significantly stronger association of O_3 with depression was observed among individuals in Midwest. This difference may due to the smaller sample size in the north and relatively higher O_3 concentration in Midwest. Similar findings were also reported in a quasi-experimental investigation on the relationship between long-term $PM_{2.5}$ exposure and depression based on CHALRS 2011–2015 (Xue et al., 2021). Meanwhile, there is also some suggestive evidence on population vulnerability in risk of depressive disorders related to mid-term (Shi et al., 2022) or short-term O_3 exposures (Lu et al., 2020). In a multi-community longitudinal investigation spanning 11 Chinese provinces (Shi et al., 2022), older adults aged 65+ years were found to

be at greater risk of depressive disorders associated with mid-term O_3 exposure; In a case-crossover analysis in 13 Chinese cities using records of 111,842 hospital outpatient visits for mental disorders, women exhibited higher vulnerability to short-term O_3-related depression (Lu et al., 2020). Given the wide lack of available population-based longitudinal evidence, more assessments in future studies are warranted to provide a better understanding of susceptible subgroups to depression related to O_3 exposure.

In the stratified analysis, we observed significantly higher risk of depression associated with long-term O_3 exposure in participants with preexisting chronic diseases. This finding was echoed with a time-stratified case-crossover study in Korea, reporting increased air pollution-associated risk of emergency department visits for depressive episode in patients with cardiovascular disease, diabetes mellitus, or asthma (Cho et al., 2014). Although the underlying mechanisms may be difficult to elucidate, a potential hypothesis has been suggested that individuals with some chronic diseases (e.g., diabetes mellitus, asthma) could be more vulnerable to O_3 related depression arising from the influence of O_3 on the psycho-endocrine-immune system through an inflammatory process (Cho et al., 2014; Patterson, 2011). Notably, the modification effects showed inconsistent direction when considering the specific classification of chronic diseases (Lim et al., 2012). Although significantly higher percentage increase in depression score associated with O_3 exposure was observed among asthma participants in our analysis, these results should be interpreted cautiously due to the limited sample size ($n = 553$) of participants prevalent with asthma. Consequently, more large-scale cohort investigations should be conducted to further explore the interactive effects of air pollution and various chronic diseases on depression.

Our study had several limitations. First, we estimated O_3 exposure for individuals at the city level due to limited access to residential address, which may lead to exposure misclassification by ignoring within-city variations in O_3 concentrations (Wu et al., 2019). Although this ignorance would not change direction of the associations, it may introduce bias into point-estimate of the association. Second, since the obvious distinction for O_3 concentrations between ambient environment and household, overestimation of individual O_3 exposure may be caused by unconsidered indoor O_3 exposure (Hu et al., 2020). Third, due to data unavailability to specific survey date for each participant, we assessed annual average O_3 exposure for each participant using monthly O_3 mean concentrations aggregated from daily estimates. This might induce the measurement bias on exposure to some extent. Fourth, unmeasured potential confounders (e.g., drug abuse and history of mental disorders) may still bias O_3-depression relationship, even though we had considered a rich set of covariates in our main analysis. In addition, the findings in our study may not be generalizable to younger population, as we only included middle-aged and older adults who had a higher prevalence of depression (Lu et al., 2021).

5. Conclusions

In summary, this study provided nationwide longitudinal evidence for positive associations between long-term O_3 exposure and depression among Chinese middle-aged and older adults. Our stratified analysis suggested higher susceptibility to O_3-related risk of depression among participants suffering from chronic diseases. These findings highlighted the significance of strengthened clean air action to reduce the global and regional burden of depressive disorders in the context of population ageing, particularly in low- and middle-income countries experiencing high-level air pollution.

Author contributions

Yang Yuan: Writing - original draft, Writing - review & editing, Methodology, Formal analysis; **Kai Wang**: Writing - review & editing, Validation, Visualization; **Zhen Wang**: Writing - review & editing, Supervision; **Hao Zheng**: Resources, Data Curation; **Zongwei Ma**: Resources, Data Curation; **Riyang Liu**: Resources, Data Curation; **Kejia Hu**: Software, Visualization; **Zhiming Yang**: Software, Visualization; **Yunquan Zhang**: Writing - review & editing, Supervision, Funding acquisition. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114185.

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A paradigm shift in cooperation between industry, legislation, and research to protect people and the environment

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1. Crossing the Rubycon

Modern human society is organized in complex ways. Today there are hardly any areas that are not somehow connected to the international trade network. This means that technologies and products manufactured anywhere can reach virtually any other point on the planet to be processed, used, or consumed. For many decades, the focus clearly was on the functionality of a product. Little attention was paid to whether it contained hazardous chemicals, whether they were released, how the product was manufactured, and how the product could be disposed of in an environmentally friendly manner. This attitude only changed when reports about environmental contamination and diseases became common. Within a short period of time, environmental legislation was enforced and the idea of recycling emerged. Suddenly, industry was confronted with various requirements, limit values, and laws, which often led to controversial and sometimes bitter discussions with authorities and environmental organizations.

The distrust was mutual. Authorities accused industry of accepting environmental damage for profit reasons, while industry in turn complained about unnecessarily high hurdles that limited or prohibited the sale of certain products, but hardly helped the environment. Unfortunately, both sides were often right. There were and are individual companies and entire branches of industry that put their economic interests ahead of ecological considerations and consciously try to undermine laws or use legal loopholes to their advantage. *Dieselgate*, with the illegal manipulations of various car manufacturers, made this very clear to us. However, there are also people in public authorities who demand or enforce disproportionately high requirements for political reasons.

Over time, however, the various interest groups have learned that it makes little sense to work against each other. As a rule, only the manufacturer of a product knows its exact composition and properties. Unilateral bans on certain components lead to substitutes, which in turn are also banned and replaced. This concept ends in a tortoise and hare contest that is unsatisfactory for all sides. In addition, the requirements and demands of consumers have grown with increasing environmental awareness in the population. It was therefore advisable for companies to establish voluntary minimum standards for their products, which required extensive investigations even before they were launched on the market. This intensified cooperation with research institutions, as small and medium-sized companies rarely have the appropriate capacities, and large companies often lack the expertise. Authorities fall back on research institutes for the investigation and evaluation of product properties, which should ultimately end in environmental recommendations or regulations. However, it is also often the results of research that trigger further measures. By far the best known example is formaldehyde – interestingly, the first publication on the release of formaldehyde from wood-based materials came from industry (Salthammer et al., 2010). Certainly, no industrial chemical has been more intensively examined and regulated with regard to its release to the indoor

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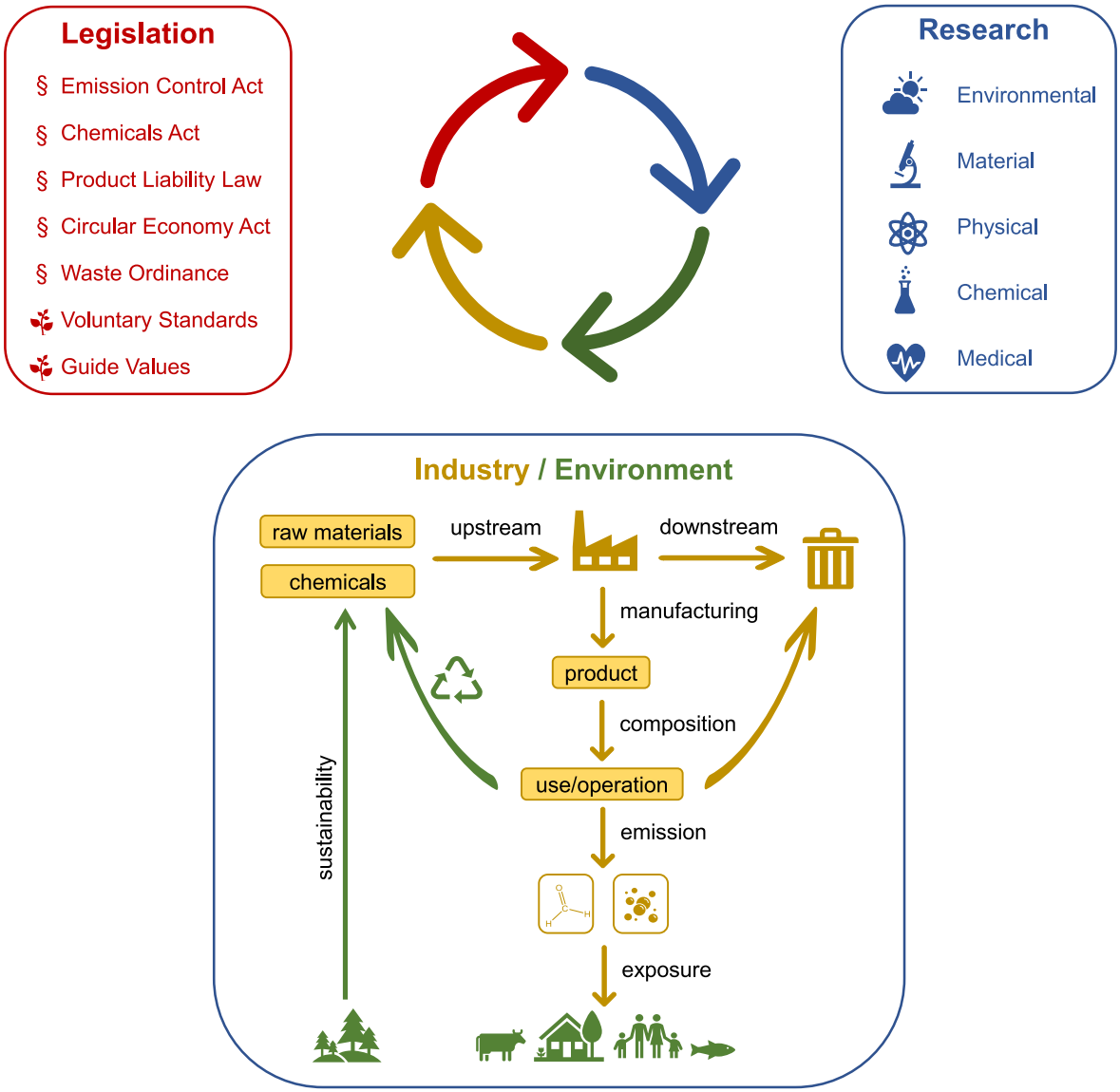

Fig. 1. A product available on the market today has to meet a number of requirements, which usually go beyond legal regulations. The product and production process should be sustainable and have no negative impact on the environment. Recycling has priority over landfill. These complex issues require interdisciplinary research and interplay with stakeholders.

environment. Nevertheless, the call for even lower guide values is heard again and again. Apart from the question of whether this makes sense for health reasons, there is a risk of losing sight of hazards from other substances.

2. The advantages of synergy

The current international regulations require a number of criteria to be observed when manufacturing and distributing products, which affect not only the manufacturer, but also the supply chains (Luthra et al., 2016). This means that the entire framework of the product design must be planned in advance with the help of internal and external expertise. The complicated, nested relationships and requirements are shown in Fig. 1 and demonstrate the necessary interaction between legislation, industry, and research.

Several interest groups have successfully set up joint ventures. For example, the German Federal Ministry for the Environment and the German Chemical Industry Association (VCI) started a cooperation to promote human biomonitoring in 2010. The main goal is to improve knowledge of substances to which the population may be increasingly exposed or which may be of particular relevance to health (Kolossa-Gehring et al., 2017). A very positive outcome of this joint venture was the market launch of the phthalate-free plasticizer DINCH, which was carried out with great transparency and with all the necessary ecological and toxicological data being available. A reliable biomonitoring method was quickly developed so that the internal exposure of the population could be monitored promptly (Kasper-Sonnenberg et al., 2019). This and other initiatives led to a very open dialogue about critical chemicals and possible substitutes (Apel et al., 2017; Salthammer, 2020).

Generally, manufacturing processes and product composition are more transparent today. In the past, the exact chemical recipe of products was often a holy grail whose secret the industry was reluctant to reveal. This has changed for the benefit of a better evaluation of products. For example, the chemical processes taking place in a candle flame could only be understood with information from the manufacturer about the candle ingredients (Salthammer et al., 2021). Such transparency is already being increasingly implemented in the food sector. The “trusted science for safe food” initiative of the European Food Safety Authority (EFSA) requests the full access to study data (EFSA - European Food Safety Authority, 2020) and this approach can in principle be

transferred to other sectors.

The establishment of emission standards for laser printers and copiers can also be seen as a success story. After we characterized the chemical and physical properties of the particles emitted (Morawska et al., 2009), criteria for environmentally friendly devices were quickly developed in research projects that were funded both by the government and by industry (Gu et al., 2020). It is noteworthy that this also changed the previously incorrect public perception that particle emissions from operating printers simply consist of small components of the toner. In fact, the mechanisms of particle formation in the printer are of very complex nature. All in all, this is an excellent example of the working triangle between research, governmental authorities, and industry (Morawska et al., 2019). However, there are also examples in which communication is significantly more difficult. Thus, it has taken an unusually long time to regulate the indoor use of tobacco and nicotine products, despite the social acceptability of protective measures.

Even lessons from wrong decisions or actions are helpful to show that it is beneficial to consider the expertise of others. In 2019, contrary to the advice of many academic and industrial scientists, the European Union classified titanium dioxide in certain forms as carcinogenic. Since then, some products have had to carry a warning label. This classification was declared void by the Court of Justice of the European Union (2022). The judges argued that the European Chemicals Agency (ECHA) had not considered all scientific aspects and therefore reached an implausible conclusion (note that France has appealed the court decision). All in all, this controversial discussion about an important raw material is as unnecessary as it is lengthy and expensive.

Probably the most glaring example of a lack of cooperation in recent times was seen during the SARS-CoV-2 pandemic. It took far too long and needed massive power of persuasion for the scientists to convince the policy makers that the virus is airborne (Morawska and Cao, 2020; Morawska and Milton, 2020). As a result, it became clear how important cooperation between different disciplines and stakeholders is. The protection of the population required an intensive exchange of knowledge. Without the expertise of the industry, the provision of air-cleaning measures (Uhde et al., 2022) would not have been possible. At the same time, however, it had to be ensured that only effective and sensible concepts reached the market and those that are ineffective or potentially dangerous, stopped. The pandemic has also relentlessly shown us the many possibilities for abuse and fraud.

3. Examples of action plans in Europe and Australia

A wide range of activities and concepts exist at different levels to improve communication between interest groups, especially with regard to the climate crisis. In 2019, the European Commission presented the “Green Deal”, which includes a commitment to achieve climate neutrality by 2050. Key points are making transport sustainable, a clean energy system, refurbishing buildings, protecting our planet and our health, and particularly transforming our economy and society. A new way is needed for European industry to take the lead in times of rapid environmental and technological change. In line with the entrepreneurial spirit of this strategy, the EU institutions, Member States, regions, industry and all other relevant stakeholders should work together (European Commission, 2020).

The Australian Life Cycle Assessment Society (ALCAS) has been in existence since 2001. It was established to promote life cycle practices and sustainable development to coordinate the rapidly growing professional community in Australia. ALCAS has members from industry, government, academia and service organizations. Membership of individuals interested in the practice, use, development, education, interpretation and advocacy of life cycle based approaches is also welcomed (ALCAS, 2023).

ALCAS and its New Zealand sister organization LCANZ developed the Environmental Product Declaration (EPD) to communicate transparent and comparable data and other relevant environmental information about the environmental impact of a product throughout its life cycle. EPD has developed into an international system. A wide range of product categories are registered by companies in many countries.

4. Our future is in collaboration

All these arguments and examples show that the days when organizations worked independently on ecological or health related criteria for products should be in the past. The knowledge of the industry is just as necessary as that of the academic scientists and the authorities. Cooperation between industry, legislation, and research is already a reality in many committees dealing with product requirements. International cooperation to overcome national interests is also important. This is the only way to create globally accepted measures and remove unnecessary trade barriers.

The trail is being followed, but the finish line is still far away, although most of the protagonists have seen the benefits of synergy. Nevertheless, the process of rethinking is happening far too slowly and time is running out. This text is therefore an appeal to stakeholders to build mutual trust and accelerate the paradigm shift, so that the concept of sustainability quickly prevails in all types of industrial products. The current experience of the SARS-CoV-2 pandemic has highlighted the importance of trusting cooperation in combating and overcoming problems. Future generations will thank us.

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Application of human biomonitoring data to support policy development, raise awareness and environmental public health protection among countries within the HBM4EU project

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ABSTRACT

Most countries have acknowledged the importance of assessing and quantifying their population's internal exposure from chemicals in air, water, soil, food and other consumer products due to the potential health and economic impact. Human biomonitoring (HBM) is a valuable tool which can be used to quantify such exposures and effects. Results from HBM studies can also contribute to improving public health by providing evidence of individuals' internal chemical exposure as well as data to understand the burden of disease and associated costs thereby stimulating the development and implementation of evidence-based policy.

To have a holistic view on HBM data utilisation, a multi-case research approach was used to explore the use of HBM data to support national chemical regulations, protect public health and raise awareness among countries participating in the HBM4EU project. The Human Biomonitoring for Europe (HBM4EU) Initiative (https://www.hbm4eu.eu/) is a collaborative effort involving 30 countries, the European Environment Agency (EEA) and the European Commission (contracting authority) to harmonise procedures across Europe and advance research into the understanding of the health impacts of environmental chemical exposure. One of the aims of the project was to use HBM data to support evidence based chemical policy and make this information timely and directly available for policy makers and all partners. The main data source for this article was the narratives collected from 27 countries within the HBM4EU project.

The countries (self-selection) were grouped into 3 categories in terms of HBM data usage either for public awareness, policy support or for the establishment HBM programme. Narratives were analysed/summarised using guidelines and templates that focused on ministries involved in or advocating for HBM; steps required to engage policy makers; barriers, drivers and opportunities in developing a HBM programme.

The narratives reported the use of HBM data either for raising awareness or addressing environmental/public health issues and policy development. The ministries of Health and Environment were reported to be the most prominent entities advocating for HBM, the involvement of several authorities/institutions in the national hubs was also cited to create an avenue to interact, discuss and gain the attention of policy makers. Participating in European projects and the general population interest in HBM studies were seen as drivers and opportunities in developing HBM programmes. A key barrier that was cited by countries for establishing and sustaining national HBM programmes was funding which is mainly due to the high costs associated with the collection and chemical analysis of human samples. Although challenges and barriers still exist, most countries within Europe were already conversant with the benefits and opportunities of HBM.

This article offers important insights into factors associated with the utilisation of HBM data for policy support and public awareness.

1. Introduction

Human biomonitoring (HBM) is a valuable tool used to quantify exposures and effects by measuring levels of chemicals and/or their metabolites in biological matrices such as blood, urine, exhaled breath, breast milk, hair, fingernails and teeth (Sexton and Pirkle, 2004; Angerer J et al., 2006; Choi J et al., 2015). HBM studies are always combined with questionnaires to evaluate exposure risk factors and sources such as food, consumer products and the environment. Additionally, markers of effect can be incorporated to facilitate our understanding of the health impacts of these exposures. The applications of the information gained from HBM are broad and include trend analysis (geographical and temporal) in the exposure or health status of a population, identification of emerging threats, undertaking chemical risk assessment, chemical incident and disaster response, addressing policy needs/development and raising awareness.

Results from HBM studies can contribute to improve public health both directly and indirectly by providing evidence of individuals' exposure and data to understand the burden of disease and costs associated with chemicals in the environment (Knudsen and Merlo, 2011a, 2011b), thereby galvanising the development and implementation of evidence-based policy. The value of national HBM programmes has been recognised in many countries across the world for decades (Choi et al., 2015). As a result, some countries have developed regional or national standalone HBM programmes (without the inclusion of health surveys or other studies) while others have sought to integrate/combine HBM with existing Health Examination Surveys (HESs)/diet/nutrition surveys or engage in targeted, specific problem oriented HBM studies. A network of European countries has been working since 2003 to progress the development and implementation of national HBM programmes as part of fulfilling the European Environmental Health Initiatives (Commission, 2020). This has culminated in the current European Human Biomonitoring project which started in 2017 and is a collaborative effort involving 30 countries, the European Environment Agency (EEA) and the European Commission (contracting authority) (HBM4EU, 2022). The European Human Biomonitoring Project (known as HBM4EU) is a multinational effort to harmonise procedures across Europe and advance research into the understanding of the health impacts of environmental chemical exposure. One of the aims of HBM4EU was to assist partner countries to develop and/or strengthen their networks of government departments/agencies/ministries, and stakeholders (from herein these groups are referred to as National Hubs (NHs). Each NH nominated a National Hub Contact Point (NHCP) that acted as the link between the project and the national institutions. The National Hubs constitution was not prescribed by HBM4EU as the national activities were not part of the project activities in HBM4EU. Thus, each country developed a NH to suit their requirements and expertise. For instance, NHs could contain all the partners who were taking part in HBM4EU plus others – such as other academic institutes, national funding bodies, government departments and other stakeholders like the industry and NGOs (Fig. 1).

1.1. HBM, policy and public health

The use of HBM to raise awareness of public health issues, steer policy development and protect population health is evident on a global level. Numerous studies directed to specific chemicals (e.g. lead, polychlorinated biphenyls (PCBs), dioxins) and geographical hotspot investigations (e.g., arsenic in drinking water, cadmium around industrial sources) have applied HBM methods to investigate excess exposures, often leading to targeted policy interventions. Also, in occupational settings, HBM has proven valuable to protect workers' health in many studies. The value of national HBM programmes is demonstrated by the National Health and Nutrition Examination Survey (NHANES) in the USA where a decline of blood lead level was detected (using HBM) following a ban on lead in gasoline (petrol) and its removal from soldered cans for food (Pirkle et al., 1994). The German Environmental

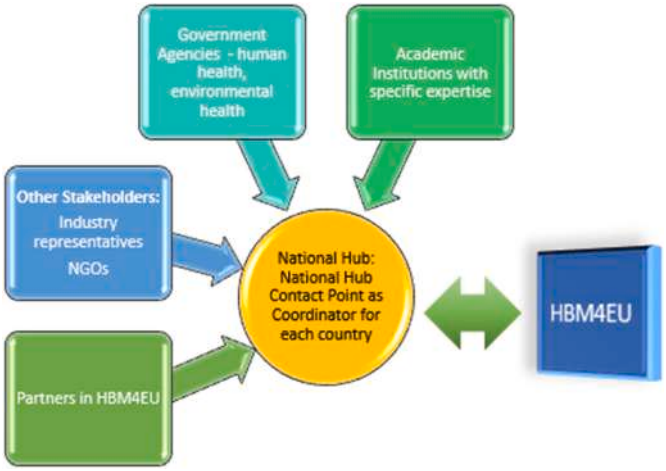

Fig. 1. Relationship between NHs, NHCPs and HBM4EU.

Survey (GerES) and the German Environmental Specimen Bank (ESB) were instrumental in initiating the avoidance of amalgam teeth fillings containing mercury in children (Kolossa-Gehring et al., 2012). Additionally, it contributed to the arguments on restriction of phthalate use in plastics (Göen T et al., 2011). In Belgium, information from the Flemish Environmental and Health Survey (FLEHS) informed the development of policy measures for persistent organic pollutants (POPs); including source-related regulation such as optimising and tightening existing Flemish legislation on open fires (Reynders et al., 2017). Biomonitoring data from the Canadian Health Measures Survey (CHMS) have been used to establish baseline concentrations of chemicals in Canadians; inform public health, regulatory risk assessment and management decisions; and fulfil national and international reporting requirements (Haines DA et al., 2017).

Action 3 of the European Environment and Health Action Plan 2004–2010 required the development of a coherent approach to biomonitoring in Europe (European Commission, 2004). Hence in 2005, the Expert Team to Support Biomonitoring in Europe (ESBIO) project supported the Implementation Group on HBM (IG-HBM) in commencing the preparation of protocols for the selection of study populations and priority chemicals. In 2009, this work was continued by the Consortium to Perform Human Biomonitoring on a European Scale (COPHES, 2009–2012) resulting in the finalisation of the protocols, selection of appropriate biomarkers and recruitment strategy. Demonstration of a study to Coordinate and Perform Human Biomonitoring on a European Scale', LIFE+, 2010–2012 (DEMOCOPHES), then tested the feasibility of a harmonised HBM approach in Europe, by successfully applying the COPHES approach in 17 European countries (Joas et al., 2012). Den Hond et al. (Den Hond E et al., 2015) provide a summary of these data for the exposure to selected chemicals, such as cadmium and phthalate concentrations in urine which are contained in consumer products and food packaging (Koch and Calafat, 2009), and mercury in hair (Grandjean and Landrigan, 2006) in mother-child pairs in those 17 countries (Wittassek and Angerer J, 2011; Budtz-Jørgensen et al., 2004; Akerstrom et al., 2013).

This work continued with HBM4EU, one of the major objectives being to create a harmonised HBM platform leading to consistent comparable and quality assured data throughout Europe. Another objective of HBM4EU is to use HBM data to support evidence based chemical policy and make this information timely and directly available for policy makers and all partners. Furthermore, the programme aims to ensure the sustainability of HBM and support capacity building through the development of a strong NHs network.

This article will assess national narratives provided by the NHCPs in countries within HBM4EU by highlighting good practice and learning gained from partner countries in the application of HBM for chemical risk assessment, policy issues and raising awareness.

2. Methodology

2.1. Research design

A multi-case research approach (a study on multiple cases to understand differences and similarities) was used to explore the use of HBM data to support national chemical regulations, protect public health, raise awareness and/or develop policy among those countries participating in HBM4EU.

Information was collated from 27 countries participating in HBM4EU (three countries did not participate due to issues primarily related to resources and time constraints). At the preliminary stages of HBM4EU the disparity with regards to the use and implementation of HBM nationally was highlighted, documented and provided the platform for this multi-case study. Hence this study has grouped countries according to the establishment of HBM programmes nationally/regionally/locally and/or the status of HBM in the country. The contributions are narratives therefore there is no formal quality control - the information has been referenced as far as possible.

The steps involved in collating and analysing the information are outlined below.

Step 1: Group Categorisation

Participating countries (see Table 1) were asked to self-select their best fit into one of the 3 categories/groups below:

- *Group 1: Countries that have not used HBM data for policy development but through HBM4EU or other initiatives/projects it has contributed to raising awareness of/addressing environmental or specific public health issues.*
- *Group 2: Countries with smaller or recently formed national hubs that have used HBM data for policy development but do not have established national or regional programmes.*
- *Group 3: Countries that have well established HBM programmes and use the data to address policy needs.*

Step 2: Data collection

Following countries' self-selection to the group most suited to their activities/status, the NHCPs (within each group) nominated a group leader to collect and summarise their respective narratives.

Guidelines and templates were developed for each group to ensure standardised/harmonised collection of the data to validate the national situation and the reason for self-selecting the group (see Appendix 1-6).

The template comprised open ended questions such as stating the

Table 1
List of the countries in the 3 groups.

Group 1	Group 2	Group 3
• Denmark	• Croatia	• Austria
• Estonia	• Cyprus	• Belgium
• Finland	• Israel	• Czech Republic
• Hungary	• North Macedonia	• France
• Iceland	• Slovakia	• Germany
• Italy	• Spain	• Sweden
• Lithuania		
• Luxemburg		
• Netherlands		
• Norway		
• Poland		
• Portugal		
• Slovenia		
• Switzerland		
• United Kingdom		

background information on the evolution and status of HBM, describing issues resulting in awareness/policy development, stating ministries, stakeholders or policy makers involved in HBM, describing barriers, challenges, opportunities regarding HBM, describing other players who would be beneficial in promoting HBM and future plans for HBM. Group leaders were also provided with a template/guideline to collate relevant data from the countries in their groups.

Step 3: Data Analysis and Scenario Development:

The first phase included analysis of the use of HBM at a national, regional or local level. In the second phase all examples of HBM use within the respective countries in a group were collated. Further summaries included areas such as ministries involved or advocating for HBM; steps required to engage policy makers; barriers, drivers and opportunities. In the final phase, a cross-case analysis was undertaken. The 3 groups were cross analysed to attempt to identify good practice and transferable lessons learned and make appropriate recommendations.

These narrative from the 3 groups were created to have an in-depth understanding of ministries/departments steering HBM and how to get/sustain the attention of policy makers. A comparison of these cases explores common themes that transcends each individual case and provides the benefit of understanding how countries can progress from using HBM data for public awareness to actual policy development.

3. Results & discussion

This section provides an overview of the information collected from the NHCPs by the group leaders. Where appropriate, the responses have been summarised in tables to show detailed information with additional findings specific to each group presented immediately below (the table). The responses affiliated with all 3 groups have also been presented in sections and text boxes (see case study boxes 1-3) that address factors associated with the utilisation of HBM data, ministries and stakeholders advocating for HBM, steps used in engaging policy makers, barriers, drivers and opportunities involved in the establishment of HBM programmes, potential groups for the promotion of HBM and the future of HBM in European countries.

The countries' self-selection into one of the 3 groups (refer to Table 1) is subjective and therefore it was noted that there are some countries which could have been placed in an alternative group. For example, Austria, which has a well-established NH and is on the verge of establishing a HBM programme, self-selected to Group 3 (well established countries with HBM programmes) rather than Group 2 and Spain, that has established national HBM studies since 2007 but recently included HBM in the Spanish Strategic Action Plan for Health and Environment could have been placed in Group 3 but is seen in Group 2. This limitation was accepted to facilitate categorisation of the groups and with the understanding that the concerned countries will have shared or similar factors.

The reported status of HBM studies/programmes/data within the countries in the 3 groups are shown in Table 1. The focus of this paper is general population-based studies, however, there are many countries which have used HBM in occupational settings hence it will be briefly discussed.

3.1. Group 1: countries that have not used HBM data for policy development but through HBM4EU or other initiatives/projects it has contributed to raising awareness of/addressing environmental or specific public health issues

In this group, relevant points with regards to the utilisation of HBM data for awareness/addressing environmental or public health issues and the establishment of a HBM programme are reported and discussed. The points addressed by countries in Group 1 include background information of HBM, the extent to which HBM data have been used (a maximum of 3 examples per country is presented) and steps used in engaging policy makers.

3.1.1. Background information of HBM and the utilisation of HBM data for awareness/addressing environmental or specific public health issues

The 15 countries in Group 1 (Table 1) reported the use of HBM data either for raising awareness or addressing environmental and public health issues dating back to more than 30 years in some cases. In

Box 1
A case study of dioxins and PCBs in Finland

The benefits of fish consumption which may be counteracted by concomitant exposure to environmental contaminants (in fish) such as dioxins and PCBs prompted the Fishermen Study and the Health 2000 Survey on fish consumption and cardiovascular health in Finland.

The professional Baltic Sea area fishermen and their family members were used to represent a population with high fish consumption (Fishermen Study) and the Health 2000 Survey participants represented the general population of Finland consuming average amounts of fish.

The work investigated:

- The ability of fish consumption biomarkers (fish derived omega-3 PUFAs and environmental contaminants) and other questions to reflect fish consumption.
- The association of fish consumption with the consumption of other foods.
- The associations of fish consumption and fish-derived serum omega-3 PUFAs and environmental contaminants with cardiovascular risk factors.
- Mortality in a population with high fish consumption and presumably high exposure to environmental contaminants.

Results showed that blood concentrations of fish-derived omega-3 PUFAs, dioxins, PCBs, functioned well as biomarkers of fish consumption, fish consumption was positively associated with eating healthy foods both among the professional fishermen and their wives and in the general population. A follow-up study from 1980 to 2005 also showed mortality from many natural causes in epidemiological studies regarding fish consumption and cardiovascular health ischaemic heart disease, was lower among the fishermen and their wives than in the general population of Finland.

In conclusion, the work added to the current advisories that the benefits of salt-water fish consumption and omega-3 PUFA intake outweigh the potential hazardous effects of fish-derived environmental contaminants even at higher levels of exposure (Epidemiological studies on fish consumption, 2000).

addition, 11 countries in this group (Denmark, Hungary, Italy, Luxembourg, The Netherlands, Norway, Poland, Portugal, Slovenia, Switzerland, UK) have participated in at least one of the previous European HBM projects (ESBIO, COPHES and DEMOCOPHES). Ministries/departments/agencies steering HBM were also highlighted of which, the Ministry of Health was the most common (Appendix 7).

Except for Estonia, all countries in Group 1 have participated in one or more of the following fieldwork activities in HBM4EU: collection of new samples from the public, use of bio-banked samples for new analyses and collection of samples from occupational settings (Appendix 1). Many countries reported having a structure that will support chemical management and policy development although they currently have no national or regional HBM programme. The use of HBM and issues which have resulted in the raising of HBM awareness or supported public health interventions in the countries within this group are summarised in Table 2.

3.1.2. Status of the establishment of HBM programme or implementation of an HBM module into Health Examination Surveys

As earlier mentioned, no national HBM programme exists within the countries in Group 1. However, Finland, Luxembourg and the United Kingdom have reported the inclusion of a subsidiary HBM module in Health Examination Surveys (HES) or the development of initiatives to align with HES as part of research activities. Other countries reported the utilisation of HBM studies in other health programmes e.g. the Estonian population health strategy (RAHVASTIKU TERVISE ARENGUKAVA 2020, 2020).

In the UK, a HBM module has been implemented in the Health Survey for England (HSfE) which is undertaken annually since 1991 and monitors trends in the population's health and care (Health Survey for England, 2021). HSfE recruits approximately 10,000 participants (8000 adults over 16years and 2000 children aged 0 to 15) annually and a subset (300 participants, aged 16–49years) of the group will be included in the HBM module.

The Netherlands had a Surveillance Programme titled, "Man, Food and Environment", that terminated in the late 20th century (Fiolet et al., 1998; Zeilmaker et al., 2003). In this programme, pesticides, PCBs and dioxins were measured periodically in mother's milk. HBM is now mainly applied in studies focused on hotspots, occupational settings, targeted research, or determinants of health/chronic or infectious diseases and an element in emergency response to chemical incidents (Behbod et al., 2017; Scheepers et al., 2011).

Norway has established a governmental Human Environmental Biomonitoring programme that utilises the Norwegian Mother, Father and Child Cohort Study (MoBa) as a basis for recruitment. MoBa is an ongoing prospective pregnancy cohort which includes 114 500 children, 95 200 mothers and 75 200 fathers (Magnus et al., 2016).

In Lithuania, the Ministry of Health approved a 2-year (2020–2021) HBM program, during which the concentrations of metals/metalloids, PAHs, dioxins/furans, and PCBs were determined in a random selection of 225 Lithuanian residents and 116 firefighters. A new HBM program for 2023–2027, as a preventive tool, is on the government's agenda.

3.2. Group 2: countries with smaller or recently formed NHs that have used HBM data for policy development

This group of 6 countries (Israel, Spain, Slovakia, Croatia, North Macedonia and Cyprus) have smaller (few participants/stakeholders) or recently formed NHs and have used HBM on an *ad hoc* basis at the local or regional level with some having plans to advance to national HBM efforts (Table 3). Croatia, Spain, Slovakia and Cyprus have participated in at least one of the three previous EU HBM programmes (ESBIO, COPHES, DEMOCOPHES), however, with the exception of Israel, none have yet established formal national/regional initiatives. This may be on the horizon as Cyprus, Spain, Croatia, Slovakia have National Strategies or Plans (including drafts) which include HBM.

The narrative for this group follows a similar pattern as seen in Group 1 except the "*policy uses of HBM data*" section which states how HBM data have been used for both awareness and policy support.

3.2.1. Policy uses of HBM data

Several countries reported using HBM for raising awareness or intervention campaigns (refer to Table 4). For example, Cyprus and Israel reported using cotinine measurement in children to raise awareness about controlling exposure to environmental tobacco smoke in this vulnerable population. In the case of Cyprus, HBM efforts were followed by an intervention campaign and direct communication with policy makers. Whereas in Israel there was media coverage of the HBM data and results were reported to the Parliament and policy makers within the Ministry of Health. In both cases, there is an understanding that even with HBM data and media coverage, there is a need for further policy actions. In Israel, HBM data on nutritional biomarkers (iodine, selenium) collected within the national HBM program was reported to Ministry of Health policy makers and raised awareness regarding iodine deficiency in a country heavily reliant on desalinated drinking water. Also, in Israel, data from a small pilot study on PFAS in blood donors was communicated to stakeholders (energy sector, Ministry of Defense, National Fire Authority) as part of government efforts to reduce use of fluorinated compounds in firefighting foams.

In Croatia, HBM data (mercury in hair) was used in the "Stay Healthy, Stop Mercury" health campaign (Janev Holcer N, 2009). Twelve women participated in the study on mercury in hair; all had detectable levels, some above reference dose level of 1 mg/g which could be linked to fish consumption. As the effects of the campaign were short term, it is recognised that further HBM data and repeated campaigns are needed to impact population behaviors.

Another potential use of HBM data for policy support is developing dietary advice. In Spain, HBM data from a national study of 2000 adults and another on mother-child pairs showed hair mercury concentrations for a fraction of the population above the recommendations proposed by the WHO (Soler-Blasco et al., 2019). This data contributed to the development of dietary advice related to the fish consumption in vulnerable populations with accompanying media campaigns (Aesan.gob.es).

HBM data can be used as part of governmental crisis management. For example, HBM was used in Cyprus to evaluate exposure and potential health risk of citizens exposed to arsenic in drinking water. Toenail arsenic concentrations were measured in hotspot and control populations; although arsenic exposure was higher in the hotspot populations, levels were below health guidance values. These findings were communicated to concerned citizens by high level government officials, and thus were part of holistic assessment, and risk communication, during an environmental crisis.

Spain, Cyprus and Israel reported using HBM data in quantitative risk assessment. In Cyprus and Spain, HBM data on mercury in hair were part of the risk asessment approach incorporating both HBM and dietary data. The Ministry of Health in Israel collected HBM data on urinary concentrations of organophosphate metabolites in children, and found that those consuming more fruit had higher exposure to these pesticides. A portion of the children in the study had estimated pesticide intake above the acceptable daily intake (Berman et al., 2020). The Ministry of Agriculture decision to phase out chlorpyrifos use in edible crops cited this HBM data (see Box 2).

3.3. Group 3: countries that have well established HBM programmes and the use of the data to address policy needs

Countries in Group 3 (Austria, Belgium, Czech Republic, France, Germany, and Sweden) either have established HBM programmes or it is in progress. They have participated in at least one of the previous European HBM projects (ESBIO, COPHES, DEMOCOPHES). All countries have played major roles in HBM4EU and some have participated in one

Table 2
Use of HBM data for awareness and addressing public health issues in countries from Group 1.

Country	Issue/Concern	Population (local/ regional/ national)	Chemical	Ministry or Institute undertaking the study/other	Outcome	Reference
Denmark	Endocrine disruptors and especially child health	National and Local	Endocrine disrupting chemicals (EDCs) such as Bisphenol A, Phthalates, paracetamol	The Danish Environmental Protection Agency (EPA) by funding a center for EDC at the Region H called CEHOS	Provided data for EU regulations and setting standards for concentration of EDCs in toys, food etc	Busch et al. (2021)
	Traffic related exposures to pollutants	National	Polycyclic aromatic hydrocarbons (PAH), particles	EPA and communities	Several monitoring activities and actions taken by the EPA and the local communities	Strak et al. (2021)
	Indoor chemical exposures	National	Polychlorinated biphenyls (PCBs)	Ministries of Health and of Buildings	Guidelines for exposure limits with action levels for evacuation. Local initiatives such as demolition of especially polluted buildings (Brøndby Strand)	Frederiksen et al. (2020)
Estonia	Investigating the impact of oil shale sector on population health	Regional	Heavy metals including mercury; PAH, BTEX	Ministry of Education and Research, Health Board, Ministry of Environment	Results informed the development of the Oil Shale Strategy and highlighted increased Hg levels in fishermen from western Estonia which was not found in oil shale workers	Orru et al. (2020)
Finland	Presence of dioxins and PCBs in fish from the Baltic Sea at levels which exceeded EU limits	National	Dioxins, PCBs	Ministry of Social Affair and Health	Demonstrated that eating the Baltic Sea fish had more positive effects than the negative ones associated with the presence of the chemicals	A. (2012)
	As a result of EFSA 2018 dioxin risk assessment an investigation was undertaken to ascertain levels in vulnerable populations	National (children 7–10 years)	Dioxin, PCBs	Ministry of Social Affairs & Health	Low levels of chemical found encouraged the development of an ongoing government programme to promote eating domestic fish	Rantakokko et al. (2020)
Hungary	Presence of lead in the environment	Local populations	Lead	National Public Health Centre	Linking of lead exposure with neurodevelopment effects (2005). This was followed by a national monitoring campaign for lead in tap water (2018–2020).	Dura Gy et al. (2011)
	Red sludge disaster in 2010	Local populations	Heavy metals	National Public Health Centre	The settled and dried red sludge had no effect on the urinary levels of metals.	Rudnai P et al. (2011)
	Endocrine disruptors	Local populations	Phthalates and certain metals (mercury, cadmium)	National Public Health Centre	The urinary metabolite levels were lower than the corresponding guidance values.	KÖZÉPESY et al. (2017)
Iceland	Arctic monitoring assessment programme investigating the effect of POPs and metals on women of childbearing age and the developing foetus	National (Artic programme)	Persistent organic pollutants (POPs), metals	Ministry of Environment	Supporting ongoing assessment of arctic pollution. Time trends showed reduction in levels since 1995	no (2021)
Italy	Exposure to polyfluoroalkyl substances (PFAS) in an area affected by water contamination	Local	PFAS	Regione del Veneto, Italian National Institute for Health	Characterisation of human exposure, definition of the most affected areas, identification of the population groups at higher risk, definition of a regional health surveillance plan	Ingelido et al. (2018)
	Concerns of citizens about the possible health risks related to the presence of a new Waste-to-Energy Plant	Local	Metals, Hydroxylated polycyclic aromatic hydrocarbons (OHPAHs), Polychlorinated dibenzodioxins (PCDDs), Polychlorinated dibenzofurans (PCDFs), and PCBs	Financially supported by the Metropolitan Area of Turin Piemonte. Realised by Italian National Institute for Health, Department of Epidemiology ASL TO3, Department of Prevention ASL TO1, Department of Prevention ASL TO3, Department of Epidemiology and Environmental Health, Regional Environmental Protection Agency.	Verification of no impact of the WTE plant on human exposure to selected contaminants, support in the definition of the Health Surveillance Program, communication to citizens involved about WTE impact, raising in public awareness	Ingelido et al. (2020)
	Concerns about exposure to contaminants of residents in an area characterised by multiple sources of exposure	Local	Metals, OHPAHs, PCDDs, PCDFs, PCBs, PBDEs, PFAS, pesticides	Ministry of Health, ASL Taranto Dipartimento Prevenzione, Italian National Institute for Health	Characterisation of human exposure, identification of the population sub-groups at higher risk, support to remediation actions taken by local and national authorities	Pitter et al. (2020) Polesello et al. (2013) del Veneto (2016)

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Table 2 (*continued*)

Country	Issue/Concern	Population (local/ regional/ national)	Chemical	Ministry or Institute undertaking the study/other	Outcome	Reference
						Bena et al. (2016) Bena et al. (2021) Bocca et al. (2016) Iamiceli et al. (2020) Iamiceli et al. (2021) Ruggieri et al. (2019) Abate et al. (2016) Comba et al. (2012) Iavarone et al. (2012) De Felip et al. (2015) Ingelido et al. (2017)
Lithuania	Carcinogenicity of cadmium in women	Regional	Cadmium	Lithuanian University of Health Sciences	Assessment of causal association between cadmium exposure and risk of breast cancer (a case-control study)	(Strumylaite et al., 2011, 2014, 2019)
	Environmental exposure of children and adults to lead	Regional	Lead	Lithuanian University of Health Sciences	Lead concentration in children and adults with various exposure levels in Lithuania	Strumylaite et al. (2004)
The Netherlands	Concerns about the emission of Perfluorooctanoic acid (PFOA) from a factory producing Teflon	Local	PFOA	Province of South-Holland; Ministry of Infrastructure & the Environment; Ministry of Health, Welfare and Sports; Ministry of Economic Affairs & Climate Policy; Ministry of Social Affairs; Ministry of Defense	Verification of previous modelling results indicating high levels of exposure to surrounding population	(van Poll et al., 2017; Gebbink WA, 2020)
	Exposure to pesticides of residents in close proximity (<250m) to agricultural fields	Local	Pesticides (asulam, carbendazim (applied as thiophanate-methyl), chlorpropham, prochloraz and tebuconazole)	Dutch Ministry of Infrastructure and the Environment; Ministry of Economic Affairs & Climate Policy	Provided evidence that exposure to pesticides is not limited to the application period but may persist throughout the year	(Figueiredo DM et al., 2020; Oerlemans A et al., 2021)
	Disaster response in victims (1788 adults & 294 children) and relief workers from across the country and neighbouring countries (n = 2114) of the Firework Disaster Enschede (2000)	Local	Metals indicative for firework-related exposures: barium, cadmium, chromium, copper, nickel, lead, antimony, strontium, titanium, zinc.	National response team GGVE, including National Institute for Public Health and the Environment (RIVM) and municipality health service Enschede	Objective was to ascertain results of initial exposure models by HBM in samples collected 2–3 weeks post-disaster. No systematic increases of blood and urine levels, found in the residential group (including children) nor in the relief workers, thus confirming model results. Chance findings, unrelated to the disaster, identified 22 people with relatively high levels on either Cu, Pb, Sr, Ba or Ni that warranted clinical toxicological follow-up.	(Roorda et al., 2004; Enschede, 2001)
Norway	The exposure of anglers to flame retardants	Regional	Flame retardants	NIPH and Norwegian Food Safety Authority	Dietary recommendations were developed following a HBM programme	Thomsen et al. (2008)
	The use of PFAS in skiwax	National	PFAS	NIPH and STAMI (National Institute of Occupational Health)	Elevated levels of PFAS were found in both amateur and professional ski waxers	(Freberg et al., 2010)
Poland	Environmental exposure to metals	National/ Regional	Lead, cadmium, chromium, arsenic, mercury	Ministry of Education and Science; Local Government Organisations	Elevated levels of chemicals in biological material. Identification of the more vulnerable	Kozłowska et al. (2019)

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Table 2 (*continued*)

Country	Issue/Concern	Population (local/ regional/ national)	Chemical	Ministry or Institute undertaking the study/other	Outcome	Reference
					groups among the study population. Search for new biomarkers of exposure, effects and sensitivity	Peplońska et al. (2020) Baszuk et al. (2021) Garí et al. (2022) Kuras et al. (2017) Kuras et al. (2018) Kuras et al. (2019)
Portugal	Population living near a chlor-alkali plant, a solid waste incinerator or a uranium mine	Local	Heavy metals, dioxins and furans	University of Aveiro, Lisbon School of Medicine, Ministry of Health	Results showed that populations presented low levels of the studied chemical agents	(Reis et al., 2007, 2009; Marinho Falcão et al., 2005, 2007)
	Population exposure to plasticisers	Regional	Plasticisers (Di-2-ethylhexyl terephthalate (DEHTP) and DINCH)	Network of Chemistry and Technology (REQUIMTE)/Associated Laboratory for Green Chemistry (LAQV)	Age and processed food consumption were associated with DEHTP exposure. Although average DEHTP exposure was of no concern at the time, increasing exposures are likely. Children are exposed at approximately 5-times higher levels of DINCH than adults. Lower DINCH exposures were observed in children with nutritional guidance.	(Lessmann et al., 2017) (Correia-Sá et al., 2017)
	Population exposure to mycotoxins	Regional	Mycotoxins	National Institute of Health Dr. Ricardo Jorge (INSA)	Exposure assessment of Portuguese population to multiple mycotoxins was confirmed. It was the first time that Alternariol was detected in Europe. Exposure above safe limits for DON.	(Martins et al., 2019, 2020)
Slovenia	Exposure of general population to chemicals	National and regional	Metals/metalloids, PAHs, Glyphosate, Dioxins, PCBs, Polybrominated diphenyl ethers (PBDEs), Phthalates, Bisphenols, Parabens, Triclosan	Ministry of Health	Estimation of exposure levels, was used to identify the main predictors of exposure, and reference values for the selected Slovenian population established	Tratnik et al. (2019)
					The results provided the first insight into the PAH exposure of study group, potential sources of exposure and its spatial variability.	Joksić et al. (2022)
					Estimation of GLY and AMPA exposure in a Slovenian study population showed much lower levels than those in similar studies worldwide.	Stajnko et al. (2020)
					POPs were detected in maternal milk, plasma, and serum; legacy of pollution still visible on a national level. Geographic location of Slovenia might have protective function.	Runkel et al. (2021)
					Exposure of men and lactating women, children and adolescents in Slovenia to a wide range of different EDCs was evaluated connecting it to exposure patterns and exposure sources and susceptibility.	(Runkel et al., 2022; Stajnko et al., 2022; Tkalec et al., 2021)
Switzerland	Political initiation to ascertain the chemical burden in the Swiss population	National	Amongst others quicksilver, glyphosate and a collection of fluorinated substances	Federal Council	Developed the process for establishing a pilot "Swiss Health Study" under the umbrella of HBM4EU	FOPH (2020)

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Table 2 (*continued*)

Country	Issue/Concern	Population (local/ regional/ national)	Chemical	Ministry or Institute undertaking the study/other	Outcome	Reference
UK	Contamination of soil, surface water and wells due to natural bedrock of area and historic tin mining	Local	Arsenic and heavy metals	Health Protection Agency (now UKHSA), British Geological Survey, University of Manchester	Elevated levels of arsenic found in the drinking water samples resulted in the development of an HBM programme to assess population exposure	(Middleton DRS et al., 2017)
	The high infant mortality rate (double that for the England & Wales) within one of the most deprived cities (Bradford) in the UK	Local	Environmental exposures	University of Edinburgh	Identification of the more vulnerable groups among the study population	Wright J et al. (2013)
	Longitudinal study of mothers and babies (referred to as Birth Cohort study) in a county in England of higher SES.	Local	Application for arising environmental exposure research/investigation	University of Bristol, Medical Research Council and Welcome Trust	Continuing evolution of information about how genes, the environment and lifestyle of the participants impact on health. These findings are often applicable to the rest of the UK population	(Bristol)

or more of the following fieldwork activities in HBM4EU: collection of new samples from public, use of biobank samples for new analyses and/or collection of samples from occupational settings.

This section presents background information on the evolution and status of the national or regional HBM programmes, issues which underpinned the sustainability of the national or regional HBM programmes and examples of the use of HBM in policy making.

3.3.1. Background information on national or regional HBM programmes

HBM programmes in Europe have been established since the 1970s. The HBM programmes reported, started in collaboration with or as a component of other surveys e.g. HES before being fully established as a primary initiative even if embedded within another (programme).

The German Environment Agency (Umweltbundesamt -UBA) and the Federal Ministry for the Environment, Nature Conservation, Nuclear Safety and Consumer Protection (BMUV) are responsible for health-related environmental monitoring and human biomonitoring studies at the federal level in Germany. The main components were established in the early 1980s and continue to be used (Schulz et al., 2007). The various components (Kolossa-Gehring et al., 2012) complement each other thereby (a) enabling a comprehensive assessment of human exposure to chemicals, (b) providing indications of sources of exposure, (c) facilitating time trend analyses and (d) helping to monitor the effectiveness of regulatory measures (Kolossa-Gehring et al., 2012). The main components of the system are a) the cross-sectional population-representative German Environmental Surveys (GerES) which combines health and HBM surveillance as well as additional environmental monitoring to enable assessment of human exposure to chemicals, b) the Environmental Specimen Bank (ESB) to facilitate time-trend analyses, c) the co-operation between the BMUV and the German Chemical Industry Association (VCI) to identify specific and sensitive markers and develop new analytical methods for the monitoring of substances to which the general population might be exposed and/or which are of health relevance (Kolossa-Gehring et al., 2017), d) the provision of assessment tools by the German HBM Commission (Apel et al., 2017) and e) the cooperation in European and international HBM networks (Schindler et al., 2014; Nakayama et al., 2019).

The French HBM programme started as a component of the nutritional and health survey. It has therefore been embedded in a wider monitoring of the French population health, nutritional behavior and exposure. More targeted studies were designed in the context of workplace or accidents assessments. In France, the National Nutrition and Health Survey (ENNS) was conducted in 2006–2007 to meet the objectives on biomonitoring, chronic disease surveillance and nutritional surveillance. Thereafter, the Grenelle I Act (No, 2009–967 of August 3, 2009) led to the development of a more sustainable French National Biomonitoring programme, implemented by Santé publique France (SpF - the French National Health Public Agency), in which two distinct studies were designed: 1) Esteban: the Health Study on Environment, Biomonitoring, Physical Activity and Nutrition, a nationwide cross-sectional survey and 2) the ELFE birth cohort (Longitudinal Study from Childhood) (Balicco et al., 2017; Dereumeaux et al., 2016; Fillol et al., 2014; Fréry et al., 2012; Ougier et al., 2021).

In Sweden, the Swedish Environmental Protection Agency (SEPA) is responsible for the monitoring of the environment which has been ongoing since the late 1970s. The monitoring consists of ten programme areas which includes physical, biological and chemical agents. Chemicals are regularly monitored in several biotic and abiotic matrices. In the early 1991 the Swedish parliament decided that there should be a program that includes the connection between the environment and human health (se, 1990). The Institute for Environmental Medicine at the Karolinska Institute developed a structure for the program in 1992 and in 1993 the national programme for health-related environmental monitoring commenced with the inclusion of HBM (Institutet., , 1992). The purpose of the programme is to enable long-term monitoring of environmental factors that can affect population health and track

Table 3
Use of HBM data for policy purposes in countries from Group 2.

Country	Issue/Concern	Population (local/ regional/ national)	Chemical	Ministry or Institute undertaking the study/ other	Outcome	Reference
Spain	Dietary exposure to mercury in children and woman of childbearing age	National	Mercury	Spanish Ministry of Agriculture, Food and Environment	Development of dietary advice related to the fish consumption in vulnerable populations	(De Consumo De Pescado, 2022)
	National Implementation Plan for surveillance on persistent organic compound and other pollutants	National	PCBs, PBDE, OC-pesticides, PAH, PFAS Phtalates&DINCH Metals (mercury, Lead, Cadmium, Cobalt, Selenium, Thallium)	Ministry of Environment Instituto de Salud Carlos III	Monitoring and Reporting Stockholm convention National Implementation plan	(Castaño et al., 2015, 2019; Ramos et al., 2017; Bartolomé et al., 2015, 2017; Cutanda et al., 2015; Lopez-Herranz et al., 2016; Sánchez-Rodríguez et al., 2015)
Croatia	Dietary exposure to mercury in women (25–45 years)	Local	Mercury	Medical School, University of Zagreb Andrija Stampar School of Public Health Department of Environmental and Occupational Health	Development of dietary advice related to the fish consumption in women of childbearing age	Janev Holcer (2010)
	Differences in dietary mercury exposure between women and their newborn babies in coastal and continental region	National	Mercury	Croatian Institute of Public Health	Recognition of the need to implement further studies in areas where environmental pollution and higher exposure have been identified	Capak et al. (2017)
Cyprus	Pesticide exposure in children	Local	Pesticides	Cyprus International Institute for Environmental and Public Health & State General Laboratory/ Ministry of Health	Comment in EFSA public consultation	Makris KC et al. (2019)
	Exposure to arsenic in drinking water	Local	Arsenic	State General Laboratory/Ministry of Health	Risk assessment, communication with citizens	Makris et al. (2012)
	Dietary exposure to Mercury	National	Mercury	State General Laboratory/Ministry of Health	Risk assessment	KATSONOURI et al. (2019)
	Exposure of children to environmental tobacco smoke	Regional	Cotinine	State General Laboratory/Ministry of Health	Policy support	Katsonouri et al. (2009)
Israel	Organophosphate pesticide exposure in children	National (small scale)	Organophosphate pesticides	Ministry of Health	Phase out of chlorpyrifos	Berman T et al. (2020)
	PFAS exposure in general population	National (small scale pilot)	PFAS	Ministry of Health	Raising awareness, data presented in stakeholder meetings	

indicators of exposure by measuring pollutants in human matrices. SEPA and universities, research institutes and other agencies that have a structure and organisation for sample collection, biobanking and are included in a lab network for chemical analysis are included in the programme. Breast milk, blood or serum, urine and hair are monitored on a regular basis.

The Czech Republic national human biomonitoring programme is one of the 7 subsystems of the comprehensive Environmental Health Monitoring System implemented based on the Czech Government Resolution in 1991. The individual subsystems have been in routine operation since 1994. The program of human biomonitoring has been focused on monitoring the most serious substances to which the Czech population is exposed: heavy metals, persistent organic pollutants and emerging organic compounds - phthalates, per- and poly-fluorinated compounds (Batáriová et al., 2006; Černá et al., 2007, 2008, 2017, 2020; Cerna et al., 2015). The toxic substances and their metabolites have been monitored in blood, urine, hair and teeth of adults and children, and in milk of primiparas from selected regions of the Czech Republic. An overview of human biomonitoring activity is presented in Cerna et al. (1997), 2007, 2012 and 2017 (Černá et al., 1997, 2007, 2012, 2017). In addition, the CELSPAC infrastructure of population cohorts, with a biomonitoring element, was established in 2012. It consists of CELSPAC grown-up birth cohort (1991), The Next Generation (TNG) birth cohort (2016) and HAPIEE aging cohort (2002).

Presently, Belgium and Austria do not have national HBM programmes. The absence of a national HBM programme in Belgium is attributed to the division in regional and federal competences; however, the Flemish and Walloon regions have their own programmes and there is collaboration and exchange of knowledge between both regions. Since 2002 the Flemish Government decided to carry out the Flemish Environment and Health Survey (FLEHS), an extended (HBM) programme, which is integrated in the environmental health policy (https://www.milieu-en-gezondheid.be/). The FLEHS studies provided Flemish policy makers with a vast amount of data such as biomarkers of exposure and effect, exposure effect associations, time trends and geographical differences (Schoeters et al., 2012, 2017). The first Walloon biomonitoring program (BMH-WAL) is part of the Walloon environment-health plan adopted in December 2018 by the Walloon government which aims to study and limit environmental risks to human health. BMH-WAL aims to develop Exposure Reference Values of the Walloon population to substances present in the environment, food and products of daily life (P, 2020; Pirard et al., 2020; Jacques et al., 2021).

Table 4
Background information on the regional/national HBM programmes in the countries in Group 3.

Country	Sweden	Belgium	France	Germany	Austria	Czech Republic
Year of establishment of the HBM programme	*1993: Swedish HBM programme*	2002: FLEHS (I-IV) 2019: start BMH-WAL	2006: ENNS 2011: ELFE 2014: Esteban	1985: GerES (I – V) 1981: Environmen-tal Specimen Bank 2010: BMU - VCI Cooperation for further develop-ment of human biomonitoring	*2007: Austrian HBM platform*	1994: Czech HBM programme
Which entities finance/steer the programme?	*financed by the government, coordinated by the SEPA under the ministry of Environment*	FLEHS I-III: financed by dep. science, environment and health, coordinated by the Center on Environment and Health (CEH) BMH-WAL: financed by environment department, coordinated by ISSeP	financed by the ministry of health; coordinated by Santé Publique France.	financed by the ministry for the environment (BMUV), coordinated and scientifically led by UBA	*Studies mostly financed by the minister for the environment (currently named Federal Ministry for Climate Action, Environment, Energy, Mobility, Innovation, and Technology (BMK)); mostly coordinated by EAA*	financed by the ministry for health and ministry for education, youth and sports; coordinated by the National Institute of Public Health and Masaryk University.
HBM included in business/ strategic/ action plan?	(1) follow-up Environmental Quality objective for a non-toxic environment (2) identified chemical risks, reported to SamTox (national authorities working to prevent and manage chemical risks in society)	*instrument for policy making in the Flemish Decree on Preventive Health Care (since 2003).* *Policy use of HBM is mentioned in every yearly policy declaration of the Flemish Minister for environment* BMH-WAL is part of the Walloon environment-health plan (2018)	Strong recommendation for a French national HBM programme in Grenelle I Act (No, 2009–967 of August 3, 2009) HBM is part of the national environment and health plan PNSE 2 (2009–2013), 3 (2015–2019) and 4 (2021–2025)	*HBM is not included in a strategic plan, but data and their evaluation are asked for by ministries and also the parliament at national and Federal State level for policy decision making. Data are also fed into REACH processes, OECD activities and tasks under the Stockholm Convention*	Since 2016, the platform is the official advisory board of the minister for Environment on E&H issues 2017: resolution of the National Council: every 2 years a report *has to be published (BMK, 2019)*	Implemented on the basis of the Resolution of the Government of the Czech Republic No. 369/1991. *HMB-related activities are part of the National Portfolio of Actions implementing the Ostrava Declaration (2017)*
Which entities are involved	Steering group for the HBM programme	Steering committee of the FLEHS studies *BMH-WAL: steering committee*	*The ministry of health and Santé Publique France with the help of a college of experts from different institutions. Constituting the French national hub*	UBA, BMUV (Federal Ministry for the Environ-ment, Nature Conservation, Nuclear Safety and Consumer Protection), HBM Commission and linked third parties involved in HBM4EU	The national hub consists of the members of the Austrian HBM platform	National Institute of Public Health and Masaryk University, national hub

Box 2
A case study of organophosphate (OP) pesticides in Israel

Screening values have not been established for assessing biomarker concentrations of organophosphate (OP) pesticide metabolites. There are also few studies using HBM data on urinary OP concentrations to assess human health risk. In Israel, a study was undertaken to measure OP exposure in a sample of children (103) and explore associations between dietary patterns and OP exposure.

Demographic and dietary information together with urinary samples were collected. Creatinine and dialkyl phosphate (DAP) concentrations were measured in the samples. Urinary DAP concentrations were compared to international populations and its associations with fruit and vegetable consumption were analysed. Using urinary DAP concentrations, estimated daily intakes (EDI) of OP pesticides in each child were calculated and compared to the acceptable daily intake (ADI).

The result showed that children consuming more fruit had higher levels to these pesticides. A portion of the children in the study had estimated pesticide intake above the acceptable daily intake (Berman T et al., 2020)-the levels of dimethyl metabolites were also high compared to other international populations; and that fruit consumption was associated with higher urinary DAP levels.

With the DAP concentration data, it was found that some children in the study may be exposed to OP pesticides above levels that are considered safe. The Ministry of Agriculture's decision to phase out the use of chlorpyrifos in edible crops cited this HBM data.

In Austria, a platform for the establishment of a HBM programme was founded in 2007. The aim was to create a network of the relevant Austrian institutions and to share knowledge to promote health and environmental protection, support national prevention goals and develop national competence in human biomonitoring. So far, several studies on priority substances have been carried out and persistent organic pollutants in mothers' milk are regularly monitored.

A summary and further details relating to regional/national HBM programmes for the countries in Group 3 are provided in Table 4.

3.3.2. Issues which propelled the sustainability of the national or regional HBM programmes

Most countries in Group 3 have reported the utilisation of HBM as a tool to assess levels of pollutants in specific or general populations since the early 1970s. In the early 80s, HBM activities at the federal level have been closely connected to the risk assessment of chemicals and their regulation in Germany and there are many examples of policy translation of such results (Kolossa-Gehring et al., 2012). HBM studies were also initiated by environmental and health crises in most countries. For example, in Belgium, a PCB and dioxin incident (van Larebeke et al., 2001) propelled the establishment and sustainability of the FLEHS studies and in Germany, the lead contamination in the vicinity of a lead smelter (Kolossa-Gehring et al., 2012; Englert et al., 1987) was a prominent driver of the first human biomonitoring studies.

Austria and the Czech Republic reported issues on POPs. In Austria, regular breast milk monitoring for POPs informs an indicator in the frame of the health target "securing sustainable natural resources such as air, water and soil and healthy environments for future generations", which is part of a set of 10 Austrian health targets officially approved by the Federal Health Commission and the Council of Ministers (Bundesministerium für Arbeit, 2019). Additionally, a report on human biomonitoring is submitted to the National Council every 2 years (Bundesministerium für Klimaschutz, 2019). The Czech Republic's long-term goal to have a sustained HBM initiative to evaluate chemical exposure of the Czech population to priority chemicals was set out in a national strategy document "Set up of the national POPs monitoring" (first edition 2009, last updated 2020).

HBM is mentioned in several business, strategic or action plans, which galvanises the sustainability of the existing HBM programmes (see Table 4). In France, the HBM programme is part of the successive national Environmental Health Plan (PNSE) since 2009, following the recommendations of the Grenelle I Act (LOI n° 2009, 2009). Several countries report HBM results in the National Implementation Plan for the Stockholm Convention (e.g. Sweden, Austria, Czech Republic, Germany) or in the Arctic Monitoring Assessment program (Sweden). In most countries implementing HBM programmes, HBM data are used to follow-up environmental quality objective to achieve a "non-toxic environment" (se, 2021) and in Sweden, the need for the programme is further stressed in the governmental strategic document "Towards a non-toxic everyday life - platform for chemical policy" (proposition, 2013).

Societal and political interest in the environment and health have further supported the sustainability of the existing HBM programmes. For example, the results of FLEHS studies provided agenda setting opportunities for politicians and pressure groups and informed the societal debate about environmental health problems in Flanders. Since 1995, the results of the HBM programme and the phased action plans have provided answers to 193 questions addressed to the ministers for environment or health from the Flemish parliament. The HBM program also actively responds to specific (local) concerns and public perceptions. Positive feedback on the FLEHS programme and a call for its continuation from a wide variety of stakeholders, ranging NGOs to industry, has further supported the sustainability of the FLEHS studies. In Germany, the human biomonitoring results, particularly relating to PFAS and glyphosate, have recently made an important contribution to the public and scientific discourse (Duffek et al., 2020; Conrad et al., 2017; Lemke et al., 2021).

3.3.3. Policy translation

Data from the HBM programmes of the countries in Group 3 have been used for a wide variety of local, regional, national or European/international policy actions. In general, HBM outputs have been used to; quantify exposure levels of general or specific populations to harmful substances from the environment, assess health effects of such exposures and long-term trends in exposure and health impacts, identify problems requiring measures to reduce/eliminate exposure, and evaluate the effectiveness of remediation plans. Such results demonstrate the use of objective data for health risks management and the development of health policies and strategies. HBM data are also provided to initiate and support risk management measures under REACH and international conventions (Stockholm Convention on POPs and Minamata Convention on Mercury). Many countries also reported that data on persistent organic pollutants are an important part of reporting in the framework of the Global Monitoring Plan of the Stockholm Convention on POPs and that it enables the effective evaluation of measures on the basis of trends and baselines.

In Germany, the HBM Commission establishes assessment values (reference and HBM values) for selected substances according to defined criteria. These values serve to provide a comprehensive and uniform assessment of HBM results, whether they are derived from the German Environmental Surveys, time-trend analyses, or collected occasionally at the level of the Federal States. The reference values are derived by means of statistical methods and describe the basic exposure of the population. Measured values that are higher than the reference values are an indication of increased exposure compared to the general population but are not apt to interpret health risks (Apel et al., 2017; Angerer et al., 2011). In the HBM4EU context, a scientific exchange took place between HBM4EU staff and members of the HBM Commission on derivation options and methods for reference values, which was also reflected in a corresponding publication (Vogel et al., 2019). The assessment values relevant for a health risk assessment are the HBM-I and II values, which are toxicologically and/or epidemiologically derived. The HBM-I value is defined as the concentration of a substance in human biological material (e.g. urine, blood, hair) at and below which no risk of adverse health effects is to be expected and consequently there is no need for action. The HBM-II value represents an intervention level, where an increased risk for adverse health effects is assumed (Apel et al., 2017; Angerer et al., 2011; der Referenz, 1996; des Umweltbundesamtes, 2007). Building on this concept as well as the concept of Biological Equivalent (BE) values and the work of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES, 2014) on guidance in the occupational field, the strategy to derive HBM-GV under HBM4EU was developed (Apel et al., 2020; Hays et al., 2007; Hays SM et al., 2008; Aylward et al., 2013). The assessment values can be used by policy makers as a basis for possible risk management measures (Choi et al., 2015). UBA regularly reports data and recommendations to the BMUV so that the Ministry of the Environment can take legislative (regulatory) action on the basis of this scientific information if necessary. In addition, other ministries, federal and Federal State authorities are informed by UBA or via the HBM Commission so that they can act accordingly. For example, the provision of HBM-I and II values for PFOA and PFOS was helpful for the Federal States to initiate and control measures in hotspot areas (Hölzer et al., 2021; Schümann et al., 2021). Additionally, they supported the initiative to propose a ban for the whole substance group. Parliamentarians were also informed at their request. Reference values for lead were also useful for local authorities in assessing the body burden of a population in an area with elevated soil lead levels. Additionally, results from the German HBM studies inform voluntary exposure mitigation activities and supply a broad variety of targeted materials for information campaigns to inform citizens and behavioral choices.

In Belgium, a phased action-plan was developed collaboratively by

FLEHS researchers and policy makers to facilitate the science policy process (Keune et al., 2009), and was implemented after each (FLEHS) study. This phased action-plan combines scientific analysis and societal deliberation in a structured and participatory approach. In several successive phases HBM results are prioritised for policy action, explanatory factors are identified and targeted policy interventions are developed.

With this approach, the FLEHS studies have resulted in several action plans, with a diversity of policy actions in addition to existing policies and in cooperation with various national and regional actors (e.g. on POPs, PFAS). Successive action plans were developed for; elevated plasma levels of POPs in rural areas, the increased asthma prevalence in urban areas, exposure to PAHs of the general population and for local increased chemical exposure in the industrial hot spots Menen and Genk-Zuid (Reynders et al., 2017; Colles et al., 2021) and also for lead and arsenic in Hoboken. Each action plan was coordinated by a policy advisor on environment and health leading to targeted policy actions communicated by the ministers for environment and health. The HBM results on endocrine disruptors (pesticides, phthalates, PFAS, BPA) contribute to the yearly revision of the Flemish strategy on endocrine disruption and feed into the national action plan on endocrine disruptors which was initiated in 2021. Recently, HBM results on PFAS (e.g. exceedance of HBM-I values for PFOS and PFOA in almost 80% of adults in FLEHS III, statistical associations with several effect markers and associations between locally grown food (eggs, vegetables) and higher exposure to PFAS) led to the development of a PFAS action plan which is a dynamic plan and involves both generic and hot spot related actions. Wallonia is in the process of developing exposure reference values for the Walloon population and an action plan to achieve targets for reducing exposure to substances of concern will need to be established. For HBM hotspots such as Liège (urban gardeners), the local authorities have been supported to organise an information and awareness campaign for the concerned population (SPW, 2020).

The French population surveys (ENNS, Esteban and ELFE) have highlighted the exposure of the population to a variety of environmental contaminants. These surveys showed a significant level of exposure to endocrine disruptors: Bisphenols (A, S and F), parabens, flame retardants and some metabolites of phthalates and perfluorinated compounds (PFCs-PFOS and PFOA) (Dereumeaux et al., 2017; Fillol et al., 2021). The results from the surveys triggered the strategy on endocrine disruptors in France resulting in stricter regulation of some of these compounds.

Additionally, since 1981 French HBM studies have focused on specific populations or pollutants to gain a better understanding of exposure to environmental chemicals to help regulators to reduce exposure and monitor existing policies of specific concerns. These French HBM studies have been implemented to better understand; the influence of living near an incinerator on serum dioxin and polychlorinated biphenyl (PCB) levels (2005–2007); the influence of consuming fish from contaminated rivers on serum PCBs of fishermen (2009–2011); the evolution of blood lead levels in children from 1 to 6 years old since 1995 (2008–2011) (Fréry et al., 2012) and more recently HBM was combined with community involvement in Southern France to manage soils polluted with lead, arsenic and cadmium in the surroundings of closed metal mines (2015–2017) (Cochet et al., 2020). Another example is a case study in the French West Indies. A HBM study was conducted in 2013–2014 with focus on chlordecone, a legacy pesticide used in the past (until 1993) for the treatment of banana trees in the French West Indies. Results suggest that exposure to chlordecone is persistent and widespread. Chlordecone impregnation appears to have decreased between 2003 and 2013 for most of the population. However, various subgroups of the population remain highly exposed mainly through consumption of contaminated foodstuffs, like fresh fish (all species combined). Supply procedures, mainly those from informal channels, are also associated with exposure to chlordecone (Dereumeaux et al., 2016; Guldner et al., 2010). Since 2002, the Ministry of Health and the Ministry of the Overseas Territories have mobilised significant resources, in the framework of the Chlordecone Action Plans, leading to the raising of awareness and protection of the population (including several HBM studies: KANNARI, HIBISCUS, TIMOUN, KARUPROSTAT), the support of impacted professionals and the improvement of knowledge on this substance (Plan chlordécone 3, 2014).

In Austria, HBM in specific pollution hotspots supported risk assessment and was used to monitor the success of minimising risks (see Box 3). In the Austrian valley Görtschitztal, hexachlorobenzene (HCB) was detected in milk products and meat after waste treatment in a cement plant. An HBM study revealed that affected populations had increased plasma concentrations of HCB. Consequently, several risk managements measures were implemented (e.g. elimination of HCB emission, feed exchange, exchange of animals as well as nutritional recommendations for the affected population) (Steinwider et al., 2019). Additionally, HBM-data are fed into risk management activities of chemicals by the Austrian Competent Authority.

Many examples on the policy use of HBM data exist in Sweden. Noteworthy is the time trend studies to follow up the efficiency of regulatory measures or to detect emerging chemicals of concern and levels of chemicals (PCBs, PFAS, PNDE, DDE, PCDD/F) in mothers' milk and blood. These are used as indicators in the follow-up of the Swedish environmental quality objective "Non-toxic environment" (se, 2019). Data from the Swedish HBM programme has been used, together with other available HBM studies, to provide supporting scientific evidence for human exposure in proposals for different regulatory measures under REACH. For example, several studies of PFAS, time-trends as well as snap-shot studies (Gyllenhammar et al., 2015, 2016; Glynn et al., 2012; Bjermo et al., 2013; Axmon et al., 2014; Gebbink et al., 2015) were used in the REACH Annex XV restriction reports for PFHxS (ECHA, 2020) and C9-C14 PFCAs ((RAC) and E.-C.f.R.A. and C.f.S.-e.A. (SEAC), 2018). In addition, HBM data were used in the risk management option analysis (RMOA) of bisphenol-F and in the REACH Annex XV dossier to identify lead as a Substance of Very High Concern (SVHC).

3.4. Ministries and stakeholders advocating for HBM

3.4.1. Ministries and stakeholders in group 1

The countries in each group listed stakeholders who were included in their national hubs. The Ministries of Health and Environment seem to be the most prominent entities among the 3 groups - all NH had representatives from their ministries/departments of health and environment. It is not possible to evaluate the contribution from each stakeholder or their role in the NHs but it is interesting to see the variety of stakeholders. The level of engagement of health and environment agencies appears to impact on factors that contribute to the establishment of a long-term HBM programme. Ministries and stakeholders are limited in Group 1 in comparison to Groups 2 and 3 which probably indicates that wide networking is the key in raising the profile and status of HBM nationally.

3.4.2. Ministries and stakeholders in group 2

Most countries in Group 1 cited the Ministry/Department of Health as the foremost ministry advocating for HBM. Additionally, some countries cited environment ministries such as the Department of Environment, Food and Rural Affairs-DEFRA (UK), Ministry of Environment and Science (Portugal), Ministry of Infrastructure and the Environment as well as the Ministry of Economic Affairs & Climate Policy (the Netherlands) as their main advocates for HBM. Other ministries supporting HBM include, Ministry/Department of Environment, Social affairs and Food Safety Agencies, Chemical Authorities, University Institutes and Medical Faculties. Other entities who have supported HBM in a smaller scale are, the Artic programme and pesticides research.

The main governmental stakeholders cited by different countries in Group 2 include Ministry of Health, Ministry of Environment, Parliament, Commissioner of the protection of children's rights, Institute of Public Health, Ministry of Agriculture and Rural Affairs and Ministry of

Box 3
A case study of Hexachlorobenzene in Austria

In an Austrian valley called Görtschitztal, Hexachlorobenzene (HCB), a banned persistent organic pollutant, was detected in milk products and meat. The source of the contamination was attributed to waste treatment in a cement plant.

The dietary risk assessment revealed that the minimum risk level to human health for HCB, which is comparable to the usual tolerable daily intake (TDI) value in Europe, of 0.07 μg/kg bw/d exceeded the average and high consumption by all population groups up to 4 and 8-times, respectively. A human biomonitoring survey of the affected population also revealed plasma concentrations of HCB in a broad range (0.1–5.29 μg/l blood plasma). This was above the levels of the reference group of 0.15–0.6 μg/l.

A precautionary warning was issued for the consumption of food with high concentrations of HCB in the affected region. Several risk managements measures e.g. more than 5000 tonnes of contaminated feed was removed from the farms and substituted by HCB-free stock and farmers were advised to interrupt the on-farm cycle of HCB and to rehabilitate the farms and their products to minimize risks. The rather early detection of the HCB release and the risk management measures led to a limited duration of increased uptake of HCB in the population.

Dietary recommendations were established to enable the decrease of the internal HCB burden. Guidance levels for HCB in food for the affected population were calculated. These levels were significantly stricter than the maximum residue levels in EC regulations (Steinwider et al., 2019).

Agriculture, Food and Environment. Additional stakeholders involved in steering and financing of HBM cited by countries include the Ministry of Ecological Transition and the Demographic Challenge, Ministry of Science and Innovation, and Ministry of Consumer Affairs. Additional *potential future* stakeholders include Sanitary and Health Inspectorate, Agriculture Inspectorate, Ministry of Environment and Physical Planning and Ministry of Economy (North Macedonia).

3.4.3. Ministries and stakeholders in group 3

Countries in Group 3 all have a multidisciplinary team who follow-up and give advice on the respective HBM programmes. The formation or expansion of NHs under HMB4EU has enabled more partners to be involved and has strengthened existing collaborations within these countries.

In Sweden, it includes the Swedish Chemicals Agency, the Swedish National Food Agency, the Public Health Agency of Sweden, the County Administrative Board, the National Board of Housing, Building and Planning and the Karolinska Institute. The steering group has an advisory function and meets on a regular basis 4 times per year.

In Belgium, a steering board is also connected to the HBM programme. The steering committee of the FLEHS studies is composed of representatives of the Flemish policy domains on environment, health, agriculture, education, science and innovation. The results are also transferred to the federal administrations on health and environment through the national cell on environment and health (NEHAP). Representatives of the Walloon Ministry of Health are involved in the BMH-WAL steering committee as well as Sciensano, the only federal organisation involved in health studies.

The German HBM Commission which has been in existence for more than 20 years steers the German National Hub. It consists of independent experts from different fields, representatives from the higher scientific federal authorities responsible for chemical risk assessment, management and health surveillance, as well as from universities, hygiene institutes and clinics. In addition to the members, representatives of the BMUV, the Federal Ministry of Health, the Robert Koch Institute, the Federal Institute for Risk Assessment, the Working Group of the Supreme State Health Authorities, and UBA are involved in the work of the HBM Commission as permanent guests, so that a transfer of information between different departments and the national and Federal State level is ensured. The HBM Commission has been providing expert advice to the German Environment Agency on all issues relevant for human biomonitoring studies, be it study design, analytics or the evaluation of collected data. With the inclusion of the research institutions taking part as linked third parties in HBM4EU, the National Hub was extended and further developed, and a further connection between HBM4EU and national activities created.

The French HBM steering board is coordinated by the ministry of health and Santé Publique France. It also includes representatives of the ministries of environment, research and education and scientists from various institutions such as INSERM as well as stakeholders-from industry and non-governmental organisations, constituting the French National Hub. A newly established board has been set up recently for future HBM studies and is expected to meet four times a year to coordinate HBM activities.

In the Czech Republic, the national HBM hub was established in 2013. It is coordinated by the National Centre for Toxic compounds, the joint establishment of the Ministry of Environment and Masaryk University and supervised by the inter-ministerial science-to-policy Board composed of representatives of all relevant ministries. Active involvement of the Ministry of Education, Youth and Sports (MEYS) responsible for the European research programmes enabled mobilisation of resources of academic institutions, and those of large infrastructures for research and innovation as a source of co-financing.

The members of the Austrian NH are experts from ministries and agencies responsible for health, environment, food safety, occupational health and related research institutes and universities. They have committed to a mission statement (Austrian Platform for Human Biomonitoring, 2022) (Umweltbundesamt, 2022). Since 2016, the platform has been the official advisory board of the Federal Ministry for Climate Protection, Environment, Energy, Mobility, Innovation and Technology on issues at the interface between environment and health and usually meets twice a year. The organisation of the platform enabled the participation of an Austrian national hub in the framework of HBM4EU and will find continuation in the upcoming Partnership on the Risk Assessment of Chemicals (PARC).

All countries in all the 3 groups have stated their interest in participating in PARC, the NHs in PARC will have to expand to cover environmental protection as well as institutes with a remit for Human Health risk assessment. The role of the NH will be similar but the remit will expand considerably.

3.5. Steps/processes needed or used to engage policy makers

Almost all the countries in Group 1 highlighted ways used/needed to promote the utilisation of HBM data. Noteworthy, is the association and involvement in HBM4EU which significantly influenced the ongoing process of a national authority's approval for a national HBM programme in Lithuania.

Other steps include the involvement of several authorities/institutes in the NH which can create an avenue to interact, discuss and gain the attention of policy makers through respective channels (Norway). Portugal highlighted the following processes: proceeding further with

the HBM dissemination activities, better communication tools to involve the civil society; extending the national laboratory network and developing research, infrastructures, and capacity building. Portugal and Norway suggested establishing more partnerships/interactions with national bodies. Denmark and Estonia advocated having forum discussions on environmental pollutants of concern. Another mechanism was through the organisation of conferences as in the case of Hungary. Additionally, Switzerland recommended promoting the need for good quality HBM data for political interventions.

For those countries in Groups 1 and 2 who do not have formal national programmes in place, HBM4EU provided a good platform to stimulate discussions and engagement with national authorities to get HBM on the research agenda. The NHs have also sought to establish communications with national authorities as a mechanism for helping to engage with relevant policy makers. Some countries have invited relevant policy leads to participate in conferences where the benefits of engaging and working together can be demonstrated in a more tangible way.

The use of focus groups to galvanize civil society and raise general awareness has also been utilised in some countries. However, in those countries where the profile of HBM is not high, there may be a requirement to have additional disseminating activities. The development of better communication tools could also be helpful in the process - HBM activities communicated to the wider society can be of immense benefit. This process can spur societal and political interest in the environment and health concomitantly supporting the establishment and sustainability of existing HBM programmes as affirmed by most countries in all 3 groups.

Countries in Group 2 have made progress in engaging some policy makers as many describe national plans, programmes or strategies which have included HBM in some way.

Most countries in Group 3 have a strong network involving policy makers and the lessons learned and good practices could be shared through workshops/meetings or in the form of a guidance document or similar.

3.6. Barriers, drivers and opportunities

3.6.1. Group 1

All the countries in Group 1 reported the lack of funding as the major barrier for HBM activities. Other barriers include the difficulty of adding modules to already established cohort studies. Many countries alluded to the fact that setting-up a HBM program is expensive and challenging. The shortage of HBM experts has resulted in underrepresentation of HBM in the national authorities as in the case of Lithuania. In Iceland the recruitment process for HBM studies is becoming difficult because young people are unwilling to participate/complete long questionnaires, most policy makers are not aware of human biomonitoring activities, analytical capacity is dispersed to universities, research centres and government laboratories and there are difficulties in inter-institutional and inter-sectorial collaboration. Participation in HBM4EU was reported to be a major driver for raising political awareness and an understanding of the importance of HBM as a tool in public health protection and chemical management. For example, HBM4EU created an opportunity for the establishment of a UK HBM Steering Group (including health, environment and food ministries/agencies); in Denmark and the Netherlands networks have been strengthened nationally/internationally; and in Luxembourg and the Netherlands it has been the driver of HBM initiatives and future participation in such projects have been welcomed and appreciated by many governmental institutions.

3.6.2. Group 2

For Group 2, the main obstacles for establishing National HBM Programs include lack of funding and resources (Spain, Croatia, Cyprus, North Macedonia, Israel). In Croatia, HBM is included in the Ministry of Health and Ministry of Economy and Sustainable Development Strategy but there is no allocated funding. The National Biomonitoring Programme in Israel was co–funded by a non-governmental agency, with government matching funding, but government funding is not ensured beyond 2021. North Macedonia has mostly received funding for HBM from international organisations such as the UN and work on a public health crisis (COVID-19 pandemic) has more recently diverted technical and human resources from HBM. Lack of legislative framework and permanent allocated resources were described as barriers by Cyprus.

Spain reported the dearth of funding being compounded by lack of clear definition of HBM competences at ministerial level (Environment – Health). Cyprus raised the issue of competing obligations in the fields of chemicals management and environmental health.

North Macedonia cited an additional barrier such as insufficient technical equipment, a slow as well as expensive process of accreditation of research methods, and challenges in recruitment and special training of research staff. Establishment of accredited HBM laboratories was stated as a major goal. In Croatia, cross government collaboration and challenges linking environmental data with exposure measurements are highlighted barriers. Israel described challenges engaging Ministries (Environment, Agriculture) and raising awareness about potential uses of HBM data within the government.

Despite the barriers to advancing national HBM programs, all countries described opportunities. For example, in Croatia participants and the general population showed an interest in participating in HBM studies, and were interested in results. In North Macedonia, HBM4EU, with focus on material for general citizens ("citizens corner"), raised interest in the public and among researchers, demonstrated the need for a national programme, and provided published materials (protocols) to be used in the future. Joining the Horizon Europe Partnership for the Assessments of Risks from Chemicals (PARC; https://www.anses.fr/en/content/european-partnership-assessment-risks-chemicals-parc) provides (a) motivation for developing a national programme in North Macedonia and (b) an opportunity for engaging additional goverment ministries (focus on Ministry of Environment) in HBM acitivities, and increasing political interest (in HBM) in Israel.

The drivers for developing a national HBM programme varies across the countries in this group. In Croatia, the national programme is being developed within the Ministry of Economy and Sustainable Development Climate Change Adaptation Action Plan. This plan (still in draft at the time of writing) will include a component on strengthening the capacity of key existing public health laboratories towards human biomonitoring, with the aim of integrating epidemiological data with the results of chemical analyses in environmental and human samples (hair, serum, urine, etc.) and strengthening the capacity to assess human exposure, including to environmental factors related to climate change. Slovakia's participation in the WHO Europe Ministerial Conference on Environment and Health was a driver for developing a national HBM programme whereas in North Macedonia, concern about exposures at industrial hotspots (chemical industry, mines, lead smelter) is the key motivator.

In Spain, participation in EU projects such as COPHES, DEMO-COPHES and HBM4EU has been a major driver in advancing HBM and has supported strong collaborations with all actors involved including competent authorities and administrations, universities, research institutions and other stakeholders. Participation in HBM4EU led to the creation of a HBM programme in the Strategic Action Plan for Health and Environment, as well as the drafting of a Royal Decree for the establishment of an Inter-Ministerial Commission as basis for the National HBM Hub. Participation in PARC will contribute to the development of the HBM programme in Spain as the Ministry of Health, primary driver of the activity together with the Instituto de Salud Carlos III, is a member of the Governing Board.

The detection of high concentrations of some chemical substances in the population of Spain has also propelled the development of a national HBM programme; exemplified by the high levels of mercury found in

humans associated with food consumption and their potential health risks to vulnerable populations, in particular children. In North Macedonia, the motivation was the need to gather data on exposure of the population to prioritised chemcials including pesticides (Lindane and other HCH), PAH, PCBs, heavy metals (Cd, Hg, As) and mycotoxins. In Israel, non-governmental funding was a major driver for establishing a national programme, combined with interest within the Ministry of Health to collect data on prioritised exposures (environmental tobacco smoke, organophosphate pesticides) and nutritional biomarkers (iodine and selenium).

3.6.3. Group 3

Although the HBM programmes for countries in Group 3 have proven to be useful for chemicals policy (see Table 5), the sustainability of most is hindered by several barriers and challenges. The lack of governmental support and long-term funding for the regional or national HBM initiative is mentioned by most countries. Although in France there has been a general agreement that HBM campaigns should be undertaken regularly, the start has been delayed due to the dependence on secured funding. In the Czech Republic, the HBM programme lacks sufficient funding as it is not the highest priority at the Ministry of Health. Due to increased government saving, funding of the FLEHS studies has decreased by almost 50% for the 4th FLEHS study in comparison to the first 3 rounds. Given the presence of other high impact policy issues (e.g. climate, COVID), it is becoming increasingly more difficult to allocate the necessary budget to continue the Flemish HBM programme.

Several countries stated that fragmentation of (i) competences, (ii) monitoring programmes and (iii) related databases among several ministries (Health, Environment, Agriculture) complicate analysis and interpretation of available data. Improved communication and collaboration between the different relevant ministries/agencies dealing with various aspects of chemical risk assessment is required. The issue was further compounded in the Czech Republic by significant organisational changes in a system of public health institutes and related services (lack of necessary capacities). This resulted in the closure of some reference laboratories and a need to subcontract external partners for the chemical analyses. The HBM samples were collected annually during the earlier years, however, collection is now reduced to once in several years and in smaller sample sets.

Many countries also mentioned the lack of regional, national, and European infrastructure for analyses and storage of samples collected within the HBM programme.

Another challenge relates to the recruitment and active involvement of participants. It is becoming increasingly challenging to engage participants to gather a multitude of data and information. In Belgium, active engagement of participants in the evaluation of the FLEHS studies is crucial to improve participants recruitment and involvement and stimulates their understanding and use of personal HBM-results (Morrens et al., 2021). A decreasing participation rate was also mentioned by Sweden. An underrepresentation of populations groups with lower socioeconomic status is likely. Recruitment of socially vulnerable groups is still difficult and requires extra efforts to include them in a representative manner. By investing in direct, person-to-person contact with trusted buddies and supported by practical advice about cultural and linguistic sensitivity, it was possible in FLEHS III to increase study participation of socially disadvantaged people (Morrens et al., 2017).

Financial and recruitment issues are compounded by the statistical power (of the study) often being too low for detailed statistical analyses due to the limited sizes of the study groups. The connection between HBM and health effects requires large population groups and access to medical documentation. The issues on statistical power can partially be addressed and improved by combining and aligning HBM studies across Europe. The same holds true for combining HBM and health studies and in bringing existing cohorts together to increase the power of statistical analyses between exposure to chemicals under study in HBM4EU and selected health effects.

Another hurdle relates to the fact that the level of chemicals in the body is a result of the total exposure from all sources. More information on specific sources is needed to inform the use of the correct measures to reduce the exposure level. In addition, identifying new threats is a complex task as banned chemicals are replaced by new ones with similar properties, making it challenging to target investigations. Chemicals are mostly studied individually, but we are exposed to mixtures, and we need more knowledge to understand that synergistic effect on human health.

Many countries cited HBM4EU as being crucial in addressing a number of these barriers and, the continuity of HBM at European level in the frame of PARC is also important in the sustainability of their existing HBM programmes. The HBM4EU co-financing to regional or national programmes has been of utmost importance in overcoming decreased budgets and has created a mutually beneficial situation for both the national/regional and the European level as results are useful for policy on both levels. In France, it is expected that the national and European programmes (such as PARC) will be further aligned. In Sweden for example, HBM4EU was supported by the government, which resulted in an extended budget for the national programme health related environmental monitoring since 2017.

HBM4EU provided better communication of HBM-related issues across the consorts and more collaboration within countries between the departments, competent entities and research institutes involved in different aspects of HBM. The concept of NH under HBM4EU has been helpful for national players to join their efforts and mobilize capacities to make the regional or national HBM programmes more sustainable. For instance, the RECETOX's open-access research infrastructure in Czech Republic listed in the national Roadmap mobilised sufficient capacities for the analyses of samples from the alignment studies at the national and European levels and also those collected in predecessor DEMOCOPHES project. Countries in this group anticipated that the PARC partnership will play an important role in strengthening collaborations at the national and European level, setting up standards and establishing processes and motivating the project partners to participate in surveys.

The EIRENE RI - research infrastructure for environmental exposure assessment in Europe which was recently added to the 2021 Update of the European (ESFRI) Roadmap could be another important gateway and pillar of sustainability of the HBM programmes in Europe in providing technical solutions and supporting pan-European collaborations.

On open policy questions such as action on new emerging chemicals, mixture effects or identification of sources of exposure, national programmes benefit from the large network of experts in HBM4EU. For example, the German system as a longstanding and well-established tool has proven itself at the national level and is also increasingly benefiting from the international activities and networks, as the many open questions can be solved more quickly and precisely by large number of actors. This is demonstrated by the Europe-wide quality assurance of analytical methods and laboratories driven by the linked third parties as well as the harmonised SOPs for study preparation, sampling and storage of human samples, the knowledge gain regarding adverse outcome pathways and non-target screening do not only provide valuable results for the HBM4EU project but will also be important for the further work of the German National Hub, including the HBM Commission and the UBA.

Countries in Group 3 have indicated that they actively use HBM4EU products in their national settings. In the Czech Republic for example, materials were not only used at the regional and national levels to promote awareness, but also to get stakeholders to prioritise issues and phase out certain chemical uses - i.e dental amalgam etc. Germany indicated that the monthly newsletter 'HBM4EU Science Digest', is particularly appreciated for a quick overview of specialist topics. HBM4EU results and conclusions are continuously used to inform the general population, stakeholders and other players concerned via the

Table 5
Use of HBM data for policy purposes in countries from Group 3.

Country	Issue/Concern	Population (local/ regional/national)	Chemical	Ministry or Institute undertaking the study/other	Outcome	Reference
Austria	Regular monitoring of POPs in breast milk	National	POPs	BMK	Assess time trends to feed the health target "securing sustainable natural resources such as air, water and soil and healthy environments for future generations"	Bundesministerium für Klimaschutz (2019)
	Dietary exposure to HCB	Local (hot spot)	Hexachlorobenzene		Support for risk management measures and monitoring of the success of the minimisation measures	Steinwider et al. (2019)
Belgium	Elevated plasma levels of POPs in rural areas and hot spot Menen	Regional (Flemish population)	Dioxines/PCBs/DDT	CEH, DOMG	POPs action plan with measures on source, monitoring and sensibilization to further reduce POPs body burdens and meet the objective of the Stockholm convention	(Reynders et al., 2017; Keune et al., 2009; Colles et al., 2021)
	Increased asthma prevalence in urban areas	Regional	(indoor) air pollution	CEH, DOMG, AZG	Action plan to reduce asthma prevalence	
	Local increased chemical exposure in the industrial hotspots Menen, Genk-Zuid and the harbour of Ghent	Local	Multiple pollutants	CEH, DOMG, AZG, VMM	Local policy action plans	Colles et al. (2021)
	Exposure to endocrine disruptors in the general population and associations with bio-markers of ED effects	Regional (Flemish population)	Recent pesticides, Phthalates, PFAS, BPA	CEH, DOMG	Contribution to the Flemish strategy on endocrine disruption, to the national action plan endocrine disruptors (2021) and to the development of a Flemish PFAS action plan (generic and hot spot related actions) which is regularly updated.	
Czech Republic	HBM data on POPs		POPs		Evaluate effectiveness of measures in the Stockholm convention on POPs on the basis of trends and baselines	
France	Exposure to endocrine disruptors in the general population	Population surveys	Endocrine disruptors	SpF, ANSES,	Contributed to the launching of the French strategy on endocrine disruptors which led to stricter regulation of some of these compounds. Report on high priority EDCs.	www.ecologie.gouv.fr and 2nd National strategy on (2019)
	HBM study with focus on chlordecone	In the French West Indies	Chlordecone	INSERM, SpF	Contribution to the Chlordecone Action Plans (e.g. awareness and protection of the population, support of impacted professionals but also the improvement of knowledge on this substance)	(Dereumeaux et al., 2016; Guldner et al., 2010)
	Sources and modes of exposure for soil pollution (lead, arsenic and cadmium)	Local: in the surroundings of closed metal mines	Arsenic, lead, cadmium	SpF, Occitanie ARS (Agence Régionale de Santé)	The study has led to a series of appropriate operational measures.	Cochet et al. (2020)
Germany	Provision of HBM-I and -II values for PFOA and PFOS	National	PFAS (PFOS, PFOA)	UBA	Initiate and control minimisation measures. Support for the initiative to propose a ban for the whole substance group.	(Hölzer et al., 2021; Schümann et al., 2021)
	Provision of representative and time trend HBM data on PFAS	National and regional	PFAS	UBA	Support EFSA with data compilation, support Federal States to evaluate HBM results from populations in hot spot areas	Göckener et al. (2020)
	Provision of representative and time trend HBM data on Phthalates	National and regional	Phthalates	UBA	Initiate and control minimisation measures. Support of REACH activities	(Schwedler et al., 2020; Koch et al., 2017)
	Annual routine measurement of selected substances in human samples from the environmental specimen bank	Regional	Arsenic, Lead, Cadmium, Copper, Mercury, Organohalogen compounds, Polycyclic aromatic hydrocarbons	UBA	Continuous control	(Bartel-Steinbach et al., 2022; Lermen et al., 2021)
Sweden	POPs in mothers' milk and blood	Local	PCBs, PFAS, PNDE, DDE, PCDD/F	SFA and financed by SEPA	Indicators in the follow-up of the Swedish environmental quality objective "Non-toxic environment".	se (2019)
	Time trends for PFAS in breast milk of first time mothers	Local	PFAS	SFA and financed by SEPA	Increasing levels of PFHxS led to the discovery of exposure from the municipal drinking water. As a result, measures were taken for treatment of the water	(Gyllenhammar et al., 2015, 2016)
	Exposure to PFAS contaminated drinking water	Employers at an airport	PFAS	Dep. of Occupational and Environmental Medicine, Sahlgrenska University Hospital	Sampling of blood was initiated among employers	(Xu et al., 2020)

(*continued on next page*)

Table 5 (*continued*)

Country	Issue/Concern	Population (local/ regional/national)	Chemical	Ministry or Institute undertaking the study/other	Outcome	Reference
	Time trend and snap-shot studies for PFAS		PFAS		PFAS results were used in the REACH Annex XV restriction reports for PFHxS and C9-C14 PFCAs	(ECHA, 2020; Reade and Pelch, 2020)
	Bisphenols in urine from first-time mothers		BPF	SFA and financed by SEPA	Used to demonstrate human exposure in the risk management option analysis (RMOA) of bisphenol	Bjermo et al. (2019)
	Time trend studies of lead in blood of children and adults		Lead	Performed by Lund University and financed by SEPA	Used in the REACH Annex XV report to identify lead as a Substance of Very High Concern (SVHC)	(Skerfving et al., 2015; Lundh et al., 2020; Wennberg et al., 2017)
	PFAS in drinking water		PFAS	Performed by SFA and financed by SEPA	Results showed that low levels in drinking water may be an important source of exposure among children	Glynn et al. (2020a)
	POPs in HBM and aquatic monitoring program		POPs	Performed by Swedish University of Agricultural Sciences and financed by SEPA	The importance of chemical regulation was clearly shown using temporal trends.	Glynn et al. (2020b)

UBA website, social media and other communication products. In Belgium, the HBM4EU videos and factsheets were used in communication to the press in support of their reports.

3.6.4. *Overview of all 3 groups*

Overall, the primary obstacle cited by countries for establishing and sustaining national HBM programmes is funding; due to the high costs associated with the collection and chemical analysis of human samples. Although it is included in national strategies in some countries in Group 2, there is no associated ring-fenced funding. Those in Group 3 have acknowledged the usefulness of HBM programmes for chemicals policy but recognise that there are barriers and challenges that could affect the sustainability of such initiatives. These barriers include the lack of governmental support and long-term funding for the national/regional programmes due to the high associated costs.

A summary of the major barriers and challenges cited by countries in the three groups include:

- All groups mentioned competing priorities as a major barrier. For example, currently in most countries Covid-19 is a priority and resources are being targeted in that area. Furthermore, developing a national programme may never become a priority in some countries who may choose to adopt a different approach to investigating population exposure to chemicals.
- Organisational changes, and decommissioning/closure of some required infrastructure e.g. reference laboratories, to support the programmes (Group 3).
- Recruitment of participants and the collection of vast amounts of data are hugely onerous. Recruitment of vulnerable groups and those from lower socio-economic status (SES) is particularly evident in these programmes (Group 3).
- Lack of required competencies at ministerial level stifles the discussions needed to develop and implement HBM programmes (Groups 1 and 2).
- Fragmentation/dispersal of competencies, fieldwork, chemical analysis and associated databases between different ministries/ agencies makes data analysis and interpretation more challenging (Group 3).
- When the sample size is too small the statistical power of the study is reduced thereby reducing data analyses to brief descriptive statistics (Group 3).
- The level of chemicals in the body is a result of the total exposure from all sources therefore more information on specific sources is needed to inform the use of the correct measures to reduce the exposure level. Often, this information is not available because it is difficult to make an informed decision on an individual route of exposure as HBM generally assesses exposure by quantitating a biomarker of exposure in either blood, urine or other biological media thus, making communication with policy makers difficult.

The drivers tended to be varied among the countries in the three groups. The most noteworthy drivers cited include:

- Investigating exposures at hotspots (industrial, traffic etc.).
- Investigation of elevated levels of chemicals found in the population (from food, water, air, consumer products).
- Strengthening relevant capacity with the aim of integrating epidemiological data with environmental and human exposure data.
- Participation in HBM projects such as COPHES/DEMOCOPHES and HBM4EU has been cited by many as a positive way to raise public and political awareness and engage with stakeholders.
- Establishing and strengthening national hubs through HBM4EU has proved especially useful in raising the profile of HBM and getting it on the national/regional agenda in some cases.

The opportunities of an HBM programme are similar for most

countries and primarily include:

- Knowledge on the internal exposure of the population to chemicals.
- The information/data gleaned can be used to influence relevant stakeholders, policy makers and politicians to prioritise chemical issue(s) identified.
- These programmes can be used to monitor the impact of new and emerging chemicals.
- On open policy questions such as new emerging chemicals, mixture effects or identification of sources of exposure, national programmes benefit from the large network of experts in HBM4EU.

3.7. Potential useful non-governmental groups/players for the promotion of HBM

The suggestions of players who would benefit in some way from HBM were varied as highlighted in only Group 1. Hungary suggested that science communication experts and behavioral scientists will add to the success in developing policy and communication materials. Industry, NGOs, and universities were also highlighted as potential players. The UK suggested the UK Chemicals Stakeholder Forum (UKCSF) which enables discussion between stakeholders, government, and regulators in support of effective chemicals and waste management. Members of this forum were receptive when UKHSA (formerly PHE) presented the work of HBM4EU in June 2019. Portugal highlighted the Chemical industry, trade unions, industrial associations, and citizens collaboration. Lithuania cited private institutions would be beneficial in linking different exposure data/experimental results to the source of exposure. Switzerland suggested that the link between environmental monitoring and HBM will be strengthened by collaborating with environmental monitoring networks. Iceland mentioned journalists as important players for creating awareness.

3.8. The future of HBM in european countries

Countries in Group 1 have highlighted different ways that HBM will be considered nationally/regionally. In Lithuania, the government has included human biomonitoring in its preventative measure objectives and intend to use it as a tool for further investigation and health surveillance. The UK (led by UKHSA) will continue to develop HBM for public health protection and chemical management. The initial step will be through the HSfE and input into PARC. Additionally, there are calls from government to continue the establishment of a sustained HBM programme. Engaging the wider stakeholder through the UK Chemical Stakeholder Forum will continue and data from HBM4EU will be used as a comparator for that produced in the UK.

The Ministry of Health in Norway had presented an opinion to the Parliament in 2019 describing their visions for public health. The government wants to continue the collaboration with other European countries to survey the general population's exposure to environmental pollutants and increase the knowledge base on these exposures' potential impact on health. This was also mentioned in the chapter on "Green Health" in their International Strategy (2021–2025), as well as in the action plan for "a toxic free everyday life", just launched by the Ministry of Climate and Environment. The National Institute of Public Health (NIPH) has also funded a second collection of samples for the Norwegian Environmental Biobank (NEB; https://www.fhi.no/studier/miljobiobanken) to take place in 2022 which will be included in PARC. The aim is to implement sustainable surveillance of the populations' exposure to hazardous chemicals and changes in diet, as well as to generate a rich data source of exposures that can be linked to health information/registries and used in future risk assessments and research projects at European level.

In Finland, levels of PFAS in fish have been rising for the last 10 years; if this continues, given the governmental programme to promote the use of domestic fish, HBM in vulnerable subpopulations (women of childbearing age, 1 year old children) will be required.

Hungary is planning to investigate different group of substances (e.g. Bisphenols and biomarkers of air pollution) in previous sample collections (biobank samples) and to establish a regular HBM programme.

In the Netherlands concerns about exposure to chemicals, e.g. pesticides for residents living in close proximity to agricultural fields or PFAS and alternatives such as GenX, will promote the use of HBM for policy making. This may involve targeted HBM studies as well as suspect screening analyses.

Portugal's ambition is to develop better communication tools and to establish a national HBM-platform that will promote interaction between the relevant players particularly those with in-house analytical capacity, scientists, risk assessors, risk managers, citizens, and policy makers. HBM4EU output, including data generated from Portuguese samples collected in the national aligned studies, will be instrumental to show stakeholders and policy makers how useful the HBM activities are, in order to generate knowledge about citizens' exposure to chemicals and its potential impact on their quality of life and health. PARC will also be a driving force for the HBM activities in Portugal.

Switzerland urgently wants to establish reference values for the general population on chemicals of concern, notably metals, dioxins and other persistent legacy chemicals, as well as selected pesticides. The national study on health and bio surveillance will be launched in 2023 based on the results of a pilot phase. Another future endeavor is to establish a biobank and database that can be used in health-related matters.

Group 2 countries' vision for the future also includes HBM. Slovak Republic's National Action Plan for Environment and Health (NEHAP V) includes a long term goal of implementing a national HBM programme which will include prioritisation of chemicals included in HBM4EU. The first draft of the Slovakian national HBM programme was officially approved by the Ministry of Health of the Slovak Republic in May 2021.

The Ministry of Health in Cyprus recently approved HBM as a one of its priorities for public health & social rights protection and included it in the CY National strategy for EU affairs. European efforts to advance HBM have complemented those nationally and provided funding to promote and develop work and translate results into policy and communicaiton efforts.

Israel will incorporate the next round of the national programme into the PARC aligned studies, however, ensuring government funding remains a challenge.

In Spain, HBM is included in the Strategic Action Plan for Health and Environment. Current work is focused on implementation of the strategic plan and HBM at national level in the next years with campaigns designed to address exposure to chemical substances of interest in Spain (PFAS and mercury).

The countries in Group 3 cited their continuous support and promotion of HBM – with all countries except Sweden citing PARC as a core contributor to the sustainability of HBM.

Most of the countries projected that, in the future, environmental monitoring and HBM will be better linked to support the overarching goals of the European Commission (e.g. Zero Pollution ambition).

PARC will also provide the opportunity to utilise and build on the achievements of HBM4EU; further develop environmental and human monitoring and the continuous support for science-based policy making. The continuation of HBM activities at European level in the frame of PARC will be of great importance to sustain the national or regional programmes for countries in Group 3. In Germany, the GerES VI pilot study has been finalised and the main study will start mid-2023. Sampling and the analysis of samples of the German Environmental Specimen Bank is ongoing. The experiences from the different work areas within HBM4EU as well as from the coordination of the entire work programme are currently being evaluated for the successful implementation of planned PARC activities. In Belgium, the preparation of the 5th FLEHS study is ongoing, and the start is foreseen for 2022. The co-financing, knowledge exchange and participation in scientific research

in PARC is essential in the sustainability of the Flemish HBM programme. Moreover, the next FLEHS campaign will be embedded in a Flemish knowledge hub 'environment and health' with strong collaboration between the policy domains on environment and health. In addition, the growing acknowledgement of the importance of HBM for chemicals policy at EU level and the continuation of HBM4EU activities under PARC are important elements for the sustainability of both the Flemish and the Walloon HBM programmes in Belgium.

In Austria, great progress has been made regarding HBM. The national platform for human biomonitoring was able to successfully establish itself as a National Hub in HBM4EU. The sustainability of the activities is reflected in the fact that the National Hub in PARC unites more than twice as many partners as in HBM4EU. Also, for the Czech Republic, the PARC partnership will play a significant role in strengthening collaborations at the national and European level, setting up standards and establishing processes as well as motivating partnership members in participating in surveys.

The participatory organisational framework of HBM4EU, as well as in the French National Hub, is particularly inspiring for continued French National Biomonitoring programme as foreseen from January 2023 onwards. Santé publique France will be at the centre of the design and management of population surveys to produce future French biomonitoring data, that will help achieve the public health objectives set out in the agency, and also continue supporting French and European research, risk assessors and managers, occupational prevention experts, and policy makers (Rambaud1 et al., 2020). It will coordinate the contributions from other agencies and research institutes. PARC will ensure the continuation of the HBM4EU biomonitoring platform and this is essential for exposure assessments of the European population.

While Sweden mentions that PARC is not crucial for the sustainability of the Swedish national HBM-program, there is an ongoing discussion within the steering group about the need for an extended HBM-program where a health examination survey (HES) could also be included. A national infrastructure for data collection would be beneficial, leading to less fragmentation and a better way to join aligned studies in projects as HBM4EU and PARC.

Overall, the status and use of HBM in the future is varied for countries in Group 1; ranging from addressing specific environmental exposures to developing reference values. Many countries future plans include using the platform provided by HBM4EU to:

- develop and implement further HBM programmes.
- implement HBM in vulnerable sub-groups where an issue has been identified.
- establish biobanks and health related databases which can be utilised as required.
- utilise HBM results to develop population guideline reference values.
- include biomonitoring in government's objectives to develop/implement preventative public health measures.
- continue collaboration with EU countries to survey populations' exposure to environmental pollutants.
- develop better communication tools and establish national HBM platforms to promote interactions between relevant parties such as scientists, laboratory analysts, risk managers, policy makers etc.
- encourage engagement in PARC which can become the driving force for undertaking HBM activities.

The countries in Group 2 are focussed on including HBM in their various national action plans, using the prioritisation of chemicals in HBM4EU as a guide and implementing initiatives through the PARC project. In Group 3, the countries cited their continuous support and promotion of HBM and their belief that PARC will contribute to its longevity and sustainability. Given that they already have HBM programmes in place, their plans include linking environmental and human biomonitoring data to support the overarching goals of the European Commission. PARC is viewed as an initiative which can support this goal as well as the sustainability of their national programmes and the associated collaborations and partnerships.

The co-financing available through the PARC project is important and in cases essential for the sustainability of national initiatives. There is the appetite to further align national HBM programmes thereby strengthening the exposure assessment of European populations to chemicals component of the PARC project. Regardless of the status of HBM in countries in HBM4EU, all are unified in the desire to strengthen the scientific basis for chemical risk assessment and transition to the next phase (of evidence-based RA) through PARC.

4. Summary

4.1. How HBM can be used to raise awareness

The utilisation of HBM data to raise awareness is mostly affiliated with countries in Groups 1 and 2. Most countries in all the groups stated that environmental and public health issues/disasters resulted in either raising awareness for HBM or getting the attention of policy makers.

The Netherlands and Italy cited the industrial emission of PFAS, the issue of higher levels of dioxin in Baltic fish and fly ash from incinerators in Finland and England, the health impact in oil shale sector in Estonia and disasters like the collapse of the giant wall reservoir of an aluminium factory in Hungary all raised the awareness of HBM and its value in risk assessment and public health protection.

Similar claims of using environmental and public health issues for HBM awareness were also made by countries in Group 2. In Cyprus and Israel, cotinine measurement in children was used to assess the exposure to environmental tobacco smoke followed by intervention campaign and media coverage. It was also stated that HBM data was used for crisis management in Cyprus.

Environmental/public health issue or minor disaster should not be the only driver to gain the attention of policy makers. Other methods of raising awareness can be explored, such as in the case of Portugal where human biomonitoring workshops and focus groups were organised to promote networking possibilities and knowledge exchange and media appearances can be utilised.

4.2. Factors that influence the establishment of HBM programmes for policy development

Some countries in all three groups highlighted the inclusion of HBM module into HES as a prerequisite for establishing a HBM programme. Countries in Group 3 stated that the inclusion of a HBM module into a national health survey progressed to a wider monitoring programme that included human health, nutritional behaviour and exposure. The combination of environmental and human health monitoring can also be a good foundation.

Other factors that influence the establishment of HBM programmes include:

- The use of HBM data for risk assessment.
- HBM studies focused on specific populations or pollutants to gain better understanding of exposure to environmental chemicals.
- The use of HBM data to support scientific evidence for human exposure in chemical restriction.
- Monitoring the public health impacts of new chemicals coming into the market daily to inform chemical regulations.

- Greater collaboration between ministries and government departments to facilitate coordinated data analysis and interpretation and the timelier publication of results to inform policy development/implementation.
- The inclusion of HBM in a national chemical strategy or action plan.
- Exploring the public interest in the environment via focus groups and media campaigns.
- Monitoring and publishing the success of using HBM data to minimize risks.
- The lag between exposure and evaluation of internal exposure using HBM means that the evaluation is always retrospective 'after the event'. Many studies create a biobank of human as well as environmental samples, these can be used for trend analysis or setting baselines.
- HBM data are an integration of all routes and sources of exposure, however, for policy development it is necessary to understand the source of exposure and the use of environmental matrices should not be ignored. There are many occasions where HBM data would or should not be recommended.

4.3. Strengths and limitations

Countries within HBM4EU that have used HBM data either for raising awareness, policy development or chemical management have been assessed using the multi-case qualitative analysis. Each NHCP self-selected their group: this means they chose to describe their national HBM structures as:

- No national programme of HBM to support policy but more *ad hoc* reacting to needs as they arise mainly for awareness raising to get the attention of policy makers or the public.
- National hubs in their infancy with the use of HBM data in policy development.
- Well established national structures which are used to support chemical management at a national level.

Review of the narratives using the templates (as published in National hubs page-HBM4EU), shows that the division between the groups is blurred, not unexpectedly. It is also important to note that progression from Group 1 to 2 or 2 to 3 is not implied, this was just a means of dividing the NHs to allow for some exploration of the issues.

The breadth of HBM related studies reported in Group 1 are reflected in Group 3 as well as Group 2, this means that it is not a prerequisite to have a national programme before studies can be carried out. However, there may be more opportunity to carryout repeat surveys by those in Group 3 as the infrastructure is already present. Those in Group 3 also emphasised that the continuation of these national programmes is still dependent on competitive justification for funding and is not guaranteed-thus they have similar challenges to those in Group 2 or 1.

Also, by describing and understanding the relevant factors that led each country to choose their different groups, we have uncovered common themes in each of the three groups and identified factors that could be beneficial to all countries.

4.4. Recommendation

Narrative research collects and reviews experiences by those writing the narrative. The aim of this paper was to describe the experience of the NHCPs focusing on the use of HBM data in policy development or awareness raising in the countries which took part in HBM4EU. It is not an exhaustive exploration but a means to show how different structures and levels of engagement can be used to further chemical management. As with most qualitative research, the information gathered and analysed are based on personal interpretations which may be subjective. One of the issues faced is the interpretation of the simple template used to gather the evidence. The templates could be tested in one or two countries before the main study. This may help reduce misinterpretation of the data required. Such a study should be planned early in conception of a multi-national study such as the one described in this paper.

Consequently, intrinsic methods/steps on the utilisation of HBM data may sometimes appear vague or inconsistent. However, the narratives provided a holistic view of the utilisation and benefits of HBM data for policy support or raising awareness. We also acknowledge that one-time, reflective summaries may not fully capture key information or issues that are required. Additional research methods are required to provide a more detailed/robust outline of the steps/process needed for policy support. For example, the incorporation of other qualitative research methods such as focus groups or interviews with key players and monitoring/evaluating the level of awareness of HBM for policy development among policy makers and the investigation of how HBM4EU has impacted on the behaviour of European citizens or Public Health professionals may give additional insight. These can be explored in subsequent projects e.g PARC.

5. Conclusion

The value of a HBM programme or use of HBM data in any country has been extensively demonstrated throughout this article. Although challenges and barriers still exist, most countries within Europe are already conversant with the benefits and opportunities of HBM.

Many HBM and other population-based studies report the difficulty in recruitment - this could be those at-risk groups, such as lower socio-economic status, young children or the elderly. Thus, it is imperative to interpret the data in line with the demography covered in the study. Additionally, sampling frames that focus on "hotspots" and at-risk groups are important. However, there is a need to have baselines for population levels of exposure biomarkers, these allow us to develop reference values. HBM reference values also support communication to the public of their exposure level and allows public health professional to monitor exposures reflecting on the potential effects of such exposures.

Unsurprisingly, funding is a major barrier to the establishment of national HBM programs. However, there are many other factors, from lack of appropriate expertise to (lack of) public engagement, that are important. HBM4EU has had a positive impact on the level of awareness crucially for the utility of HBM data. Understanding the true cost of *ad hoc* or national programmes balanced with the impact of reducing exposure to chemicals would be a powerful message to support the ever-present battle for funding.

To propel HBM onto political agendas, countries must continue to raise their profiles, strengthen and extend engagement with relevant ministries and groups as highlighted in this article. The NHs established in HBM4EU supply an excellent basis for their further development in PARC.

The HBM boat has definitely left the harbour armed with many provisions; however, the journey is a long one and requires resilience and tenacity to ensure that the full benefits are achieved at the end.

Appendix 1

Group 1 National Hub Template

Introduction:	
• Background information on the evolution and status of HBM in your country e.g COPHES/DEMOCOPHES and EU programs.	300 words maximum for only the Introduction.
Main text - Results and Discussion ENSURE YOUR NARRATIVES ARE REFERENCED AS FAR AS POSSIBLE • Description of issue(s) which have resulted in the raising of awareness. • Include brief description of sample population, substances of concern and whether local/regional/national. • Give example of cases and specific studies • Description of HBM programme if it exists e.g. implementation of a HBM module into HES or relevant other activities funded by the government. • Describe which ministries (Environment, Health etc.)/policy makers and stakeholders involved/steering/financing the HBM programme. • Give examples - specific chemicals or outcomes. • Steps/processes needed or used to get the attention of policy makers. • Describe barriers e.g funding; challenges e.g. participant recruitment; opportunities e.g. enhancing cross government working and linking of env data with exposure measurements currently at play in your country with regards to HBM. • Have any of these barriers been addressed by HBM4EU? If yes - describe. • Other players who would be beneficial in raising awareness and working together to promote HBM	
Future plans - • Are there plans to use HBM data in the future for policy or awareness - give clear examples. Will the data from HBM4EU be used?	

Appendix 2

Group 2 National Hub Template

Introduction:	
Background information on the evolution and status of HBM in your country.	300 words maximum for only the Introduction.
Main text - Results and Discussion ENSURE YOUR NARRATIVES ARE REFERENCED AS FAR AS POSSIBLE • Description of HBM programme if it exists e.g. implementation of a HBM module into HES or development of a standalone HBM programme. • Examples of HBM data for policy development. Please specify chemicals or chemical groups. • Describe which ministries (Environment, Health etc.)/policy makers and stakeholders involved in/steering/financing the HBM programme. • Describe steps/processes used in involving policy makers. • Is HBM included in their business/strategic/action plan. • State which ministry is HBM data reported to or its being utilised. • Describe barriers e.g funding; challenges e.g. participant recruitment; opportunities e.g. enhancing cross government working and linking of env data with exposure measurements currently at play in your country with regards to HBM. • Have any of these barriers been addressed by HBM4EU? If yes - describe. • Elaborate on issues which propelled the HBM data of choice e.g. disaster, pollution, incidence/prevalence of a health-related issue	
Future plans - • Are there plans to increase the use of HBM data in the future for policy give clear examples. • Will the data from HBM4EU be used? • Has HBM4EU re-enforced the need for a National programme? • What are your future plans? • Do you think PARC will be crucial to the development of your HBM programme?	

Appendix 3

Group 3 National Hub Template

Introduction:	
Background information on the evolution and status of your National HBM programme in your country. Include year of establishment -Who pays for the programme of work? Give web links.	300 words maximum for only the Introduction.
Main text - Results and Discussion ENSURE YOUR NARRATIVES ARE REFERENCED AS FAR AS POSSIBLE • Describe which ministries (Environment, Health etc.)/policy makers and stakeholders involved/steering/financing the HBM programme. • Involvement with HBM and Steps/processes used in involving policy makers. • Is HBM included in their business/strategic/action plan. • State which ministry is HBM data reported to or it is being utilised. • Describe barriers e.g funding; challenges e.g. participant recruitment; opportunities e.g. enhancing cross government working and linking of env data with exposure measurements currently at play in your country with regards to HBM or other things of note. • Have any of these barriers been addressed by HBM4EU? If yes - describe. • Elaborate on issues which propelled the establishment and sustainability of your HBM programme.	

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(*continued*)

Introduction:
• Detailed information of HBM priority substance used for policy development e.g. disaster, pollution, incidence/prevalence of a health-related issue. • Give examples where the work has led to policy implementation, monitoring, or control of chemical exposures etc • Have HBM or other monitoring activities been linked or adapted. Give examples in detail. • Other players who would be beneficial in the continued support of HBM at a governmental level and working together to promote HBM in your country. • Have you used HBM4EU data e.g newsletter, videos to support policy? Future Plans • Ways/process used in maintaining the programme • What are your future plans? • Do you think PARC will be crucial to the sustainability of your HBM programme?

Appendix 4

Guideline for group 1 Lead:

NH narratives should include the following	Group Lead will then collate and include the following
Introduction:	
• Background information on the evolution and status of HBM in your country.	• State purpose of group 1 narrative. • On background information of HBM, you can use infographics to state how/when/why it evolved (maybe via HBM4EU) for each country.
Main text - results and discussion ENSURE YOUR NARRATIVES ARE REFERENCED AS FAR AS POSSIBLE	
• Description of issue(s) which have resulted in the raising of awareness. • **Include brief description of sample population, substances of concern and whether local/regional/national.**	• Write HBM data (chemical) of interest. Indicate if it is in the list of priority substances. Identify and compare case studies.
• Description of HBM programme **if it exists** e.g. implementation of a HBM module into HES	• State countries which have HBM programmes and countries which have implemented a HBM module into HES or countries which have done both.
• List ministries (Environment, Health etc.) and stakeholders advocating for HBM.	• Analyse if it is the same for all the countries or different. • Again, an infographic may be possible- % with ministries 5 with other stakeholders etc
• Policy makers involvement with HBM or level of awareness of HBM. • Give examples - specific chemicals or outcomes	• Analyse level of awareness, policy makers involvement (look at what they are mostly interested in) for each country. • Summarise with infographics if need be.
• Steps/processes needed or used to get the attention of policy makers.	• Analyse steps used by each country. Compare countries and see if a group of countries use the same method.
• Describe barriers e.g funding; challenges e.g. participant recruitment; opportunities e.g. enhancing cross government working and linking of env data with exposure measurements currently at play in your country with regards to HBM.	• List all barriers, challenges, and opportunities for all countries. • Compare and state which is more prominent. • See if you can detect why these challenges, opportunities and barriers arise.
• Have any of these barriers been addressed by HBM4EU? If yes - describe.	•See if there are any patterns emerging
• Other players who would be beneficial in raising awareness and working together to promote HBM	• List players and state why they were suggested.
Future plans -	• Evaluate the level of future work
• Are there plans to use HBM data in the future for policy or awareness - give clear examples. Will the data from HBM4EU be used?	• How may hubs will use HBM4EU data in the future?

Appendix 5

Guideline for group 2 Lead:

NH narratives should include the following	Group Lead will then collate and include the following:
Introduction:	
Background information on the evolution and status of HBM in your country.	• State purpose of group 2 narrative. • On background information of HBM, you can use infographics to state how/when/why it evolved (maybe via HBM4EU) for each country.
Main text - results and discussion ENSURE YOUR NARRATIVES ARE REFERENCED AS FAR AS POSSIBLE	
• Description of HBM programme if it exists e.g. implementation of a HBM module into HES or development of a standalone HBM programme. • Examples of HBM data for policy development.	• State countries which have HBM programmes and countries which have implemented a HBM module into HES or countries which have done both. • Compare HBM data of all countries and policies. Use infographics if needed. • These could be the case studies
• List ministries (Environment, Health etc.) and stakeholders advocating for HBM.	• Analyse if it is the same for all the countries or different. • Present the ministries and stakeholders in a pictorial format.

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NH narratives should include the following	Group Lead will then collate and include the following:
• Describe barriers e.g funding; challenges e.g. participant recruitment; opportunities e.g. enhancing cross government working and linking of env data with exposure measurements currently at play in your country with regards to HBM. • Have any of these barriers been addressed by HBM4EU? If yes - describe. • Elaborate on issues which propelled the HBM data of choice e.g. disaster, pollution, incidence/prevalence of a health-related issue Future plans - • Are there plans to increase the use of HBM data in the future for policy give clear examples. • Will the data from HBM4EU be used? • Has HBM4EU re-enforced the need for a National programme? • What are your future plans?	• List all barriers, challenges, and opportunities for all countries. • Compare and state which is more prominent. • See if you can detect why these challenges, opportunities and barriers arise. • See if there are any patterns emerging • Write HBM data (chemical) of interest. Indicate if it is in the list of HBM4EU priority substances. Identify and compare case studies. • Evaluate the level of future work • How may hubs will use HBM4EU data for policy in the future? • Will this build on current platforms or start from scratch

Appendix 6

Guideline for Group 3 Lead

NH narratives should include the following	Group Lead will collate and include the following
Introduction: Background information on the evolution and status of your National HBM programme in your country. Include year of establishment -Who pays for the programme of work? Give web links. Main text - Results and Discussion ENSURE YOUR NARRATIVES ARE REFERENCED AS FAR AS POSSIBLE	• State purpose of group 3 narrative. • On background information of HBM, you can use infographics to state how/when/why it evolved (maybe via HBM4EU or not) for each country.
• List ministries (Environment, Health etc.) and stakeholders involved in the HBM programme.	• Analyse if it is the same for all the countries or different. • Present the ministries and stakeholders in a pictorial format.
• Policy makers involvement with HBM and Steps/processes used in involving policy makers.	• Analyse policy makers involvement (look at what they are mostly interested in) access level of commitment for each country. • List the steps/processes used for their involvement and compare similarities with other countries. • Summarise with infographics if need be.
• Describe barriers e.g funding; challenges e.g. participant recruitment; opportunities e.g. enhancing cross government working and linking of env data with exposure measurements currently at play in your country with regards to HBM. • Have any of these barriers been addressed by HBM4EU? If yes - describe. • Elaborate on issues which propelled the establishment and sustainability of your HBM programme. • Detailed information of HBM priority substance used for policy development e.g. disaster, pollution, incidence/prevalence of a health-related issue. • Give examples where the work has led to policy implementation, monitoring, or control of chemical exposures etc	• List all barriers, challenges, and opportunities for all countries. • Compare and state which is more prominent. • See if you can detect why these challenges, opportunities and barriers arise. • See if there are any patterns emerging • Write HBM data (chemical) of interest. Indicate if it is in the list of priority substances. Identify and compare case studies. • Compare HBM data of all countries and policies. Use infographics if needed. • List if it was a health-related issue or environmental or occupational.
• Other players who would be beneficial in the continued support of HBM at a governmental level and working together to promote HBM in your country.	• List all and compare similarities.
Future Plans • Ways/process used in maintaining the programme • What are your future plans?	• List all and compare similarities.

Appendix 7

Activities of countries prior and within HBM4EU and ministries involved in HBM

Country	EU HBM activities prior to HBM4EU -Research	Cohorts/studies in HBM4EU	Ministries/agencies involved
Denmark	ESBIO/COPHES/DEMOCOPHES many EU projects related to genotoxicology, EDC, POPs and methods development	MiniPub, OCC, DEMOCOPHES	EPA, Food Agency; Labour Inspection, Research and Innovation; Board of Health
Estonia	Human Biomonitoring in the Oil Shale Industry Area in Estonia—Overview of Earlier Programmes and Future Perspectives		Ministry of Social Affairs, Health Board, Ministry of the Environment
Finland	EU project EDC Mix Risk Involvement in several EU-projects related to the risks of nanomaterials	FINRISK, FinHealth, Occupational	Ministry of Social Affairs and Health
Hungary	ESBIO/COPHES/DEMOCOPHES	DEMOCOPHES Specimen, In Air Quality	Ministry of Human Capacities and National Public Health Center
Iceland	Arctic monitoring program	Mercury, nutrition	Health and Environment
Ireland	ESBIO/COPHES/DEMOCOPHES		Environmental Health Service
Italy	National activities and EU projects	Analytics, statistics	Northern Adriatic cohort, occupational
Lithuania	Children		Ministry of Health
Luxembourg	ESBIO/COPHES/DEMOCOPHES	DEMOCOPHES Occupational	Ministry of Health
Netherlands	Metals, PFAS, Pesticides, occupational	Occupational, Specimen, Dutch Youth cohort	Ministry of Health, Welfare and Sports, occupational studies fall under the Ministry of Social Affairs
Norway	National platform since 1980 and EU projects. COPHES	MobA, IES, NEBII	Ministry of Health and Care

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Country	EU HBM activities prior to HBM4EU -Research	Cohorts/studies in HBM4EU	Ministries/agencies involved
Poland	ESBIO/COPHES/DEMOCOPHES	Occupational	Lodz
Portugal	ESBIO/COPHES/DEMOCOPHES Multiple national projects	Mercury, INSEF-ExpoQuim occupational	Ministries of Health, Environment and Science
Slovenia	ESBIO/COPHES/DEMOCOPHES EU Project: PHIME, CROME	DEMOCOPHES, HBM, SLOCRP	Chemicals Office (CORS) as part of Ministry of Health
Switzerland	DEMOCOPHES	Health Study	Swiss Federal Office of Public Health (FOPH), Food safety and vet (FSVO), Environment (FOEN)
United Kingdom	ESBIO/COPHES/DEMOCOPHES	Occupational	Health and Safety Executive, UKHSA, EA, Department of Health & Social Care (DHSC) and DEFRA

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Assessing the impact of coexposure on the measurement of biomarkers of exposure to the pyrethroid lambda-cyhalothrin in agricultural workers

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ABSTRACT

There are few published data on the impact of combined exposure to multiple pesticides (coexposure) on levels of biomarkers of exposure in workers, which may alter their toxicokinetics and thus the interpretation of biomonitoring data. This study aimed to assess the impact of coexposure to two pesticides with shared metabolism pathways on levels of biomarkers of exposure to pyrethroid pesticides in agricultural workers. The pyrethroid lambda-cyhalothrin (LCT) and the fungicide captan were used as sentinel pesticides, since they are widely sprayed concomitantly in agricultural crops. Eighty-seven (87) workers assigned to different tasks (application, weeding, picking) were recruited. The recruited workers provided two-consecutive 24-h urine collections following an episode of lambda-cyhalothrin application alone or in combination with captan or following tasks in the treated fields, as well as a control collection. Concentrations of lambda-cyhalothrin metabolites – 3-(2-chloro-3,3,3-trifluoroprop-1-en-1-yl)-2,2-dimethyl-cyclopropanecarboxylic acid (CFMP) and 3-phenoxybenzoic acid (3-PBA) – were measured in the samples. Potential determinants of exposure established in a previous study, including the task performed and personal factors were documented by questionnaire. Multivariate analyses showed that coexposure did not have a statistically significant effect on the observed urinary levels of 3-PBA (Exp (β) (95% confidence interval (95% CI)): 0.94 (0.78–1.13)) and CFMP (1.10 (0.93–1.30). The repeated biological measurements ("time variable") – defined as the within-subjects variable – was a significant predictor of observed biological levels of 3-PBA and CFMP; the within-subjects variance (Exp(β) (95% (95% CI)) for 3-PBA and CFMP was 1.11 (1.09–3.49) and 1.25 (1.20–1.31). Only the main occupational task was associated with urinary levels of 3-PBA and CFMP. Compared to the weeding or picking task, the pesticide application task was associated with higher urinary 3-PBA and CFMP concentrations. In sum, coexposure to agricultural pesticides in the strawberry fields did not increase pyrethroid biomarker concentrations at the exposure levels observed in the studied workers. The study also confirmed previous data suggesting that applicators were more exposed than workers assigned to field tasks such as weeding and picking.

1. Introduction

The assessment of risks associated with pyrethroid exposure is among the priorities of major government agencies such as Health Canada, the United States Environmental Protection Agency (U.S. EPA), and the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), as several *in vitro* cellular and *in vivo* animal studies have shown that exposure to repeated high doses of these chemicals induces early biological alterations, such as oxidative stress, immune alterations, and endocrine disruption (Barrón Cuenca et al., 2019; Costa et al., 2013; El Okda et al., 2017; Lee et al., 2020; Ravula and Yenugu, 2021; Shearer et al., 2019; Wang et al., 2016; Zepeda-Arce et al., 2017). Cases of acute intoxication or incidents in workers exposed to pyrethroids have also been reported, including respiratory and neurological symptoms (Amoatey et al., 2020; Curl et al., 2020; de Graaf et al., 2022; Ismail et al., 2017; Lucero and Muñoz-Quezada, 2021; Mattila et al., 2021; Ratanachina et al., 2020). The U.S. EPA has classified some pyrethroids, including permethrin, as possibly carcinogenic to humans, based on observations of (benign) lung and liver tumors in mice exposed to high doses, although these findings are not supported

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by available epidemiological studies (Burns and Juberg, 2021; De Roos et al., 2021; U.S. EPA 2018). It is therefore important to develop and apply tools to properly evaluate and control exposure to these contaminants, which are still insufficiently evaluated in some agricultural workplaces. To assess internal exposure to pesticides such as pyrethroids, urinary measurements of metabolites is considered a preferred tool (Arcury et al., 2018; Buchholz et al., 2021; Curl et al., 2021; Maule et al., 2019).

However, our latest work has raised the issue of multiple exposures to several pesticides and the impact that this coexposure (concomitant or combined exposure on the same day or on sequential days (OECD, 2018)) could have on the interpretation of biomonitoring data used to assess exposure to pyrethroids (Bossou et al., 2020; Bouchard et al., 2016; Khemiri et al., 2017; Ratelle et al., 2015a, 2015b). Currently, there is a lack of data on the impact of multiple pesticide coexposure on levels of exposure biomarkers to commonly used pyrethroids in agricultural settings.

Overall, the data published in the scientific literature on the impact of coexposure on the biological behavior of pyrethroids and their metabolites used as biomarkers of exposure are very limited in real-life context such as agricultural settings. Experimentally or in controlled studies, some studies reported decreased urinary excretion of pyrethroid exposure biomarkers in animals or volunteers coexposed with organophosphate insecticides (Hirosawa et al., 2011; Sams and Jones, 2011; Wielgomas and Krechniak, 2007). However, the doses administered were relatively high in relation to worker exposure levels and the exposure scenarios (pyrethroid/organophosphate coexposure) were not very representative of the exposure context of workers. Recently, our team experimentally evaluated the influence of coexposure to the fungicide captan on the kinetic profiles of the main metabolites of the pyrethroid lambda-cyhalothrin in a rat study, 3-(2-chloro-3,3,3-trifluoroprop-1-en-1-yl)-2,2-dimethyl-cyclopropanecarboxylic acid (CFMP), 3-phenoxybenzoic acid (3-PBA) and 4-hydroxy-3-phenoxybenzoic acid (4-OH3PBA) (Bossou et al., 2020). The *a priori* hypothesis was that captan could interfere with the CYP450 metabolism pathway of pyrethroids or the excretion mechanisms (Paolini et al., 1999) in a dose-dependent manner. More specifically, lambda-cyhalothrin metabolism to its main metabolites used as biomarkers of exposure (CFMP and 3-PBA) is catalyzed by CYP450 enzymes also implicated in the metabolism of captan (Kaneko, 2011; Paolini et al., 1999; Scollon et al., 2009). The results of the animal study of Bossou et al. (2020) showed that captan and lambda-cyhalothrin coexposure resulted in a trend toward lower levels of metabolite excretion, in particular on the benzyl metabolite pathway leading to 3-PBA formation. This was observed in the higher dose group exposed to the "Lowest-Observed Adverse Effect Level" (LOAEL) of 12.5 mg/kg bw/day reported by the U.S. EPA (2004) but not in the lower exposure group exposed to the "No-Observed Adverse Effect Level" (NOAEL) of 2.5 mg lambda-cyhalothrin/kg bw/day. Again, these animal results cannot be directly extrapolated to humans for interpretation of biomonitoring data because the experimental exposure doses were much higher than those estimated under human occupational exposure conditions (<1 μg/kg bw/day) (Chester et al., 1992). In addition, there may be interspecies differences in toxicokinetics.

In real-life exposure situations in workers, the impact of coexposure to different pesticides in workers on variability in levels of biomarkers of exposure, relative to other factors, remains to be verified. In order to properly interpret the significance of a measurement of these exposure biomarkers, it becomes necessary to fully understand the influence of parameters such as coexposure to other pesticides. We hypothesize that, at a certain exposure level, combined exposure to multiple pesticides may alter biomarker concentrations in urine used to assess internal exposure in agricultural workers and hence may have an impact on the interpretation of biomonitoring data. This research thus specifically aimed to evaluate the impact of coexposure on biomarkers of exposure to pyrethroid pesticides in agricultural workers and to identify the contribution of this factor to the variability in biological monitoring data. The pyrethroid lambda-cyhalothrin (LCT) and the fungicide captan were used as sentinel pesticides, since they are widely sprayed concomitantly on agricultural crops and share common metabolism pathways (Paolini et al., 1999).

2. Methods

2.1. Study population, crops and targeted pesticide active ingredients

A biomonitoring study was conducted in agricultural workers exposed to lambda-cyhalothrin alone or in combination with captan. Strawberry workers were targeted because this crop represents an important production in Quebec; it involves a large number of workers and pyrethroids and fungicides are widely used in these fields (MAPAQ, 2020, 2021). The workers were recruited using the Quebec Directory of Horticultural Producers obtained from the Quebec Fruit and Vegetable Growers' Association. From this list of farms organized by city and crop type, strawberry farm owners within a 100 km radius from the University of Montreal were contacted by telephone (using a standard text) to assess their willingness to solicit their field workers to participate in the study.

A total of 87 workers assigned to different tasks (application, weeding, strawberry picking) were recruited, and evaluated under their usual working conditions. The target workers were exposed to lambda-cyhalothrin alone, or alternatively, in combination with captan. In the case of combined exposure in applicators, lambda-cyhalothrin and captan were mixed and spayed at the same time; in the case of field workers, they entered an area previously treated with both chemicals. This sample size was based on the number of workers used in a previous study, which assessed the impact of various personal factors and exposure determinants on the kinetics of biomarkers of exposure to cypermethrin in vegetable crop workers by statistical multivariate analysis (Ratelle et al., 2016).

The recruitment strategy used for this study was the same as that described in Bossou et al. (2022). Eligibility criteria were: i) the worker anticipated being exposed to formulations containing the active ingredient lambda-cyhalothrin (Matador®, Silencer®, Demand CS®, Warrior®) alone or in combination with captan fungicide (Captan®, Supra Captan 80 WDG®, Captan 80-WP®, Maestro®) during the summer as part of their normal activities; ii) they were willing to provide a 24-h urine collection prior to application (-24 - 0 h prior to application) and two consecutive 24-h urine collections (0–24 h and 24–48 h) following the onset of an exposure episode (after spraying the pesticide or working in a treated field).

Subjects who participated in the study signed a free and informed consent form after receiving all necessary information about the project. Each participant was free to withdraw at any time. The study protocol, consent form, and other relevant documents were approved by the Clinical Research Ethics Committee (CERC) of the Université de Montréal. The anonymity of the subjects was also respected by coding the samples.

2.2. Urine collections and measurement of exposure biomarkers

Recruited workers were asked to provide a first full 24-h urine collection prior to an exposure episode to establish baseline exposure levels as well as two consecutive 24-h urine collections following an episode of lambda-cyhalothrin spaying alone or in combination with captan or tasks in treated fields (weeding and strawberry picking). Each 24-h samples were collected in 1.5 L polypropylene Nalgene® bottles. Workers were asked to write down the date and time of each micturition on the identification label affixed to the Nalgene collection bottles. The samples collected were kept in coolers with ice packs provided by our team. Samples were picked up on a daily basis at the workplace by a member of our research team and directly brought to the laboratory

where urine volumes were measured using graduated cylinders. On days when the team were not able to arrive before the end of the workday, workers transferred samples into the farm cold room until our team members arrived. Two aliquots of 120 mL per collection bottle were prepared and placed in polypropylene Sarstedt tubes for storage at – 20 °C until analysis.

All participants were well-informed of the importance of complete urine collections without omissions. They were also compensated for their time and efforts to ensure compliance to the protocol and limit the proportion of incomplete urine collection. However, one participant failed to collect all his 24 h-urine samples and was excluded in analyses.

The urine samples (5 mL) were subjected to an enzyme hydrolysis with β-glucuronidase/arylsulfatase enzyme to obtain the sum of free and glucurono- and sulfo-conjugated metabolites followed by solid-phase extraction where the metabolites were recovered in methanol (1 mL). The concentrations of lambda-cyhalothrin metabolites used as biomarkers of exposure, 3-(2-chloro-3,3,3-trifluoroprop-1-en-1-yl)-2,2-dimethyl-cyclopropanecarboxylic acid (CFMP; otherwise known as ClF_3CA) and 3-phenoxybenzoic acid (3-PBA), were then analyzed in the methanolic extracts using a high performance liquid chromatography coupled to triple quadripole (QQQ) mass spectrometry (UHPLC-MS/MS) method validated in our laboratory and published elsewhere (Bossou et al., 2022; Khemiri et al., 2018).

The limit of detection (LOD) was calculated to be 0.7–2.5 pmol/mL of methanolic extract for 3-PBA and 3.9–6 pmol/mL for CFMP. The limit of quantification (LOQ), which represents the lowest level of the calibration curve that is quantified with a less than 20% error, was 6 pmol/mL and 12 pmol/mL of methanolic extract for 3-PBA and CFMP, respectively. Corresponding LOQ values in urine are 1–2 pmol/mL of urine considering that 5 mL of urine were analyzed and the residue obtained after solid-phase extraction and evaporation was redissolved in 1 mL of methanol. This method allowed quantifying 3-PBA and CFMP metabolites in 88% and 43% of the analyzed samples, respectively.

Creatinine concentrations were also measured in urine using the Jaffé method, an alkaline picric acid method with deproteinization (PAP enzymatic colorimetric assay from Boehringer Mannheim, Germany). Concentrations of lambda-cyhalothrin metabolites corrected for creatinine (μmol/mol creatinine) were then established for each sample. For values below the LOQ, they were treated using a robust method called "regression on order statistics" (ROS) (Helsel, 2005). This method was used because it is less sensitive to small sample sizes, low censoring percentages and is more resistant to non-normality in the data (Huston and Juarez-Colunga, 2009).

2.3. Questionnaires and field observations

Potential determinants of metabolite levels used as biomarkers of exposure or potential confounders were assessed by questionnaire in addition to coexposure to lambda-cyhalothrin and captan. These included the main work tasks performed, work practices and hygiene, personal protective equipment as well as personal factors and lifestyle habits (see supplementary file S1). Main questions were used in a previous work on cypermethrin exposure in workers (Ratelle et al., 2016). Members of the team also conducted field observations during the first day of exposure. On the third day post-exposure, research team members also checked the daily questionnaire responses, and participants were asked to record any missing urine collection.

2.4. Data analysis

The impact of coexposure on variations in urinary levels of exposure biomarkers was established using *linear mixed effects models* (MIXM). The focus was on 3-PBA which is the most measured metabolite of pyrethroids and CFMP which is more specific. The potential determinants of exposure established in a previous study (Ratelle et al., 2016), including the task performed and personal factors, were considered in the models. Specifically, the subject variable was set as a *random effect* and a *compound symmetry* covariance *structure among repeated measurements* was considered. The levels of CFMP and 3-PBA metabolites expressed as concentrations (μmol/mol creat.) were considered as dependent variables in the models. Since the biomarker levels showed a log-normal distribution and not normal after analysis by the Kolmogorov-Smirnov test, the exposure biomarker levels (CFMP and 3-PBA) were expressed as log-transformed values to obtain a normal distribution with constant variance.

Potential determinants of biological levels of 3-PBA and CFMP were considered, including: 1) coexposure, *i.e.* exposure to lambda-cyhalothrin alone or lambda-cyhalothrin in combination with captan; 2) main occupational task performed (application including mix preparation and equipment cleaning, weeding, or picking); 3) time since the onset of lambda-cyhalothrin spraying alone or in combination with captan ($\leq$7 days and >7 days); and 4) farm size ($\leq$10 workers or >10 workers). Potential confounding variables considered in the models included age (log-transformed continuous variable in years), body mass index (log-transformed continuous BMI), ethnicity (Caucasian or Latino American), education (primary/high school or college/university), alcohol use (yes/no during the study period), cigarette smoking (yes/no), ibuprofen or acetaminophen use, other medication use.

Associations between biomarker levels (3-PBA or CFMP metabolites) in urine of farmworkers and potential determinants of exposure including the influence of coexposure or potential confounding factors were initially assessed in univariate models (explanatory variables considered one by one in the models). Multivariate models were then constructed by first inserting all variables and then sequentially subtracting those that did not contribute to the model according to the Akaike's information criterion (AIC) following the approach proposed by Zuur et al. (2009). Only predictors and confounders contributing to the fit of the multivariate models to the data were retained in the final models. Statistical analyses were performed using SPSS plus (SPSS Inc, Chicago). The level of statistical significance for the final multivariate models was set at $p \leq 0.05$.

3. Results

3.1. Study of the impact of coexposure versus other factors on the measurement of biomarkers of exposure in workers

After a screening telephone interview in early 2019 and 2020 to identify potentially eligible workers followed by a farm visit to recruit farmers, a total of 87 workers of strawberry fields – where lambda-cyhalothrin alone or in combination with captan was sprayed –signed informed consent to participate in the study. They provided urine samples during summer 2019 and 2020. Workers were recruited from 13 farms in three regions of the Province of Quebec (Montérégie, Laurentides, Lanaudière) and the main professionals tasks performed were spraying of pesticides (lambda-cyhalothrin or lambda-cyhalothrin combined with captan), weeding or strawberry picking in a treated area. In total, 70 of the recruited workers had an exposure episode to lambda-cyhalothrin alone and 49 had an exposure episode to lambda-cyhalothrin in combination with captan over the two-year study period. Some workers were thus monitored more than once since they performed different tasks in the fields (weeding versus picking) or were evaluated for more than one exposure scenario (exposure to lambda-cyhalothrin alone or in combination with captan) at different periods.

Table 1 shows the main personal characteristics of the 87 workers included in the study who provided serial urine collections following an exposure episode to lambda-cyhalothrin alone (70 workers) or in combination with captan (49 workers). A participant may have been evaluated for more than one task or more than one exposure scenario. Only 1% of all study participants were women and 82% of them were of Latin origin (from Guatemala, Honduras and Mexico); the median age was 33 years and their education level was low, with 84% reporting a high

Table 1
Characteristics of participants.

	All participants (n = 87[a])	LCT exposed (n = 70[b])	LCT + Captan exposed (n = 49[c])
Sex: n (%)			
Women	1 (1.1%)	0 (0%)	1 (2.0%)
Men	86 (98.9%)	70 (100%)	48 (98.0%)
Age: years			
Average (SD)	34.8 (10.2)	34.1 (9.91)	36.3 (10.2)
Median [Min, Max]	33.0 [20.0, 64.0]	31.5 [21.0, 64.0]	34.0 [20.0, 64.0]
Age categories: years (%)			
20–30	35 (40.2%)	31 (44.3%)	15 (30.6%)
31–40	30 (34.5%)	22 (31.4%)	22 (44.9%)
≥ 41	22 (25.3%)	17 (24.3%)	12 (24.5%)
Body weight: kg			
Average (SD)	72.3 (14.0)	71.3 (12.4)	74.3 (14.2)
Median [Min, Max]	70.0 [50.0, 127]	70.0 [50.0, 125]	71.2 [54.5, 127]
Height: cm			
Average (SD)	165 (9.38)	165 (9.34)	167 (9.19)
Median [Min, Max]	162 [150, 198]	162 [150, 198]	165 [155, 189]
BMI: kg/m^2			
Average (SD)	26.4 (4.11)	26.1 (2.97)	26.6 (4.60)
Median [Min, Max]	26.3 [17.9, 52.5]	26.2 [17.9, 34.1]	26.3 [21.2, 52.5]
BMI categories: kg/m^2 (%)			
< 24.9	30 (34.5%)	25 (35.7%)	17 (34.7%)
25–29.9	46 (52.9%)	38 (54.3%)	26 (53.1%)
≥ 30	11 (12.6%)	7 (10.0%)	6 (12.2%)
Country of birth: n (%)			
Bosnia	1 (1.1%)	0 (0%)	1 (2.0%)
Canada	15 (17.2%)	11 (15.7%)	10 (20.4%)
Guatemala	35 (40.2%)	33 (47.1%)	13 (26.5%)
Honduras	24 (27.6%)	16 (22.9%)	20 (40.8%)
Mexico	12 (13.8%)	10 (14.3%)	5 (10.2%)
Ethnicity: n (%)			
Caucasian	16 (18.4%)	11 (15.7%)	11 (22.4%)
Latino American	71 (81.6%)	59 (84.3%)	38 (77.6%)
Language: n (%)			
French	16 (18.4%)	11 (15.7%)	11 (22.4%)
Spanish	71 (81.6%)	59 (84.3%)	38 (77.6%)
Education: n (%)			
High school or less	73 (83.9%)	59 (84.3%)	39 (79.6%)
College and University	14 (16.1%)	11 (15.7%)	10 (20.4%)

[a] Number of participants included in the study.
[b] Number of participants who provided biological samples following application of lambda-cyhalothrin alone. A participant may have been evaluated for more than one task or more than one exposure scenario.
[c] Number of participants who provided biological samples following application of lambda-cyhalothrin in combination with captan. A participant may have been evaluated for more than one task or more than one exposure scenario.

school level or less as the highest education level. In addition, during the biological sampling period, 13% of the workers (11 workers out of 87) reported smoking tobacco; 16% (14 workers out of 87) reported consuming alcohol during at least one of their biological follow-ups; 13% (11 workers out of 87) mentioned taking ibuprofen or acetaminophen; and 9% (8 workers out of 87) reported taking other types of medication.

In terms of biological follow-up, considering that some workers provided more than one set of urine collections, tobacco use was reported for 10% of the biological follow-ups (i.e., for 14 of the 139 biological follow-ups); alcohol use was indicated for 12% of the biological follow-ups (16 out of 139 biological follow-ups); ibuprofen or acetaminophen use was reported in 10% of the follow-ups (14 out of 139 follow-ups) and other types of medication were reported in 7% of the follow-ups (10 out of 139 follow-ups).

The consumption of fruits, vegetables and cereals (number of servings according to Canada's Food Guide (2011)) was documented by questionnaire, but this variable was not considered in the results, as workers did not appear to be able to adequately answer these questions. Only one person reported the use of lice treatment and no participants reported the use of animal treatment or the use of pesticides for residential purposes. None of the participants reported signs or symptoms (as mentioned in supplementary file S1) that, although not specific, could be associated with exposure to this type of pesticide.

While most workers wore long pants and long-sleeved shirts and boots, only a proportion of workers wore gloves, goggles, and a hat (Table 2). Although only 45% of workers wore gloves, no significant effect of glove wearing on urinary levels of CFMP and 3-PBA was observed ($p > 0.05$). Wearing goggles and hats was also not significantly associated with urinary levels of CFMP and 3-PBA. Other personal protective clothing or equipment (mask, raincoat, scarf) were worn by only a small number of workers such that the association between wearing the latter and urinary levels of CFMP and 3-PBA was not tested.

Table 3 presents the distribution of urinary concentrations of CFMP, the more specific metabolite of lambda-cyhalothrin, for all participants and for participants stratified by exposure group, either exposure to lambda-cyhalothrin alone or in combination with captan. The results show that the distribution of CFMP values was similar for all groups in

Table 2
Protective equipment (PPE) for all participants as well as for participants stratified by exposure group (exposure to lambda-cyhalothrin alone or combination with captan).

Type of PPE	Population			P-value
	All[a]	Exposure to the LCT[b]	Exposure to LCT + Captan[b]	
Long pants: n (%)				
Yes	125 (89)	78 (90)	47 (89)	0.857
No	15 (11)	9 (10)	6 (11)	
Long sleeve shirt: n (%)				
Yes	121 (86)	72 (83)	49 (92)	0.106
No	19 (14)	15 (17)	4 (8)	
Hat: n (%)				
Yes	94 (67)	60 (69)	34 (64)	0.558
No	46 (33)	27 (31)	19 (36)	
Goggles: n (%)				
Yes	22 (16)	8 (9)	14 (26)	0.007
No	118 (84)	79 (91)	39 (74)	
Scarf: n (%)				
Yes	2 (1)	1 (1)	1 (2)	NA
No	138 (99)	86 (99)	52 (98)	
Raincoat: n (%)				
Yes	15 (11)	10 (11)	5 (9)	0.703
No	125 (89)	77 (89)	48 (91)	
Gloves: n (%)				
Yes	63 (45)	27 (31)	36 (68)	0.00002
No	77 (55)	60 (69)	17 (32)	
Boots: n (%)				
Yes	111 (79)	70 (80)	41 (77)	0.662
No	29 (21)	17 (20)	12 (23)	

[a] The n is the number of biomonitoring for all participants across all tasks (application, weeding, and harvesting) and all exposure scenarios (lambda-cyhalothrin alone versus lambda-cyhalothrin in combination with captan). A participant may have been evaluated for more than one task or more than one exposure scenario. The percentage represents the proportion of workers who reported wearing the equipment over the entire biological monitoring.
[b] The n is the number of biomonitoring for all participants across all tasks (application, weeding and harvesting) but for a given exposure scenario (lambda-cyhalothrin alone or in combination with captan). A participant may have been assessed for more than one task. The percentage represents the reported proportion of workers who reported wearing the equipment for all biological monitoring but for a given exposure scenario.

Table 3
Distribution of CFMP concentrations in urine for all participants as well as for participants stratified by exposure group (exposure to lambda-cyhalothrin alone or in combination with captan).

Time since the onset of an exposure episode (h)	Exposure group	N of samples[a]	CFMP concentration (μmol/mol creat.)						
			Geometric mean	Percentile					
				5th	10th	25th	50th	75th	95th
−24–0[b]	All	134	0.132	0.011	0.018	0.067	0.180	0.290	0.776
	LCT	85	0.141	0.012	0.025	0.076	0.182	0.309	0.799
	LCT + Captan	49	0.117	0.010	0.015	0.053	0.173	0.259	0.691
0–24	All population	138	0.146	0.010	0.023	0.056	0.147	0.389	1.598
	LCT	85	0.132	0.012	0.024	0.061	0.129	0.295	1.417
	LCT + Captan	53	0.173	0.008	0.018	0.039	0.180	0.780	2.829
24–48	All	135	0.122	0.019	0.031	0.063	0.133	0.281	0.582
	LCT	84	0.115	0.013	0.028	0.061	0.139	0.267	0.554
	LCT + Captan	53	0.136	0.028	0.035	0.064	0.131	0.309	0.620

[a] Some workers performed biological monitoring for more than one exposure scenario. This number represents the number of biological samples per exposure period and exposure scenario.
[b] Control urine collection in workers to be assessed for exposure to LCT or LCT + captan.

control urine as well as in 24–48 h urine collections post-exposure (for all participants, participants exposed to lambda-cyhalothrin alone and participants exposed to lambda-cyhalothrin plus captan). In 0–24 h collections post-exposure, CFMP metabolite values for the upper extremes of the distribution (75th and 95th percentiles in the table) were higher than in control urine or in urine collected 24–48 h after the onset of an exposure period (application or working in a field treated with lambda-cyhalothrin alone or in combination with captan). For the extremes of the distribution (75th and 95th percentiles), CFMP values in urine collected after the onset of an exposure episode to lambda-cyhalothrin combined with captan were also higher than in urine collected after exposure to lambda-cyhalothrin alone. Table 4 shows that the trend was the same for 3-PBA metabolite, which is not specific to lambda-cyhalothrin as it is a common metabolite of several pyrethroids. However, exceptionally, a high 3-PBA value was obtained for the 95th percentile of the distribution in control urine of workers to be later assessed for exposure to lambda-cyhalothrin plus captan.

Tables 5 and 6 present the potential determinants of urinary 3-PBA and CFMP levels in exposed strawberry workers, in particular the variable "exposure group" or so called "coexposure" (exposure to lambda-cyhalothrin alone or in combination with captan) but also potential confounding factors documented by questionnaire. Urinary metabolite levels showed a log-normal distribution as did age and body mass index (BMI); univariate and multivariate statistical analyses were therefore performed on the log-transformed values for these variables. When considered individually in the univariate model, age, ethnicity, education, farm size, main occupational task, and time since pesticide application showed a statistically significant association ($p < 0.05$) with 3-PBA levels and, in the case of CFMP, ibuprofen or acetaminophen use. For the 3-PBA, using the linear mixed effects model (MIXM), farm size, main occupational task and time since pesticide application as well as alcohol consumption, ibuprofen or acetaminophen use, and other medication use were retained for adjustment of the multivariate model, according to the Akaike's information criterion (AIC). These variables were considered as variables contributing to the final multivariate model. The variable "time," representing repeated measurements and defined as a within-subjects variable, was a significant predictor of observed biological levels of 3-PBA; the within-subjects variance (95% confidence interval (95% CI)) was 1.11 (1.09–3.49), $p < 0.001$. However, coexposure did not have any statistically significant effect on observed urinary 3-PBA levels (0.94 (0.78–1.13); $p = 0.48$). Table 5 shows that the main occupational task (pesticide application, weed control, or picking), time since exposure, and farm size were the main three predictors of observed biological levels in the final model. Compared to the picking task, the weeding and pesticide spraying tasks were overall associated with higher urinary 3-PBA concentrations ($p < 0.05$). However, the pesticide application task had a greater effect than the weeding task. No statistically significant associations ($p < 0.05$) were detected with the other factors assessed in the multivariate model (alcohol consumption, ibuprofen or acetaminophen use, or other

Table 4
Distribution of 3-PBA concentrations in urine for all participants as well as for participants stratified by exposure group (exposure to lambda-cyhalothrin alone or in combination with captan).

Time since the beginning of the exposure (h)	Exposure group	N of samples[a]	3-PBA concentration (μmol/mol creat.)						
			Geometric mean	Percentile					
				5th	10th	25th	50th	75th	95th
−24–0[b]	All	134	0.125	0.016	0.034	0.059	0.129	0.231	0.670
	LCT	85	0.136	0.015	0.029	0.082	0.151	0.263	0.589
	LCT + Captan	49	0.107	0.018	0.034	0.044	0.101	0.175	1.979
0–24	All population	138	0.151	0.021	0.032	0.081	0.160	0.260	1.418
	LCT	85	0.146	0.022	0.040	0.084	0.161	0.247	0.998
	LCT + Captan	53	0.159	0.015	0.031	0.067	0.148	0.322	3.020
24–48	All	135	0.129	0.014	0.029	0.057	0.133	0.305	0.729
	LCT	84	0.122	0.015	0.025	0.058	0.138	0.263	0.647
	LCT + Captan	51	0.140	0.010	0.030	0.057	0.104	0.408	2.064

[a] Some workers performed biological monitoring for more than one exposure scenario. This number represents the number of biological samples per exposure period and exposure scenario.
[b] Control urine collection in workers to be assessed for exposure to LCT or LCT + captan.

Table 5
Predictors of 3-PBA levels in workers' urine using a linear mixed effects model (MIXM).

Predictors		3-PBA concentration (µmol/mol creat.) (n = 139)[a]		
		Univariate Analysis [b,c]	Multivariate analysis [c,d,e]	
		Exp(β) (CI95%)	Exp(β) (CI95%)	P-value
Coexposure	LCT	1.02 (0.87–1.18)	0.94 (0.78–1.13)	0.48
	LCT + captan	Reference	Reference	
Age	Years (log)	1.93 (1.03–3.61)		
BMI	(kg/m^2) (log)	0.49 (0.13–1.89)		
Ethnicity	Caucasian	1.36 (1.12–1.65)		
	Latino American	Reference		
Education	Primary or High school	0.74 (0.62–0.90)		
	College or University	Reference		
Alcohol consumption	No	0.97 (0.76–1.23)	1.15 (0.90–1.45)	0.26
	Yes	Reference	Reference	
Cigarette consumption	No	0.81 (0.63–1.05)		
	Yes	Reference		
Ibuprofen or acetaminophen	No	1.18 (0.92–1.53)	1.14 (0.82–1.60)	0.43
	Yes	Reference	Reference	
Other medications	No	0.84 (0.62–1.13)	0.82 (0.60–1.12)	0.21
	Yes	Reference	Reference	
Size of the farm	≤10 workers	1.23 (1.01–1.51)	0.69 (0.50–0.95)	0.02
	>10 workers	Reference	Reference	
Main professional task	Pesticide application	1.38 (1.13–1.67)	2.49 (1.74–3.56)	<0.001
	Weeding	0.89 (0.76–1.04)	1.29 (1.00–1.67)	0.053
	Picking	Reference	Reference	
Time since pesticide application	≤7 days	0.87 (0.75–1.01)	0.56 (0.44–0.71)	<0.001
	>7 days	Reference	Reference	

[a] This number represents the number of biological monitoring for all participants across all tasks (application, weeding, and harvesting) and all exposure scenarios (lambda-cyhalothrin alone or in combination with captan). A participant may have been evaluated for more than one task or more than one exposure scenario.
[b] All variables were tested individually in the model. The variable "time" (urine collections at -24-0, 0–24, and 24–48 h following an exposure episode) was considered a repeated measure.
[c] The β estimates and 95% CIs were exponentially transformed from log values.
[d] The variable "time" was considered a repeated measure and a within-subject variable in the multivariate model. The within-subject variance (95% CI) was: 1.11 (1.09–3.49); $p < 0.001$.
[e] Intra-class correlation coefficient (ICC) was 0.54.

Table 6
Predictors of CFMP levels in workers' urine using a linear mixed effects model (MIXM).

Predictors		CFMP concentration (µmol/mol creat.) (n = 139)[a]		
		Univariate Analysis[b,c]	Multivariate analysis[c,d,e]	
		Exp(β) (CI95%)	Exp(β) (CI95%)	P-value
Coexposure	LCT	0.966 (0.84–1.10)	1.10 (0.93–1.30)	0.26
	LCT + captan	Reference	Reference	
Age	Years (log)	0.964 (0.55–1.69)		
BMI	(kg/m^2) (log)	1.837 (0.55–6.10)		
Ethnicity	Caucasian	1.050 (0.88–1.26)		
	Latino American	Reference		
Education	Primary or high school	0.875 (0.88–1.26)		
	College or University	Reference		
Alcohol consumption	No	1.038 (0.85–1.27)	1.13 (0.91–1.40)	0.26
	Yes	Reference	Reference	
Cigarette consumption	No	1.133 (0.91–1.41)		
	Yes	Reference		
Ibuprofen or acetaminophen	No	0.771 (0.62–0.96)	0.80 (0.59–1.08)	0.14
	Yes	Reference	Reference	
Other medications	No	1.018 (0.79–1.30)	1.22 (0.92–1.63)	0.16
	Yes	Reference	Reference	
Size of the farm	≤10 workers	1.041 (0.87–1.25)	0.94 (0.70–1.27)	0.69
	>10 workers	Reference	Reference	
Main professional task	Pesticide application	1.17 (0.98–1.40)	1.41 (1.01–1.96)	0.04
	Weeding	1.04 (0.91–1.21)	1.13 (0.89–1.43)	0.30
	Picking	Reference	Reference	
Time since pesticide application	<7 days	1.09 (0.95–1.24)	1.04 (0.84–1.30)	0.70
	>7 days	Reference	Reference	

[a] This number represents the number of biological monitoring for all participants across all tasks (application, weeding and harvesting) and all exposure scenarios (lambda-cyhalothrin alone or in combination with captan). A participant may have been evaluated for more than one task or more than one exposure scenario.
[b] All variables were tested individually in the model. The variable "time" (urine collections at -24-0, 0–24 and 24–48 h) was considered a repeated measure.
[c] The β estimates and 95% CIs were exponentially transformed from log values.
[d] The variable "time" was considered a repeated measure and a within-subject variable in the multivariate model. The within-subject variance (95% CI) was: 1.25 (1.20–1.31); $p < 0.001$.
[e] Intra-class correlation coefficient (ICC) was 0.20.

medications).

For CFMP, farm size, main occupational task, and time since pesticide application as well as alcohol consumption, ibuprofen or acetaminophen use, and other medication use were also included in the multivariate MIXM model, using Akaike's information criterion (AIC). The variable "time", representing repeated measurements and defined as a within-subject variable, was a significant predictor of the observed urinary CFMP levels; the within-subject variance (95% CI) was 1.25 (1.20–1.31), $p < 0.001$. However, coexposure had no significant effect on observed urinary CFMP levels (1.10 (0.93–1.30); $p = 0.26$) (Table 6).

In the final model, only the main occupational task (pesticide application, weeding or picking), was significantly associated with observed urinary CFMP levels. Compared to the weeding or picking task, the pesticide application task was overall associated with higher urinary CFMP concentrations. No statistically significant associations ($p < 0.05$) were detected with the other factors assessed in the multivariate model (farm size, time since pesticide application, alcohol consumption, ibuprofen or acetaminophen use, or other medications).

4. Discussion

4.1. Impact of coexposure and other factors on urinary 3-PBA and CFMP levels

This study is the first to assess the impact of pyrethroid-fungicide (lambda-cyhalothrin-captan) coexposure on levels of urinary biomarkers of exposure in workers, while accounting for other determinants. It is also the first to assess the impact of the work tasks on levels of biomarkers of exposure to lambda-cyhalothrin. Multivariate statistical analyses showed that coexposure to lambda-cyhalothrin and captan was not a significant contributor to the variability in CFMP or 3-PBA concentrations in urine, used as biomarkers of exposure. Only the main task was consistently associated with variations in urinary levels of CFMP and 3-PBA; the pesticide application task was associated with higher levels of metabolites in urine compared to the weeding or picking tasks. These results are very similar to those reported in a previous study on exposure to another pyrethroid, cypermethrin, which is metabolized to 3-PBA, as is lambda-cyhalothrin (Bouchard et al., 2019; Ratelle et al., 2016). In the latter study, some individually assessed personal or occupational characteristics were associated with the excretion of the metabolite 3-PBA after exposure to cypermethrin, but only the main occupational task was associated with the excretion of exposure biomarkers in the multivariate MIXM model. Similar to the present study, cypermethrin pesticide applicators had higher overall urinary levels of 3-PBA than workers performing tasks such as weeding, harvesting, or inspecting fields in an area treated with this pesticide.

In a German study, Hardt and Angerer (2003) also reported higher levels of the pyrethroid metabolites DCCA and 3-PBA in the urine of pesticide sprayers compared to the overall workers assessed (farmers, greenhouse workers or building exterminators). In a Japanese biological monitoring study of pyrethroid exposure in pesticide sprayers, geometric mean concentrations of 3-PBA in the urine of operators who sprayed in the two days preceding the survey were significantly higher than those who did not perform any spraying (5.4 μg/g creat. vs. 0.9 μg/g creat.) (Wang et al., 2007). However, a significant association between urinary 3-PBA levels and pyrethroid spraying was observed only in winter and not in summer. Hence, the fact that all biomonitoring data were collected during summer in our study excludes to some extent the seasonal variability.

In the present study, time since pesticide application (lambda-cyhalothrin alone or mixed with captan) as well as farm size were also associated with urinary 3-PBA levels in the multivariate MIXM model (but the result did not come out significant for CFMP). In the study by Ratelle et al. (2016), farm size – when considered individually in the MIXM model – showed an association with urinary 3-PBA excretion, but this association was not significant in the multivariate model (*i.e.* in combination with other factors).

Also, in the present study, when considered individually in the MIXM model, ethnicity, age, and education were associated with urinary 3-PBA levels, but these variables did not contribute significantly to the fit of the final multivariate model. On the other hand, alcohol consumption and the use of ibuprofen, acetaminophen or other medications contributed to the fit of the multivariate model (according to the AIC criterion), but did not show a statistically significant association with urinary 3-PBA or CFMP levels in the multivariate model.

In an exposure biomonitoring study conducted by our group in the general population of Montreal, Québec, Canada, prescription and over-the-counter drug use was associated with higher urinary excretion of pyrethroid metabolites, including 3-PBA (Fortin et al., 2008). In addition, Barr et al. (2010) reported that urinary excretion of 3-PBA in individuals from the general U.S. population was significantly associated with ethnicity and age in a multivariate model. In our study, the sample size was relatively small, and the group evaluated was composed mainly of healthy Latino workers.

Lopez-Galvez et al. (2018) evaluated pesticide exposure in 20 migrant farmers in a study in Sonora, Mexico and the impact of different factors, based on urine metabolite measurements including 3-PBA. Farmworkers age, language, personal protective equipment, time spent on the farm and season were important determinants of exposure. In a biomonitoring study of 50 textile workers in eastern China, Lu et al. (2013) also reported an effect of age and task on biological levels of pyrethroid metabolites (*trans*-DCCA, *cis*-DCCA and 3-PBA). In addition, in a study among farmers and their families, Trunnelle et al. (2014) reported a positive association between poor housing conditions and levels of urinary 3-PBA metabolites. They reported that poor housing conditions was a contributing factor to the higher levels of 3-PBA observed in the urine of these farmworker families.

Furthermore, in the present study, information on clothing worn was fairly consistent among workers (long pants (89%) and long-sleeved shirt or sweater (86%) and boots (79%) for the majority of workers); the small number of workers who reported wearing half-masks or helmets with filters (only nine pesticide sprayers) did not allow for a specific assessment of the impact of this protective equipment on biological levels of CFMP and 3-PBA. Only 45% of workers wore gloves, but there was no effect of glove wearing on urinary levels of CFMP and 3-PBA. Wearing goggles and hats was also not significantly associated with urinary levels of CFMP and 3-PBA. In contrast, in a recent biomonitoring study in which the metabolites 3-PBA and 4F-3PBA were measured in the urine of 45 farmers in northwestern Catalonia, Spain (Bravo et al., 2022), the use of specific personal protective equipment among farm workers, such as the use of gloves and masks during mixing, was associated with lower biological levels, although the differences were not statistically significant. However, a positive association was found between the use of a cap during mixing and during application. The authors reported that these caps were primarily used for sun protection, and when not cleaned after handling pesticides, they could represent a continuous source of exposure through dermal contact. In the same study, farm workers using tractors with cabin also had statistically lower concentrations of the metabolite of another pesticide, 2-diethylamino-6-methyl pyrimidin-4-ol, than those using tractors without cabin. In the present study, most applicators used a tractor for pesticide spraying, with cabin in eight cases and without cabin in four cases; only one applicator used a backpack sprayer. Because there was little difference in application conditions in our study, it was not possible to associate spraying equipment with biological levels.

Furthermore, in our study, there was no significant difference between the wearing of personal protective equipment (PPE) for the group exposed to lambda-cyhalothrin alone or in combination with captan, except for wearing gloves and goggles, which was more worn in the group coexposed to lambda-cyhalothrin and captan. Occupational hygiene practices in each exposure group (lambda-cyhalothrin alone or in combination with captan) were therefore relatively homogeneous, and the variability that could be associated with PPE between exposure groups was low.

4.2. Comparison of 3-PBA and CFMP levels with reference values in the general population or in exposed volunteers

The study also provided an overall indication of the magnitude of exposure of strawberry workers relative to the general population. In order to assess the importance of this exposure of all workers in the study, concentrations of 3-PBA metabolites in urine (24-h) from workers in our study were compared with those reported (in spot urine collections) in the general Canadian population and collected in the CHMS Cycle 6 (Health Canada, 2021). The 95th percentiles of the distribution of 3-PBA concentrations in the urine of our study workers reached values (3.02 μmol/mol creat. in urine collections performed 0–24 h after spraying lambda-cyhalothrin in combination with captan; seeTable 4) similar to the 95th percentile (CI) of 3.1 (1.06–5.02) μmol/mol creat. reported in the CHMS (Health Canada, 2021). The CHMS sample may have included workers exposed to pesticides. CFMP was not measured in

the CHMS study but lower 95th percentile values (0.145 and 0.261 μmol/mol creat.) were reported in spot urine samples collected from Sweden adolescents (Norén et al., 2020) and German individuals from the general population (Schettgen et al., 2016). Conversely, the 95th percentile concentration value of CFMP reported in the urine of the UK general population (0.839 μmol/mol creat.) (Bevan et al., 2013) was close to the values reported in our study (0.554–0.799 μmol/mol creat.) at -24-0 h prior to exposure and 24–48 h post-exposure.

Urinary levels of 3-PBA in the present study were comparable to those of Ratelle et al. (2016) where 34 patterns of 3-PBA excretion were observed in vegetable crop workers. Depending on the profile, median urinary 3-PBA values for a given individual ranged from 0.073 to 1.28 μmol/mol creat. and the 95e percentiles were as high as 9.07 μmol/mol creat. In the latter study, 16 profiles had geometric mean urinary 3-PBA concentrations above those reported in the CHMS at that time (Health Canada, 2013), and five profiles had 95th percentile values of 3-PBA higher than those reported in that survey. The observed levels of 3-PBA in our study were also comparable to those reported in German and Japanese studies where spot urine measurements (Panuwet et al., 2008; Wang et al., 2007) or 24-h collections were made (Hardt and Angerer, 2003). Pesticide exposure and uptake, and thus observed biological levels, may vary depending on several factors, including work habits, protective equipment, climate (heat and humidity) (Havenith, 1999).

The levels of 3-PBA and CFMP observed in the urine of workers can also be compared to the maximum urine values obtained from eight volunteers for whom the temporal profiles of CFMP and 3-PBA in urine were determined over a period of 84 h following the administration of an acute oral dose of 0.0025 mg lambda-cyhalothrin/kg bw, which is the EFSA acceptable daily intake (ADI) value (2014) or 0.025 mg lambda-cyhalothrin/kg bw (Khemiri et al., 2017). In the study of Khemiri et al. (2017), the maximum concentration of 3-PBA in urine was 10 μmol/mol creat. in the volunteer exposed to the ADI and ranged from 60 to 211 μmol/mol creat. in the other seven volunteers exposed to 0.025 mg/kg bw. The corresponding values for CFMP were 41 and 63–431 μmol/mol creat. In the present study, the 95th percentiles of the distribution of 3-PBA and CFMP concentrations in urine reached 2.8 and 3.0 μmol/mol creat. respectively; these values are 3.6 and 14 times lower than the urinary levels observed in the ADI-exposed volunteer.

4.3. Limitations and interest of the current biomonitoring study

In multivariate analyses, the levels of CFMP and 3-PBA between the group exposed to lambda-cyhalothrin alone and that coexposed to lambda-cyhalothrin and captan were compared while controlling for other factors that may influence urinary levels. The contribution of personal factors (age, ethnicity, BMI) and lifestyle habits (smoking and alcohol consumption or use of medication), as well as occupational exposure conditions (task, time since application, size of the farm in terms of number of employees) were therefore tested. One limitation was that some factors documented by questionnaire were not reported much so that their impact on exposure biomarker levels could not be tested (such as the impact of the "sex" variable since the farmers were mostly men, or the impact of domestic treatments or residential pesticide use since it was reported only in one case). In addition, although the questionnaires were translated in Spanish and a Spanish-speaking member of our team was available to assist and verify the participants' answers, the questions on the consumption of foods that may contain pesticide residues (consumption of cereals, fruits and vegetables, and number of servings) were not answered adequately or were poorly answered, so that they were not considered in the analyses. Also, the clothing and PPE worn by the workers were not considered in the multivariate analyses because, as mentioned above, the information on the clothing worn was fairly uniform among the workers (mostly long pants, long-sleeved shirts and boots), and the wearing of specific PPE (wearing half-masks or helmets with filters) was rarely reported. In addition, wearing gloves, goggles and a hat did not show a significant association with urinary levels of CFMP and 3-PBA.

Another limitation related to biological exposure monitoring concerns the fact that it involves the measurement of 3-PBA, the metabolite common to several pyrethroids, in addition to the more specific CFMP metabolite. As noted in a previous study on cypermethrin (Bouchard et al., 2016), the measurement of specific metabolites is important to confirm the source of exposure. It is only recently that the measurement of CFMP metabolites was conducted in large surveys (*e.g.*, Apel et al. (2023)). Furthermore, although it was confirmed that both captan and lambda-cyhalothrin were sprayed concomitantly by the applicators in our study and that field workers entered areas previously treated with both compounds, the metabolites of captan were not specifically measured in the urine of the workers under study to confirm internal exposure to both pyrethroids and captan.

Despite these limitations, this study showed that coexposure did not significantly impact biological levels of lambda-cyhalothrin metabolites, while controlling for other factors such as the main task performed, time since application, age, ethnicity, education level, medication, which may contribute to biological variability. In the study of Khemiri et al. (2017, 2018) in volunteers exposed to lambda-cyhalothrin orally and dermally under controlled conditions, variability in urine levels of CFMP and 3-PBA was significant despite identical exposure doses and the absence of coexposure. This indicates that physiological factors, related to absorption, distribution, metabolism and excretion, contribute significantly to inter-individual variability in the biological levels of metabolites used as biomarkers of exposure. The results of the present study thus suggest that at the levels of pesticide exposure observed in the targeted workers, coexposure does not contribute significantly to increasing this variability in the biological levels of CFMP and 3-PBA. At these exposure levels, coexposure therefore had no significant impact on the kinetics of lambda-cyhalothrin and its assessed metabolites used as biomarkers of exposure.

Furthermore, while the measurement of 3-PBA is not specific to lambda-cyhalothrin, the analyses carried out on the basis of this metabolite allow *a contrario* to assume that the results on the impact of coexposure to lambda-cyhalothrin and captan can be extrapolated and therefore generalized to other pyrethroids. Considering that the workers in the study were also exposed to other pesticides in their workplace (e.g. application of Roundup reported in some cases or other fungicides), the observed results indirectly point to a lack of impact of exposure to other pesticides used in the workplace on the levels of biomarkers of exposure to pyrethroids at the observed levels, although this remains to be confirmed.

Overall, this study provided novel data confirming that at the levels observed in the agricultural workers under study, biomarker concentrations in urine used to assess exposure to pyrethroids should not be influenced by coexposure to captan. As concluded from the animal results of Bossou et al. (2020), the present study thus suggests that the pyrethroid metabolites CFMP and 3-PBA, mostly measured in biomonitoring studies, remain useful as biomarkers of exposure in mixtures, when pesticide exposure levels are in the range of the sampled workers or at the general populational levels. Future perspectives include the use of a toxicokinetic model specific to lambda-cyhalothrin and Monte Carlo simulations to obtain the reconstructed absorbed dose possibilities for each worker, based on the amounts of the more specific metabolite CFMP measured in urine, throughout the biological monitoring period. The reconstructed daily dose results for applicators and other agricultural weed control and harvest workers can then be compared to limit values such as the *Acceptable Operator Exposure Level* (AOEL) reference value established by the European Commission (EFSA, 2014).

Author contributions

Yélian Marc Bossou: Methodology, Formal analysis, Investigation,

Writing – Original draft preparation. **Jonathan Côté:** Formal analysis, Investigation, Writing – Reviewing and Editing. **Étienne Dumais:** Formal analysis, Investigation, Writing – Reviewing and Editing. **Éloïse Morin:** Formal analysis, Investigation, Writing – Reviewing and Editing. **Clara Bianci:** Formal analysis, Investigation, Writing – Reviewing and Editing. **Michèle Bouchard:** Conceptualization, Methodology, Formal analysis, Supervision, Project Management, Writing – Original draft preparation, Writing – Reviewing and Editing, Funding acquisition.

Data availability

All data generated during this study are included in this article or are available on reasonable request from the corresponding author.

Ethics approval

The study protocol, the information and consent form, and other relevant documents were approved by Research Ethics Committee of the University of Montreal (*Comité d'éthique de la recherche Clinique de Université de Montréal* #CERC-19-007-D).

Consent to participate

The study was based on a voluntary participation. Participants received a slight financial compensation for their involvement and time. Subjects wishing to participate in the study signed an informed consent form, after receiving all necessary information about the project. Each participant was free to withdraw from the study at any time, without any prejudice.

Consent for publication

This manuscript has not been published or presented elsewhere and is not under consideration by another journal. All authors read and approved the final manuscript and consent for publication.

Declaration of competing interest

The authors have no competing interests to declare that are relevant to the content of this article.

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Appendix A. Supplementary data

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Coordination of chemical analyses under the European Human Biomonitoring Initiative (HBM4EU): Concepts, procedures and lessons learnt

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ABSTRACT

The European Human Biomonitoring Initiative (HBM4EU) ran from 2017 to 2022 with the aim of advancing and harmonizing human biomonitoring in Europe. More than 40,000 analyses were performed on human samples in different human biomonitoring studies in HBM4EU, addressing the chemical exposure of the general population, temporal developments, occupational exposure and a public health intervention on mercury in populations with high fish consumption. The analyses covered 15 priority groups of organic chemicals and metals and were carried out by a network of laboratories meeting the requirements of a comprehensive quality assurance and control system. The coordination of the chemical analyses included establishing contacts between sample owners and qualified laboratories and monitoring the progress of the chemical analyses during the analytical phase, also addressing status and consequences of Covid-19 measures. Other challenges were related to the novelty and complexity of HBM4EU, including administrative and financial matters and implementation of standardized procedures. Many individual contacts were necessary in the initial phase of HBM4EU. However, there is a potential to develop more streamlined and standardized communication and coordination in the analytical phase of a consolidated European HBM programme.

1. Introduction

The European Human Biomonitoring Initiative (HBM4EU) was launched in 2017 to develop and establish a coordinated and harmonized approach to human biomonitoring (HBM) across Europe. It built on the previous European projects *Expert Team to Support Biomonitoring in Europe (ESBIO)*, *European Coordination Action on Human Biomonitoring (COPHES)* and its demonstration project *DEMOCOPHES*, and on national or regional HBM programmes of some European countries (Kolossa-Gehring et al., 2012; Schindler et al., 2014; Den Hond et al., 2015; Joas et al., 2015). One of the characteristics of HBM4EU was a high degree of diversity, as it encompassed partners from 30 countries with different levels of HBM experience. Furthermore, it supported national as well as European authorities in chemical risk assessment, and it addressed a variety of chemicals, exposure scenarios and health outcomes (Ganzleben et al., 2017; Kolossa-Gehring et al., 2023). Consequently, coordination points had a vital role in HBM4EU in advancing the initiative from distinct activities to a coherent programme.

The chemical analyses in HBM4EU included human samples from four complementary approaches: Aligned national and regional HBM

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studies, connections to previous analyses in *DEMOCOPHES*, occupational exposure monitoring and an intervention study focussing on mercury (Table 1). These studies addressed two groups of priority substances. The first group had been selected in the preparation of HBM4EU according to policy-relevant questions defined by HBM4EU partner countries and European authorities, and the second group was defined in a prioritization process developed in HBM4EU (Ougier et al., 2021) (Table 1). An additional study on pesticide exposure using non-target and suspect screening, abbreviated SPECIMEn, was conducted in HBM4EU as well (Vitale et al., 2022), but not included in the coordination of analyses of the priority compounds. Except for some existing data from *DEMOCOPHES* or national and regional HBM programmes, new chemical analyses of the priority compounds were conducted in HBM4EU, based on the prerequisite of coordinated and harmonized approaches (Ganzleben et al., 2017).

For that purpose, a comprehensive quality assurance and control (QA/QC) programme was designed in HBM4EU, open to all European laboratories with an interest in performing HBM analyses of exposure biomarkers of the priority substances (Esteban López et al., 2021). It was organized and coordinated by the Quality Assurance Unit (QAU) established in HBM4EU and involved different HBM4EU partners taking responsibility for interlaboratory comparison investigations (ICIs) and/or external quality assurance schemes (EQUAS) in their field of expertise. This approach ensured greatest scientific expertise for the priority chemicals, substance-tailored ICI and EQUAS approaches with a common design and optimized timelines in parallel ICIs/EQUAS. The programme specified the criterion of at least two successful rounds of ICI/EQUAS participation to qualify for the analysis of specific biomarkers in HBM4EU.

The QA/QC programme resulted in the qualification of 75 laboratories from 25 countries for HBM analyses of different biomarkers related to the priority substances in Table 1. It was up to the sample owner, providing the samples to HBM4EU, to select a laboratory for the chemical analysis, on an informed basis. Collecting and conveying this information was part of the coordination of the analytical phase in HBM4EU, under the responsibility of Aarhus University (AU). The coordination also included progress monitoring of the chemical analyses. The Covid-19 pandemic required communication efforts beyond regular updates as well as adjustments of work plans that affected the overall HBM4EU timelines.

The objective of this article is to present and discuss the coordination of the chemical analyses in HBM4EU, starting from the conceptual approach and subsequently detailing the main activities. The article also includes the challenges encountered in the process and possible solutions for future projects.

2. Conceptual approach

The first step in the coordination of the analytical phase was to connect the qualified laboratories, i.e. laboratories with successful participation in the HBM4EU QA/QC programme, and the sample owners, with the purpose of providing the sample owners with the necessary information to select laboratories for the planned analyses (Fig. 1). In addition, this connection should give the laboratories the possibility to prepare for potential analytical tasks in their work plans. This first step required inputs from other tasks and work packages (WPs) in HBM4EU, including lists of qualified laboratories (Esteban López et al., 2021) and of the sample owners with their specific analytical interests (Gilles et al., 2021).

Once the sample owners had selected a laboratory, AU assisted with potential questions about administrative and technical issues. When the samples had been shipped to the selected laboratory, the monitoring phase began, i.e. the second part of the coordination work (Fig. 1). It involved regular contacts to each laboratory to enquire about progress and potential difficulties, which was intensified during the Covid-19 pandemic when work conditions became unpredictable as laboratories were affected by lockdowns and/or reduction of activities. AU regularly summarized the status of the analytical work in internal progress reports for the attention of task and WP leaders.

Although consecutive in the conceptual approach, the connecting efforts and the progress monitoring proceeded in parallel and overlapped in their timing. As the chemical analyses in HBM4EU included two groups of prioritized substances (Table 1) and both were covered by the QA/QC programme, the process in Fig. 1 was applied twice. However, fewer laboratories were involved in the second round of analyses than in the first one. In addition, the chemical analyses included a comparison of concentrations at different time points and samples from occupational studies, although the number of analyses was considerably lower than in the HBM4EU Aligned Studies (Table 1).

In the HBM4EU-MOM study and the HBM4EU occupational study on exposure in e-waste management, the coordination requirements were reduced to the progress monitoring. In both studies, one central laboratory was pre-selected for each type of analysis. The occupational studies on diisocyanates further included analyses of hemoglobin adducts and urine lysine adducts, which were of exploratory nature and thus not included in the HBM4EU QA/QC programme or the coordination of the analytical phase (Jones et al., 2022).

The outputs of the analytical phase were HBM data on the priority substances in the individual studies in Table 1, accompanied by contextual QA/QC information. These data were further processed and analysed in other WPs in HBM4EU and not part of the coordination of the analytical phase (Fig. 1). However, it meant that the analytical phase

Table 1
Summary of chemical analyses in studies on priority substances under HBM4EU.

Study	First group of priority substances	Second group of priority substances	Number of analyses	Reference
HBM4EU Aligned Studies: Alignment of national and regional HBM studies	Phthalates and 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH), bisphenols, per- and polyfluoroalkyl substances (PFAS), organophosphorous flame retardants (OPFRs), halogenated flame retardants (HFRs), polycyclic aromatic hydrocarbons (PAHs), cadmium	Acrylamide, mycotoxins, pesticides, UV filters, arsenic	29,074	Gilles et al. (2021); Gilles et al. (2022); Govarts et al. (2023)
Comparisons of different time points, including *DEMOCOPHES* samples	Phthalates and DINCH, bisphenols, OPFRs, PAHs, cadmium	–	4863	Vogel et al. (2023)
Occupational exposure studies	Chromium[a], PFAS[a], OPFRs[b], HFRs[b], phthalates[b] and DINCH[b], cadmium[b]	Diisocyanates[b], mercury[b], lead[b]	8574	Galea et al. (2021); Jones et al. (2022); Santonen et al. (2019, 2022); Scheepers et al. (2021)
HBM4EU-MOM[c]: Intervention study on mercury	–	Mercury	1305	Namorado et al. (2021); Katsonouri et al. (2023)

[a] First occupational study: Exposure to chromium.
[b] Second occupational studies: Exposure to diisocyanates and exposure in e-waste management, respectively.
[c] Methylmercury-control in expectant mothers through suitable dietary advice for pregnancy.

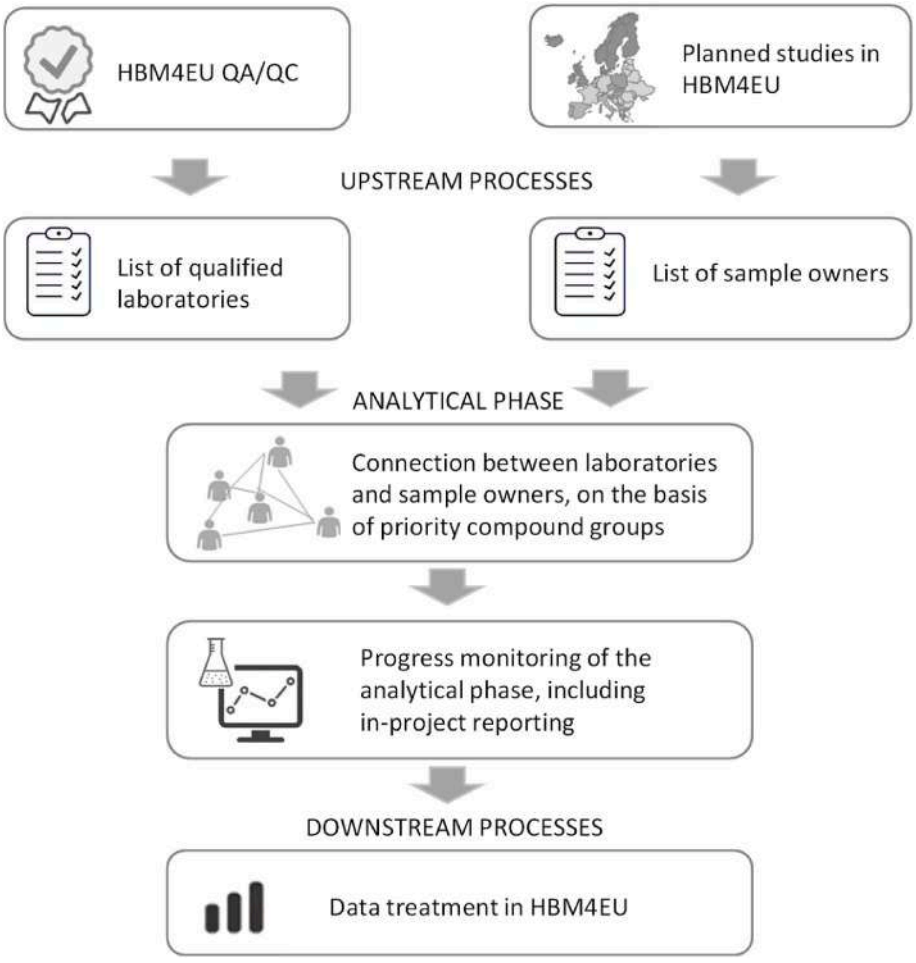


Fig. 1. Concept of coordination of the chemical analyses in HBM4EU.

had to be closely connected to upstream processes (lists of qualified laboratories, planned studies) and downstream processes (data processing and interpretation).

3. Connecting qualified laboratories and sample owners

Fig. 2 lists the activities included in the first step of the coordination work, i.e. the establishment of connections between qualified laboratories and sample owners. In order to collect information on analyses,

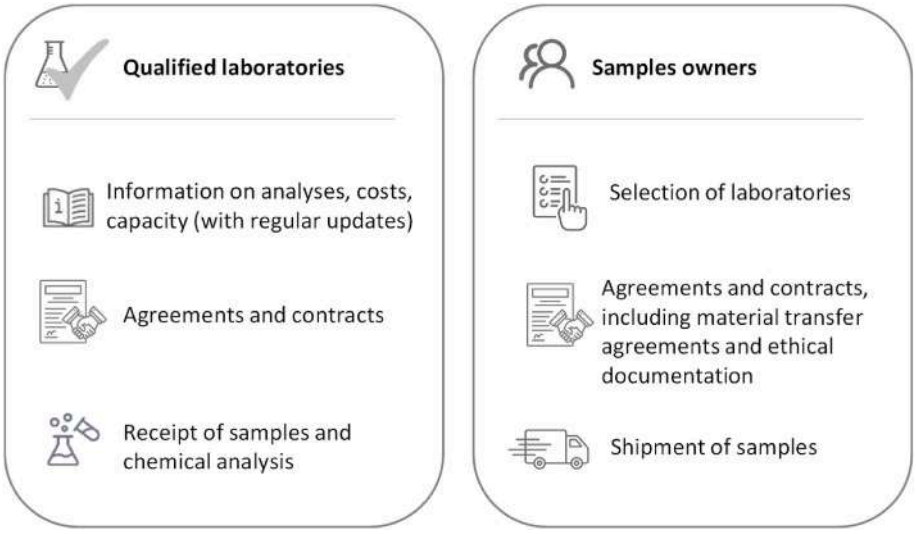


Fig. 2. Topics covered in the first step of the coordination of the chemical analyses in HBM4EU, i.e. the establishment of contacts between qualified laboratories and sample owners.

costs and capacity, a questionnaire was prepared for the qualified laboratories, including questions on the laboratory itself (e.g. use of a quality management system, involvement in HBM4EU and contact details), the specific biomarkers that the laboratory offered to analyze, the analytical methods (e.g. sample volume required, limit of quantification (LOQ), extraction, clean-up and instrumental techniques), the price and time frames for the specific analysis and whether or not the laboratory required any information from the sample owner. The questionnaire for bisphenols is shown as an example in Fig. 3. Questionnaires for the other priority substances are available in the Supporting Information (Fig. S1 - Fig. S13). The questionnaires were adapted to each group of substances to account for different biomarkers, matrices (urine, serum) and analytical methods.

Membership in the HBM4EU consortium was not a prerequisite to be selected for analysis. However, whether or not a laboratory was a partner in HBM4EU had administrative implications for the invoicing, as further discussed in Section 5. The only criterion for conducting analyses in HBM4EU was the successful participation in the QA/QC programme. The candidate list for the QA/QC programme was open for non-HBM4EU as well as HBM4EU laboratories (Esteban López et al., 2021). All laboratories were informed, as part of the questionnaire, that the analytical method had to be identical with that applied in the ICIs and EQUASs. Potential changes had to be disclosed in the questionnaire and would lead to an expert assessment of eligibility. However, no laboratory reported any changes.

Price for analyzing bisphenol biomarkers in urine

HBM4EU will investigate the human exposure to a list of prioritized substances in the European population. We kindly request your offer for analyses of bisphenol biomarkers in urine samples, to be performed in the HBM4EU project. The information in this form will be used in subsequent deliberations and negotiations between sample owners and laboratories.

Please fill in the requested information, sign the form and return it to hbm4eu@envs.au.dk.

Laboratory information	
Laboratory name	
Head of the laboratory	
Number of staff and qualifications	
Address	
Contact person	
Phone	
E-mail	
Is the laboratory member of the HBM4EU consortium?	
Is the laboratory ISO/IEC 17025 accredited?	
If not, does it work under a quality management system?	
Biomarkers	
Please indicate which biomarkers are included in the analytical method	
Bisphenol A (BPA)	
Bisphenol F (BPF)	
Bisphenol S (BPS)	
Analytical method	
Sample volume required (ml)	
LOQ (ng/ml)	
Will the analytical method be identical with that used in the HBM4EU ICI/EQUAS?	
If not, please specify any deviations, subject to evaluation by the HBM4EU Quality Assurance Unit. Please note that inconsistencies with the HBM4EU ICI/EQUAS might affect the eligibility of the laboratory for analyses in HBM4EU.	
In-house quality assurance/quality control	
Internal standards used for analysis	
Calibration method	
Extraction and clean-up	
Sample pre-treatment	
Type of deconjugation	
Enzyme used (ref number)	
SPE offline including type of column	
SPE online including type of column	
Other extraction and clean-up methods	
Derivatization (reagent)	
Instrumental analysis	
GC-MS (model, brand, column, ionisation)	
GC-MS/MS (model, brand, column, ionisation)	
LC-MS/MS (model, brand, column, ionisation)	
Other (please specify)	
Price	
Price, in Euro, for 300 samples	
Please specify person months (PM) for the analysis of 300 samples, costs of PM for different categories of staff involved in the analyses, and direct costs (consumables).	
Does the price include the determination of creatinine?	
If not, what is the price for creatinine determination in 300 samples? If possible, please specify PM and direct costs.	
Capacity and timeframe for analysis	
Time frame for 300 samples (assuming that they will be available in approximately one month).	
How much time does the laboratory need in advance to plan and prepare the analysis of 300 samples?	
Are there variations in the laboratory's capacity (due to holidays etc.)? If so, please specify.	
Does the laboratory have the capacity to analyse several sets of 300 samples in parallel?	
If yes, what is the maximum capacity?	
If no, what would be the time frame for several sets of 300 samples?	
Information required from the sample owners	
What information does the laboratory need from the sample owner for a potential contract for analyses?	
Other comments	
Validity of this document until	
Please note any additional comments.	

Date and signature

Fig. 3. Example of a questionnaire sent to laboratories qualified in HBM4EU, here for the analysis of bisphenols.

It should be noted that the design of the QA/QC programme, including the criterion of two successful rounds of participation, meant that the laboratories qualified for specific biomarker analyses at different points in time. Consequently, the collection of information from the laboratories was a rolling process, repeated after each update of the list of qualified laboratories in relation to completed rounds of ICIs and EQUAS. Furthermore, as the laboratories indicated in the questionnaires for how long this information was valid (Fig. 3), updates were requested from the laboratories when this time had passed. The information received from the laboratories was compiled in a table, with updates marked, and circulated to the sample owners on a weekly basis. The qualified laboratories, but no details on prices, capacities or methods, were also published on the HBM4EU website.

Parallel to the regular contacts with the qualified laboratories, inventories of all planned analyses were established and kept up-to-date, mainly based on information provided by the task leaders responsible for the studies in Table 1. This overview is shown in Table S1 of the Supporting Information, details are also given by Gilles et al. (2021, 2022), Govarts et al. (2023) and Santonen et al. (2019, 2022). In collaboration with the task leaders responsible for the studies in Table 1, questions were prepared for the sample owners, including information on the status of the sampling campaign, preparatory steps, such as ethical approval (Knudsen et al., 2023), and availability of auxiliary data. The selected laboratories were regularly added to the inventory, for internal use in HBM4EU. If a lack of progress was noted, the sample owners were contacted and asked if a decision had been reached on the choice of laboratory.

The sample owners were encouraged to contact AU as coordinators for the analytical phase to request information and updates according to their work plans. In some cases, sample owners had pre-selected a qualified laboratory or chosen to analyze the samples in-house, which they were also asked to communicate to AU's coordinating team. It is also worth noting that in this process of establishing contacts and exchanging information of relevance to the analytical work, including analytical costs, AU as the coordinator of the analytical phase was not involved in any deliberations of financial matters between the sample owners and the laboratories. AU and others assisted with guidance on the technicalities of budget transfer, as further specified in Section 5, but price negotiations between sample owners and laboratories or any involvement in the actual selection process were not part of the coordinating activities in the analytical phase.

Shipment of samples to the laboratories followed Standard Operating Procedures (SOPs) developed in HBM4EU (Pack et al., 2023). It was accompanied by Material and Data Transfer Agreements, which were also filed in a central HBM4EU database under the auspices of the HBM4EU ethics coordinator (Lermen et al., 2020; Knudsen et al., 2023). Thus, AU as coordinator for the chemical analyses was in close contact with the ethics coordinator to ensure correct documentation in accordance with rules for ethics and data protection. The HBM4EU coordinator, holding the main responsibility for ethics and data management in HBM4EU, was also copied on correspondence in this field.

4. Progress monitoring of the chemical analyses

In order to know the status of the HBM analyses and to assist with

potential difficulties on a one-on-one basis, close contacts to the sample owners and the selected laboratories were established. As discussed in Section 3, agreements between the sample owners and the selected laboratories were reached at different time points in HBM4EU. Consequently, sample shipment and the analyses in each laboratory had their own timelines. At a given point in time, the individual analyses in the studies of Table 1 had progressed very differently. The status was described in progress reports for internal use in HBM4EU, providing leaders and colleagues in other WPs with regular updates relevant for their work in HBM4EU. The first laboratories had passed the qualification criteria in July 2019, whereas the last analyses were completed with the end of the project in June 2022.

Monitoring the progress of the chemical analyses proceeded via e-mail communication, mainly with the responsible project leaders as the first contacts, but also, with their agreement and information, directly with the selected laboratories or sample owners. This communication was not standardized in any way, beyond carbon copying to the institutions involved in the respective study. In hindsight, standardized progress forms could have been circulated at regular intervals, but a more informal and individualized approach was chosen in HBM4EU, reflective of the close collaboration amongst most partners as well as the wish to create possibilities for open discussion and solution-oriented dialogue in case of problems or delays. Thus, a small team formed around each study, which proved efficient in finding solutions and conveying relevant information to other groups in HBM4EU.

Table 2 summarizes the analyses in HBM4EU according to the prioritized substance group, also including the number of laboratories qualified and eventually selected for the chemical analyses. As mentioned in Section 1, the total number of qualified laboratories was 75, but as one laboratory could be qualified for multiple biomarkers, the total number in Table 2 is higher. Of the 75 qualified laboratories, 34 laboratories (45%) were selected for analysis in HBM4EU, some of them for multiple analyses. Their geographical distribution is summarized in Fig. 4. A corresponding figure, stratified by priority group, is shown in the Supporting Information (Fig. S14).

The number of qualified laboratories is highest in the larger European countries as well as in those with existing HBM programmes (Fig. 4). The differences are smaller for the number of selected laboratories. However, while a large number of laboratories was selected for the chemical analyses, the number of samples analysed per laboratory varied considerably, ranging between 60 and 5198. About 30% of all analyses were conducted by three of the 34 selected laboratories, located in Germany, the Czech Republic and Denmark.

Fig. 5 shows how many samples in percentage of the total number were analysed in their country of origin. A corresponding figure with absolute numbers is presented in the Supporting Information (Fig. S15). The high percentage of cadmium analyses conducted at the national level suggests that this analysis is well-established in many European countries. Cadmium was also the biomarker with the highest number of laboratories qualified for analyses in HBM4EU (Fig. S14). However, other factors may also influence how many samples are analysed in the same country: Samples might have been chosen for HBM4EU analyses because analytical capacities were available in the country. Likewise, participation in the HBM4EU QA/QC programme might have been prioritized because samples were to be analysed from the same country. For the second group of priority substances (Table 1), only few invited expert laboratories had qualified for HBM4EU analyses, limiting the possibility for national-based analyses.

5. Challenges

5.1. Standardization

One of the central objectives of HBM4EU was the standardization and harmonization of procedures, taking into account that a large number of European countries and institutions participated in HBM4EU. While some of them had no prior experience with HBM and had to establish new routines, others with some HBM experience had to adjust their procedures to meet the standards developed in HBM4EU (Pack et al., 2023). This transition towards standardized procedures covered

Table 2
Substance groups and qualified laboratories performing chemical analyses.

Priority substance (group)	Individual biomarkers[a]	Matrix	Qualified laboratories	Selected laboratories	Analyses	QA/QC programme
Acrylamide	2	Urine	5	2	1795	Esteban López et al. (2021)
Arsenic	6	Urine	2	1	900	Esteban López et al. (2021)
Bisphenols	3	Urine	25	8	3613	Vaccher et al. (2022)
Cadmium	1[b]	Urine, blood[c]	38	11	3967	Nübler et al. (2021)
Chromium	1[b]	Urine, plasma, blood[d]	28	11	2758	Nübler et al. (2022a)
Diisocyanates	3	Urine	3	3	356	Jones et al. (2022)
DINCH	2	Urine	8	8	6160	Mol et al. (2022)
Halogenated flame retardants	10	Serum	15	5	1178	Dvorakova et al. (2021)
Mercury[e]	1	Hair	–	1	1305	-[e]
Mycotoxins	1	Urine	4	3	1304	Esteban López et al. (2021)
Organophosphorous flame retardants	4	Urine	5	5	2856	Dvorakova et al. (2021)
Per- and polyfluoroalkyl substances (PFAS)	12	Serum	21	6	1663	Nübler et al. (2022b)
Pesticides	9	Urine	2	2	2188	Esteban López et al. (2021)
Phthalates	15	Urine	20	9	5949	Mol et al. (2022)
Polycyclic aromatic hydrocarbons (PAHs)	13	Urine	5	5	2856	Nübler et al. (2023)
UV filters	2	Urine	2	1	1975	Esteban López et al. (2021)

[a] For a full list of biomarkers, see Esteban López et al. (2021).
[b] One parameter, but several matrices.
[c] Blood analyses were included in the HBM4EU QA/QC programme, while the final HBM4EU studies only included urine samples.
[d] The HBM4EU studies analysed Cr in red blood cells and plasma. Blood was used as a surrogate in the QA/QC programme, see details in Nübler et al. (2022a).
[e] Not included in the QA/QC programme because of a pre-selected laboratory accredited for these analyses and with prior experience from *DEMOCOPHES*.

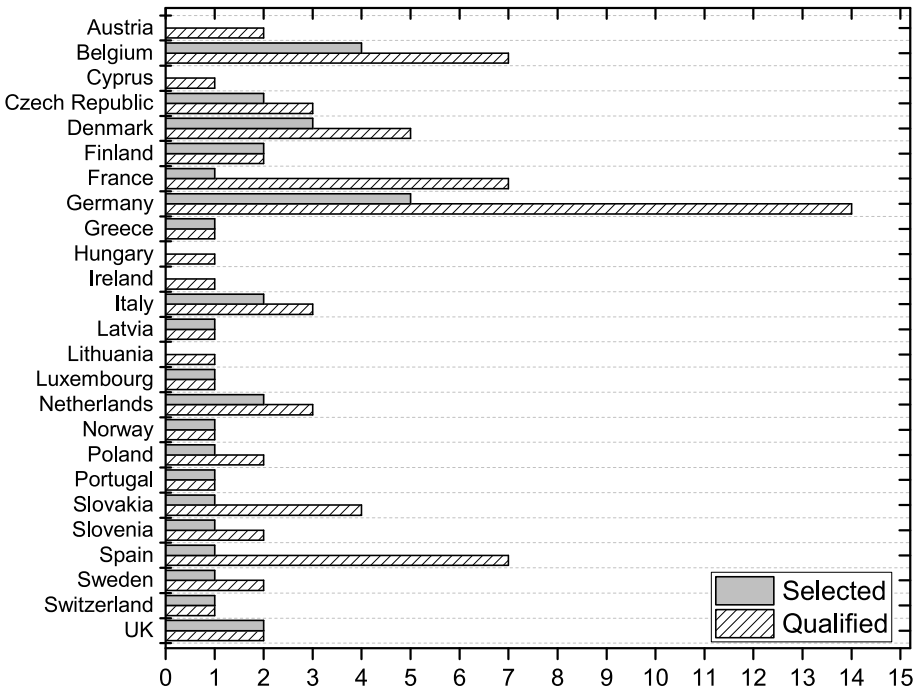


Fig. 4. Number of laboratories from different countries qualified and selected for the chemical analyses in HBM4EU.

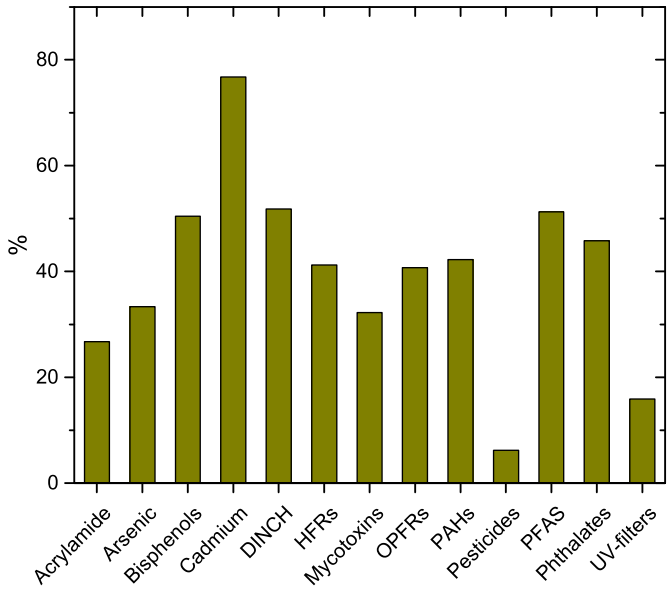


Fig. 5. Percentage of samples for different priority substances analysed by a laboratory of the same country as the sample owner, for the Aligned Studies and the time trend analyses. HFRs: Halogenated flame retardants. OPFRs: Organophosphorous flame retardants. PAHs: Polycyclic aromatic hydrocarbons. PFAS: Per- and polyfluoroalkyl substances.

many aspects of HBM4EU, including the material and data transfer. "Data" in this sense refers to (personal) data associated with the sample material, in contrast to (chemical) data as a result of the chemical analysis.

A detailed guidance document was developed in HBM4EU to ensure that procedures were in compliance with the General Data Protection Regulation (GDPR) of the European Union, also detailing terms and conditions for material transfer. This document contained a Material and Data Transfer Agreement form (Fig. S16 of the Supporting Information), to be completed by providers (sample owners) and recipients (qualified laboratories) and submitted to the ethics coordinator and HBM4EU coordinator (Knudsen et al., 2023). Establishing smooth workflows in this field proved challenging, probably reflective of an adaptation process, also including the translation of documents from national languages to English and vice versa, for example for the occupational exposure studies. An example of the difficulties encountered was the correct, standardized file naming, to ensure systematic entries in the database. Challenges related to ethics and GDPR were further discussed by Knudsen et al. (2023).

The extent of standardization in analytical chemistry is not a new question. As summarized in Table 3 and further discussed in Section 7,

Table 3
Details of analytical methods used by the qualified laboratories in HBM4EU.

Priority substance (group)	Matrix	Sample volume (mL)	LOQ (ng/mL)	Instrumental analysis
Acrylamide	Urine	0.1–2	1–5	LC-MS/MS
Arsenic	Urine	0.7–1	0.1–0.6	ICP-MS
Bisphenols	Urine	0.2–5	0.01–0.7	LC-MS/MS, GC-MS/MS
Cadmium	Urine	0.2–10	0.001–0.5	AAS, ICP-MS
Chromium	Blood, Urine	0.2–5	0.028–2.5	AAS, ICP-MS
DINCH	Urine	0.2–2	0.05–0.7	LC-MS/MS
Halogenated flame retardants	Serum	0.2–5	0.0001–2	GC-MS, GC-MS/MS, GC-HRMS
Mycotoxins	Urine	1–3	0.05–0.5	LC-MS/MS, LC-HRMS/MS
Organophosphorous flame retardants	Urine	0.3–5	0.02–0.5	LC-MS/MS, GC-MS/MS
Per- and polyfluoroalkyl substances (PFAS)	Serum	0.05–5	0.01–0.5	LC-MS/MS, LC-HRMS/MS
Pesticides	Urine	0.05–5	0.1–0.6	LC-MS/MS, GC-MS/MS
Phthalates	Urine	0.2–5	0.1–3.5	LC-MS/MS
Polycyclic aromatic hydrocarbons (PAHs)	Urine	0.5–11	0.001–6	LC-MS/MS, GC-MS, GC-MS/MS
UV filters	Urine	0.1–1	0.01–0.2	LC-MS/MS

LC: Liquid chromatography. MS: Mass spectrometry. ICP: Inductively coupled plasma. AAS: Atomic absorption spectroscopy. GC: Gas chromatography. HRMS: High resolution mass spectrometry.

different analytical methods were applied in HBM4EU. It is common practice in chemical monitoring programmes that laboratories follow general guidelines (e.g. OSPAR, 2016; EFSA, 2022), but keep some flexibility with regard to specific methods, as long as the quality of the data is ensured. Typically, laboratories document satisfactory performance in their chemical analyses by participation in externally organised proficiency testing schemes, in the same way as the ICIs and EQUAS organised in HBM4EU, and/or the analysis of certified reference materials (Arnaud et al., 2020; Göen et al., 2012a). This approach aims at the harmonization rather than the standardization of analytical methods and was the preferred approach in a multicentre HBM study like HBM4EU.

However, details in the analytical methods might require a higher degree of standardization to ensure comparability, for example the calculation of limits of detection (LODs) and LOQs, the use of either LODs or LOQs as well as the handling of concentrations below LOQs. For many chemicals, exposure levels of the general population are low or cover a relatively large range from low to higher concentrations (Göen et al., 2012b). How LOQs are defined and whether values below LOQs are considered as lower, medium or upper bound concentrations (EU, 2014; 2017), or assigned a different value, can therefore have an impact on the overall exposure level that is reported and assessed.

In addition, variability in LOQs can cause challenges in the comparability and aggregation of results (Table 3). This was experienced in the HBM4EU chromate study, where differences in LOQs in blood analyses of Cr led to considerable differences in detection frequencies between samples analysed in different laboratories (Galea et al., 2021; Ndaw et al., 2022). The variability was mainly a result of differences in the sensitivity of the analytical method, although differences in calculation methods also contributed to it.

5.2. Financial procedures

Since the chemical analyses were conducted in the framework of HBM4EU, an EU Horizon 2020 project, their invoicing followed the overall financial rules of HBM4EU. These were perceived as complex by many of the sample owners and qualified laboratories, especially the rules related to the difference in reimbursement rates between chemical analyses (50%) and other work in HBM4EU (70%). In addition, many laboratories were used to providing a total price for a service, but were now expected to differentiate person months and direct costs for the different chemical analyses. AU and other task leaders received many questions requesting clarifications on these matters. In response, the HBM4EU coordinator took the initiative, in collaboration with the relevant WP and task leaders, to prepare a guidance document that explained the administrative procedures of correct invoicing (Fig. S17 in the Supporting Information). It distinguishes three main cases:

- The sample owner and qualified laboratory are identical (i.e. in-house analysis)
- The qualified laboratory is a partner in HBM4EU
- The qualified laboratory is outside of HBM4EU (i.e subcontracting)

The second case occurred most frequently and was addressed by a budget transfer from the sample owner to the qualified laboratory, as approved by AU as part of the coordination process. However, this held the challenges that a) co-financing was necessary to cover the qualified laboratory's expenses, by either of the two partners, i.e. sample owner or laboratory, or another source, and b) a budget had to be allocated to the sample owner before the actual costs of analyses were known, as this information was collected as part of the analytical phase (Fig. 3). In order to work with realistic estimates, a survey was conducted in the first year of HBM4EU, to collect preliminary information on prices for chemical analyses. This led to a situation where some qualified laboratories felt that they were providing the same type of information repeatedly during the course of HBM4EU.

Clarifying the situation about invoicing and co-financing resulted in some delays in starting the chemical analyses, due to a combination of factors. The problems had to be understood in detail, several partners with leading functions in HBM4EU had to be involved, and a specific guidance document had to be prepared. Given the importance of this document, it passed several rounds of comments and adjustments, prior to broader communication to the sample owners and qualified laboratories.

5.3. Non-qualified laboratories

Although the prerequisite of passing the QA/QC programme to be eligible for analyses in HBM4EU and the associated criteria were clearly communicated at all levels of HBM4EU, a few analyses during HBM4EU were conducted by non-qualified laboratories (Table 4). These were usually laboratories that were qualified for other analyses in HBM4EU, possibly analysing the same samples for other priority substances, and adding more biomarkers from a cost-benefit perspective. Thus, these analyses were usually an "add-on" and did not result in a loss of information. As documented in Table 4, this was limited to very few analyses in the overall project, accounting for 2.4% of all analyses. Therefore, the main challenge was related to noticing this issue and communicating it efficiently to the downstream process (Fig. 1). These data were flagged as not quality assured through the HBM4EU QA/QC programme and disregarded in the calculations of European exposure values and geographical comparisons, as detailed by Govarts et al. (2023). This is different from the case of pre-HBM4EU data, for example for the time trend analyses, which were included if evaluated as being of acceptable quality. This was the case if the laboratory qualified in the HBM4EU QA/QC programme using the same method and documented continuous internal QA/QC measures (Govarts et al., 2023).

Furthermore, some biomarkers were novel and/or used on a more exploratory basis. In these cases, they were not covered by the full QA/QC programme and some pragmatic approaches had to be chosen to ensure analytical quality and comparability. This was the case for chromium analyses in exhaled breath condensate in the occupational studies, for which a small interlaboratory comparison was performed among the laboratories involved in these analyses (Leese et al., 2023).

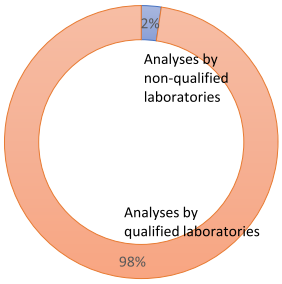
5.4. Capacity loss during the Covid-19 pandemic

The Covid-19 pandemic affected all partners in HBM4EU and caused delays in all project-related activities, in particular in sampling campaigns and laboratory work. Most research institutions and laboratories were shut down in spring 2020 and resumed work with varying capacity at different time points. However, as only few laboratories returned to full capacity immediately and all had to catch up with analyses that had been postponed in spring 2020, delays expanded. This situation required frequent contacts to sample owners and laboratories, to stay up-to-date with developments in each country and each laboratory and institution and to assess the implications for the overall work plan in HBM4EU. As Covid-19 countermeasures varied for each country and over time, these contacts and regular updates resulted in substantial additional work, which had not been foreseen in the planning of the analytical phase.

In addition to the regular progress reports, AU prepared "corona crises analysis" tables for the information of leaders in HBM4EU as well as the HBM4EU Management and Governing Boards. It soon became apparent that the Covid-19 related delays would have effects on the completion of the overall projects, as analytical results would be available later than anticipated. Based on updated information on the progress of the analytical phase, and on developments in the Covid-19 related effects on laboratory capacity, the HBM4EU Governing Board opted for a six months' extension of HBM4EU.

Table 4
Summary of analyses conducted by non-qualified laboratories during HBM4EU.

Study	Study group	Priority substances	Matrix	Analyses
HBM4EU Aligned Studies	Teenagers	PFAS[a]	Serum	300
	Teenagers	Arsenic	Urine	300
	Adults	Mycotoxins	Urine	200
Comparison of different time points, including *DEMOCOPHES* samples	Adults	OPFRs[b]	Urine	94
	Children	OPFRs[b]	Urine	99



[a] PFAS: Per- and polyfluoroalkyl substances
[b] OPFRs: Organophosphorous flame retardants

6. Lessons learnt

In general, the lessons learnt are connected to the fact that HBM4EU was a very large and ambitious project whose partners had different points of departures, in terms of previous experience. Reaching the stage of a harmonized and standardized HBM initiative across Europe was an ambitious goal and an achievement in itself.

6.1. Time buffers

Despite many years of experience in the field, a risk remains of underestimating the time required to implement certain steps in a new project. The size and diversity of HBM4EU amplified these usual time requirements. Changing established routines or building up new workflows in standardization attempts was more difficult and time-consuming than expected. It is an obvious and slightly banal lesson that time estimates should be conservative, including buffers that also allow newcomers in the field to catch up with experienced partners. However, it remains challenging to implement more generous timelines while keeping up with the rapid international development in research and monitoring, including ambitions of leading the development in some fields, as well as responding to urgent data needs for risk assessment and regulatory purposes.

6.2. Administrative guidance

The administrative and financial side of the analytical phase in HBM4EU was generally considered complex. To avoid confusions and delays, guidance should be developed and provided a priori. A help desk function was included in the WP for QA/QC and chemical analyses, which would probably benefit from an administrative counterpart, preferably staffed with administrative and financial experts rather than scientists. In general, the categorization of activities with different internal funding rates should be avoided. A uniform funding rate would have precluded the substantial additional administrative effort experienced in HBM4EU (Kolossa-Gehring et al., 2023).

6.3. Standardization

In addition to the standardized procedures around ethics and the standardization of technical elements such as LOQs, the coordination of the analytical phase could also be developed towards more standardization, provided that the HBM programme has a more permanent structure. While the same forms were used for regular updates in the phase connecting the sample owners and qualified laboratories (Fig. 3), also providing recognizability for the recipients, the monitoring of the chemical analyses was still mainly based on one-to-one correspondence. This was useful in the establishment of HBM4EU, but could be replaced by more standardized forms in a long-term perspective. Similarly, while progress reports had a recognizable format, they were prepared at varying intervals and would benefit from more regularity, perhaps aligned with HBM4EU Management Board meetings. Flexibility in the communication will still be important, to allow discussions of partner-specific questions and concerns, but developments towards SOPs in the coordination of the chemical analyses could be an option.

6.4. Connection to ethics

Although not included in the original concept (Fig. 1), it proved useful and efficient to collaborate with the ethics coordinator and to assist with the filing of Material and Data Transfer Agreements. As coordinator of the analytical phase, AU was in regular contact with sample owners and qualified laboratories and could use these communication channels to follow up on information required elsewhere in HBM4EU. In general, it is worth considering how to focus the communication, so partners do not feel that they receive uncoordinated and potentially duplicate requests. Shared sites for document exchange and communication could be an improvement to e-mail-based communication. It will be important to optimize communication both between different parts of the project and over time.

6.5. Capacity building

During HBM4EU, an increasing number of laboratories participated in the HBM4EU QA/QC scheme and obtained satisfactory results, documenting an increase in the HBM analytical capacity in Europe (Esteban López et al., 2021). However, approximately one third of the chemical analyses were conducted by only three European laboratories, leaving room for a wider implementation of high-quality HBM analyses. This extension may require a first analysis of existing obstacles. Training activities were included in HBM4EU (Kolossa-Gehring et al., 2023), but would benefit from more continuous and focused initiatives to improve technical capabilities and overcome potential obstacles. Capacity building could be linked to a set of minimum performance criteria for an HBM programme, including satisfactory results in regular proficiency testing and sufficiently low LOQs to avoid discrepancies in detection frequencies.

7. A network of laboratories – discussion of the HBM4EU experience

Different strategies exist for chemical analyses in HBM programmes around the world. In the US National Health and Nutrition Examination Survey (NHANES), for example, the analyses are centralised at the Environmental Health Laboratory of the Centers for Disease Control and Prevention (CDC) (CDC, 2022). HBM4EU has chosen a decentralized approach in its analytical phase, reflecting the European diversity as well as the wish to build transnational capacity in the field of HBM

analyses. In addition, an unprecedented high number of analyses had to be completed in a relatively short time frame, which was not possible for a single laboratory. Obviously, this strategy required a higher degree of coordination, in addition to the QA/QC programme, to ensure high-quality and comparable results as well as administrative clarity. However, many of the coordination efforts were related to the fact that HBM4EU was new and to the unexpected challenges of Covid-19 during the analytical phase. As discussed in Section 6, communication between partners could be more streamlined in an established and more permanent programme. This would reduce the correspondence that was necessary in coordinating the chemical analyses in HBM4EU.

Regarding efficiency, the network of laboratories carrying out the chemical analyses in HBM4EU has advantages and disadvantages. Laboratories that had successfully participated in the HBM4EU QA/QC programme could start the chemical analyses immediately without further method development. Distributing the work amongst several expert laboratories, according to their reported capacities, increased efficiency. On the other hand, the data analysis in HBM4EU was dependent on complete datasets, meaning that potential delays in one single laboratory carried the risk of delaying the whole downstream data analysis process. The interlinkages and inter-dependencies might need stronger emphasis in the communication with the participating laboratories. However, the different timelines between laboratories were also related to the upstream processes, which provided samples from the HBM4EU Aligned Studies at different points in the HBM4EU project.

The fact that HBM4EU only needed a six months' extension to complete its work plan, including the chemical analyses, indicates a robust design and an efficient steering that was not severely affected by Covid-19 restrictions. After the first wave in spring 2020, Covid-19 countermeasures began to vary between countries and over time, ranging from temporary lockdowns to near-normal work routines. This diversity made the decentralized analytical strategy more robust than the concentration on few expert laboratories would have been. Some progress was always possible with the chemical analyses, and the close contact between laboratories, sample owners and the coordinators for the analytical phase ensured regular updates and individually optimized solutions.

For some of the priority substances, a variety of methods was applied by the 75 laboratories qualified for the chemical analyses in HBM4EU (Table 3). Most suitable analytical methods had been discussed and recommended in HBM4EU (Vorkamp et al., 2021), but the laboratories were free to use a method of their choice provided it had generated satisfactory results in the HBM4EU QA/QC programme (Esteban López et al., 2021). This diversity of methods increased robustness and might also favour methodological developments as different methods are tested and optimized. In addition, it has a strong capacity building component since laboratories can learn from each-other and implement procedures needed for chemical monitoring. Laboratories with less experience in HBM analyses were given the opportunity to establish and improve their analytical capabilities. However, some method standardization may be advisable, for example in terms of minimum performance criteria, as discussed in Section 5. Furthermore, the harmonization of different methods requires external QC, in terms of regular proficiency testing exercises and certified reference materials. Given the large number of compounds and laboratories, this is a considerable effort, but with the obvious benefit of creating long-term structures for coordinated and harmonized HBM chemical analyses in Europe.

8. Conclusions and outlook

The analytical phase in HBM4EU included a large number of participants in terms of sample owners (providing samples to the HBM4EU Aligned Studies from national and regional cohorts and collections) and laboratories having passed a comprehensive QA/QC scheme to qualify for chemical analyses in HBM4EU. This required a high degree of coordination, also ensuring connections to upstream and downstream processes in HBM4EU, i.e. the preparation of the analytical phase and the data treatment, respectively. A central coordination point was essential in HBM4EU, also regarding the unexpected challenge of managing consequences of Covid-19 measures. Given the novelty and complexity of the HBM4EU project, it initially operated largely on an individualized communication basis. There is potential to further develop streamlining and standardization of the coordination process in a long-term and consolidated programme, in close collaboration with experts in administrative, financial, ethical as well as data-related questions.

The decentralized approach of chemical analyses involving a network of laboratories appears to be the best solution for a European HBM programme, generating high-quality and comparable data in a harmonized, efficient and robust framework. It has the potential to be consolidated in a group of national and European reference laboratories in the HBM field. Certain aspects of the chemical analyses, for example LOD and LOQ calculations, would benefit from more standardization, and a set of minimum performance criteria will ensure better comparability between laboratories. Thus, the coordination of the chemical analyses should be linked to general QA/QC questions, as addressed in the HBM4EU QAU. Regular proficiency testing and certified reference materials for HBM are points where more discussion has been initiated to overcome current lacks.

Combining and formalizing the chemistry-related structural elements of HBM4EU, such as the laboratory network, the QA/QC programme, the QAU and the coordination of the chemical analyses, would create a cornerstone of a European HBM programme. These structures should be sufficiently flexible to include possibilities of extensions, towards other chemical substances, novel biomarkers and emerging scientific questions. An obvious extension could be the connection to chemical analyses in exposure media and the environment, as envisaged in the Horizon Europe Partnership for the Assessment of Risks from Chemicals (PARC).

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Appendix A. Supplementary data

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Decay rate estimation of respiratory viruses in aerosols and on surfaces under different environmental conditions

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ABSTRACT

Majority of the viral outbreaks are super-spreading events established within 2–10 h, dependent on a critical time interval for successful transmission between humans, which is governed by the decay rates of viruses. To evaluate the decay rates of respiratory viruses over a short span, we calculated their decay rate values for various surfaces and aerosols. We applied Bayesian regression and ridge regression and determined the best estimation for respiratory viruses, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), severe acute respiratory syndrome coronavirus (SARS-CoV), middle east respiratory syndrome coronavirus (MERS-CoV), influenza viruses, and respiratory syncytial virus (RSV); the decay rate values in aerosols for these viruses were 4.83 ± 5.70, 0.40 ± 0.24, 0.11 ± 0.04, 2.43 ± 5.94, and 1.00 ± 0.50 h^{-1}, respectively. The highest decay rate values for each virus type differed according to the surface type. According to the model performance criteria, the Bayesian regression model was better for SARS-CoV-2 and influenza viruses, whereas ridge regression was better for SARS-CoV and MERS-CoV. A simulation using a better estimation will help us find effective non-pharmaceutical interventions to control virus transmissions.

1. Introduction

Emerging infectious diseases have a significant impact on public health and economies (Jones et al., 2008). Approximately 15 of 57 million (>25%) annual global deaths are related to infectious diseases (Morens et al. 2010). Out of these, the majority (3.96 million) are due to respiratory infections (Morens et al., 2010). Acute respiratory diseases are the most widely reported infections among individuals of all age groups (Monto 2002). Respiratory viruses replicate in the respiratory tract and are subsequently transmitted by respiratory secretions, causing infections ranging from asymptomatic to symptomatic (Kutter et al., 2018; To et al., 2020; Zhao et al., 2020). Respiratory tract infections are caused by various respiratory viruses, including severe acute respiratory virus coronavirus 2 (SARS-CoV-2), severe acute respiratory syndrome coronavirus (SARS-CoV) (Casanova et al., 2010; Darnell and Taylor 2006; Lai et al. 2005), Middle East respiratory syndrome (MERS-CoV) (Chu et al., 2020; van Doremalen et al., 2021; Gabutti et al., 2020; Su et al., 2016), influenza viruses (Harper, 1961; Hirose et al., 2022; Izumikawa, 2019; Sutton et al., 2013), and respiratory syncytial virus (RSV) (Moreira et al., 2018; Paynter 2015).

Most cases of outbreaks are associated with indoor environments (Chau et al., 2021; Qian et al., 2021). Superspreading events have been reported in indoor environments such as restaurants (Y. Li et al., 2020; Majra et al., 2021; Qian et al., 2021), households (W. Li et al., 2020; Qian et al., 2021), buses (Majra et al., 2021; Mangili and Gendreau 2005; Shen et al., 2020; Tsuchihashi et al., 2021), airplanes (Flight et al., 2020; Mangili and Gendreau 2005), trains (Pestre et al., 2012), classrooms (Charlotte 2020; Rothamer et al., 2020), and healthcare facilities (Bin et al., 2015). Most transmissions led to clusters within an exposure period of less than 12 h. For instance, during a 10-h nonstop commercial flight, a cluster of 16 infected individuals was reported from one probable index case (Flight et al., 2020). The above-mentioned studies concluded that clusters of infections were established within 2–10 h, highlighting that a critical time interval for successful transmission may

Abbreviations: SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; SARS-CoV, severe acute respiratory syndrome coronavirus; MERS-CoV, middle east respiratory syndrome coronavirus; RSV, respiratory syncytial virus; VIF, variance inflation factor; AIC, Akaike's information criterion; RH, Relative humidity.

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be governed by the decay rate of the viruses in indoor environments.

Infected individuals expel large droplets and aerosols into the air via expiratory activities such as coughing, sneezing, breathing, and speaking (Klompas et al., 2021; Yin et al., 2022). There are three dominant modes of respiratory virus transmission (1) droplet transmission: the direct inhalation of relatively larger droplets (>5 μm); (2) airborne transmission: the inhalation of tiny droplets, aerosols, and droplet nuclei floating in the air (<5 μm); and (3) fomite transmission: viruses can remain viable on inanimate surfaces for hours to even days and cause indirect contact through fomites (Castaño et al., 2021; Chaudhuri et al. 2020; Delikhoon et al., 2021; Kutter et al., 2018; Patel et al., 2020; Pease et al., 2021). A previous study reported that the sizes of 87% of exhaled particles are less than 1 μm, emphasizing the importance of considering aerosol transmission in long-range transmissions (Zhang et al., 2020). Recently, the World Health Organization and U.S. Centers for Disease Control and Prevention have issued scientific declarations on the importance of aerosols in SARS-CoV-2 transmission (Klompas et al., 2021). Meanwhile, droplet transmission causes short-range transmission (<1 m), and these droplets remains in the air for a short period (<17 min) (Kutter et al., 2018). Droplets travel directly from the mouth or nose of the infected individual to the nostrils or mouth of susceptible individuals and cause deposition on the upper respiratory tract and mucous membranes (Arslan et al., 2020; Biryukov et al., 2020a; Kutter et al., 2018; Miller et al., 2021). Fomite transmission occurs by the rapid deposition of larger droplets on inanimate surfaces (Castaño et al., 2021; Karia et al., 2020). Influenza virus is predominantly transmitted via fomites (Nicas and Jones 2009) and causes infection by gaining entry via hands and subsequently through facial membranes such as the nose, mouth, or eyes.

Environmental factors such as temperature, humidity, and solar radiation can affect the stability of viruses in aerosols and surfaces (Casanova et al., 2010; Gamble et al., 2021; Paynter 2015; Schuit et al., 2020; Wood et al., 2010). The relative humidity (RH) around the surface can affect the evaporation rate and concentration of compounds such as salts and proteins in the droplets, which influence the decay rate of viruses (Guo et al., 2021). At a low humidity, owing to the high evaporation rate, respiratory droplets reduce in size and form tiny droplets and droplet nuclei (Paynter 2015). Conversely, respiratory droplets are larger at a high humidity and settle faster on surfaces because of their lower evaporation rate (Paynter 2015). Higher temperature and higher humidity levels have a synergistic effect on virus decay compared to lower temperature and humidity (Chan et al., 2011). However, few studies have indicated an increased daily incidence at lower temperatures and humidity (Chan et al., 2011). Additionally, solar radiation affects the viability of viruses in the environment; ultra violet light is a natural environmental virucide which disrupts viral replication by causing the formation of photodimers (Sutton et al., 2013). Therefore, determining viral decay rates under different environments is vital for adopting interventional strategies and decisions initiated by policymakers and government health authorities (Dublineau et al., 2011).

Although we found a few prediction modeling approaches used in wastewater epidemiology (Kadoya et al., 2021; Zhu et al., 2022) and virus transmission dynamics (Vuorinen et al., 2020), there have been limited modeling studies that predict viral decay rates in indoor environments (Guillier et al., 2020). To the best of our knowledge, no study has focused on viral decay rates at shorter time intervals or on virus-specific or surface-specific decay rate estimation based on environmental conditions. Therefore, the objectives of our study were to predict the decay rates of respiratory viruses (SARS-CoV-2, SARS-CoV, MERS-CoV, influenza virus, and RSV) and develop estimation models for decay rates on different surfaces under diverse environmental conditions (temperature, relative humidity, and solar radiation), which would help determine effective control measures.

2. Materials and methods

2.1. Article screening

Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines used in clinical medicine were employed to identify the articles providing details about the decay rates of viruses (Fig. 1) (Moher et al., 2009). Here we focused on specific respiratory viruses, including SARS-CoV-2, SARS-CoV, influenza viruses, MERS-CoV, and RSV. Articles were screened using the Google Scholar and PubMed search engines. Articles collected from PubMed coincided with those collected from Google Scholar; therefore, the articles mentioned hereafter are based on the results from Google Scholar. Articles were screened between March 2021 and January 2022. The keywords used were "X" AND ("inactivation" OR "decay" OR "stability" OR "viability") AND ("surface" OR "environment") where X was each virus type ("SARS-CoV-2," "SARS-CoV," "Influenza," "MERS-CoV," or "RSV"). Several criteria were considered before selecting the eligible papers. The inclusion criteria were (1) not a review paper; (2) a peer-reviewed paper; (3) published in English; and (4) contained experimental data on the decay of the target virus and presentation of the extractable data related to the virus decay rate (containing data for at least two-time points).

During the first screening, non-English articles, dissertations, book chapters, and conference reports were eliminated from the collected articles. After the first screening, the titles of the selected papers were checked. Then, the abstracts of those articles were further evaluated to check whether the selected paper could specify the decay rate values of the target virus. The assembled articles were then subjected to a full-text review for data extraction.

The select eligible articles were then used to calculate viral decay rates using a first-order reaction. In a first-order reaction, the rate of the reaction is proportional to the concentration of the reacting substance (HIATT 1964). The exponential form of the decay is as follows:

$$\frac{C}{C_0} = e^{-kt} \tag{1}$$

since

$$\log\frac{C}{C_0} = -\frac{kt}{2.303} \tag{2}$$

where C_0 is the initial virus concentration ($TCID_{50}$/ml or PFU/ml), C is the virus concentration at time t, t is the duration of the virus on the surface or in the aerosol, and k is the virus decay rate on the surfaces or in the aerosols. The decay rate was calculated based on the ratio proportion of the virus concentration to the initial concentration. When the plot of log C/C_0 versus time is a straight line with a slope of $k/2.303$, the decay rate is given by the following equation:

$$k = -\frac{2.303\log C/C_0}{t} \tag{3}$$

The decay rate was calculated as the regression slope of ln (C/C_0) versus time (in hours) using linear least-squares regression. The R^2 and p values of each curve were reported separately. Out of the calculated decay rates from the eligible articles, decay rates with $p < 0.1$ were used in this study. If k was determined by the regression slope of log (C/C_0) versus time (in hours), it was transformed into ln (C/C_0). If a study presented first-order k values, they were directly extracted from the paper, along with any reported errors or R^2 values. Unit conversions were appropriately performed where needed. The calculated decay rates were then transformed into $\log_{10}$ values; this would enable readers to make intuitive comparisons of the decay rates, which differ by several orders of magnitude owing to differences in experimental conditions. Several methods, such as cell culture and PCR techniques (Bischoff et al., 2013; Johnson et al., 2021; Jones et al., 2020; Lindsley et al., 2015),

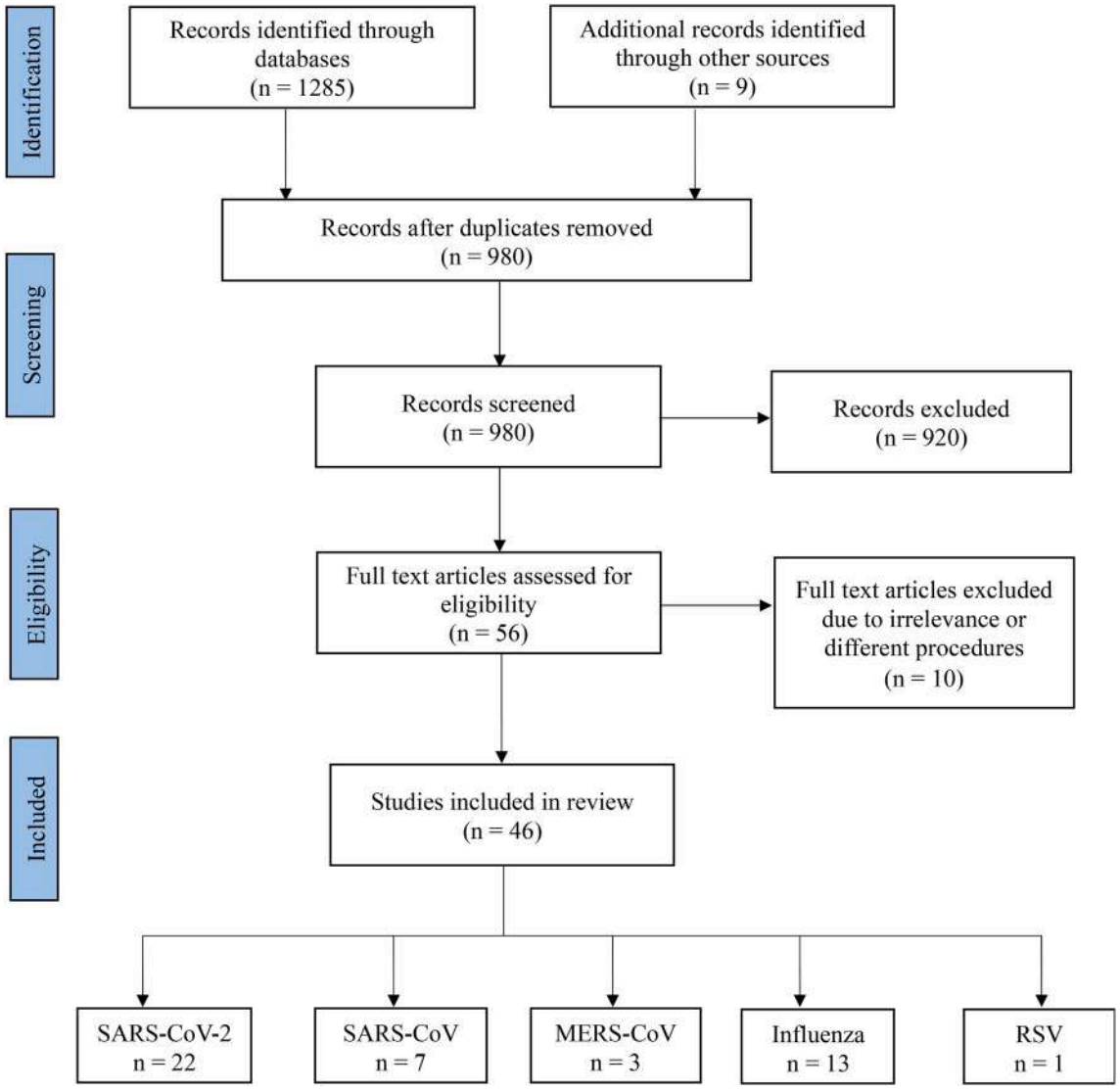

Fig. 1. Flowchart depicting the literature selection steps followed in this study.

have been used to determine viral concentrations in suspensions. In this study, we used experimental data generated from cell culture methods ($TCID_{50}$/mL or PFU/mL) to calculate decay rates using Equation (3).

The first-order decay rate (k) in units per hour (h^{-1}) was calculated for a maximum period of 12 h. If the values of the first-order decay rate were not provided in the papers, but the plots were available as the virus concentration changed over time, the virus concentration for each time point of the curve was digitized using the Web plot digitizer version (AssessmentUS EPA National Center, 2009).

In addition to the decay rate values of the target virus, information related to the surface type and environmental conditions (temperature, humidity, and solar radiation) were collected separately for each virus. For example, if a range of temperature or humidity range was provided, the given mean of the reported range was recorded. To address the environmental differences in each experiment (temperature, relative humidity, and solar radiation), we included temperature and humidity as continuous variables which can reflect the experimental conditions. In addition, due to the lack of quantitative information in the previous studies, we assumed solar radiation as a categorical variable to reflect the presence and absence of solar radiation for data input in the simulation and predictions. Before model development, the multicollinearity of the variables was evaluated using the variance inflation factor (VIF). In basic regularized regression analyses, the multicollinearity problem is solved so that all the explanatory variables can be used in the model (Kadoya et al., 2021).

Bayesian formulations are widely used in diverse environmental applications. There are several advantages over other data aggregation methods: Bayesian approach incorporates quantitative prior information about the distributions and range of model parameters or measurements. All parameters are treated as probability distributions. This approach uses whole dataset simultaneously to fit the model parameters, which share details across datasets and minimize the uncertainty by up to 95% (Britten et al., 2021). To address the multicollinearity, ridge regression shrinks the absolute values of the coefficients of the model variables, which all explanatory variables can be used (Kadoya et al., 2021). Thus, two modeling approaches—ridge regression and the Bayesian regression model —were used in this study.

2.2. Bayesian regression model

We used a Bayesian regression model based on the Markov Chain Monte Carlo method (MCMC) (Kadoya et al., 2021). Before developing the Bayesian regression model, probability distributions of decay rates were determined using Akaike's information criterion (AIC) using the "fitdistrplus" package in the R software. External factors affecting viral decay rates were considered explanatory variables: environmental

factors (temperature, relative humidity, and solar radiation) and surface type (Chan et al., 2020; Raiteux et al., 2021; Rockey et al., 2020). Categorical variables (solar radiation; surface type: aerosol, paper, steel, banknote, mask, skin, cloth, cardboard, plastic, glass, and metal) were assigned a value of 1 (with sunlight or with assigned surface type) or 0 (without sunlight or assigned surface type). The relationship between the variables was expressed as shown in equation (4). The model parameter coefficients were then estimated using an MCMC-based Bayesian regression model. The structure of the Bayesian regression model used in this study is shown in Fig. S1.

$$\text{Population parameter of decay rate} = a + b \cdot \text{Temperature} + c \cdot \text{Humidity} + d \cdot \text{Solar radiation} + e \cdot \text{Aerosol} + f \cdot \text{Paper} + g \cdot \text{Steel} + h \cdot \text{Bank note} + i \cdot \text{Mask} + j \cdot \text{Skin} + k \cdot \text{Cloth} + l \cdot \text{Cardboard} + m \cdot \text{Plastic} + n \cdot \text{Glass} \quad (4)$$

$$X(a\text{-}n) \sim \text{Normal}\ (\mu(a\text{-}n), \sigma(a\text{-}n)) \quad (5)$$

where X represents the intercept (a) and coefficients for each variable named from b to n, μ represents mean and σ represents standard deviation. Based on data availability, the number of explanatory variables (types of surfaces) was selected for the model. Therefore, the total number of explanatory variables used in the models varied for each virus type. We ran 4000 simulations to ensure the model convergence for all parameters. The statistical analysis and parameter estimation were performed using the statistical software R (version 3.6.1) and R Stan (Stan Development Team (2022). "RStan: the R interface to Stan." R package version 2.21.7 n.d.; Statistical and computing. R Foundation for Statistical Computing, Vienna n.d.). The R code for the Bayesian regression model is available in the supplementary materials (code S1).

2.3. Ridge regression model

There are three basic regularized regression analyses—lasso, elastic net, and ridge regression (Kadoya et al., 2020). Regularization helps us avoid multicollinearity and model overfitting, which ensures generalization of new data (Alkinani et al., 2021; Kadoya et al., 2020). Lasso and elastic net are sparse estimation methods that select essential variables in the model (Kadoya et al., 2020; Oishi et al., 2021); ridge regression, on the other hand, uses all explanatory variables. Furthermore, in contrast to ordinary least squares regression, which reduces the sum of the square residuals between observed and predicted values, ridge regression minimizes model overfitting by adding a penalty to the size of the coefficients (Alkinani et al., 2021).

$$L(\widehat{\mathscr{B}}) = \sum_{i=1}^{n} (y_i - x_i\widehat{\mathscr{B}})^2 + \lambda \sum_{j=1}^{m} \widehat{\mathscr{B}}_j^{\,2} = \left\| y - X\widehat{\mathscr{B}} \right\|^2 + \lambda \|\widehat{\mathscr{B}}\|^2 \quad (6)$$

λ parameter is the regularization penalty. $L(\widehat{\mathscr{B}})$ gives ridge regression estimates, where x is explanatory variables (temperature, humidity, solar radiation, and surface type: aerosol, paper, steel etc.) and y is objective variable decay rates. When λ is zero, it is same as ordinary least square method. While larger the λ is stronger the coefficients' size penalized. In this study, decay rate estimation was performed using the "glmnet" package in the R software (version 3.6.1). The R code for the ridge regression model is given in the Supplementary Materials (code S2).

2.4. Model validation

We randomly divided the datasets into training (80%) and test (20%) datasets for each virus type to evaluate the prediction: for SARS-CoV-2 65 training data and 17 test data, for influenza viruses 33 training data and 9 test data, for SARS-CoV 14 training data and 4 test data, and for MERS -CoV 28 training data and 7 test data respectively. Explanatory variables were scaled before splitting the datasets. K-fold cross-validation was used to obtain robust and accurate results. Root mean square error (RMSE), mean absolute error (MAE) and area under the curve (AUC) were calculated separately for each model to evaluate the goodness of fit. In addition, R^2 values of the train and test data of the better estimation model were calculated to check whether model overfitted data.

3. Results

3.1. Article screening

We identified 1285 papers using Google Scholar and nine from other sources; 1226 articles were removed during the first screening. After further screening (Figs. 1), 46 articles were identified as eligible for further analysis. We identified 197 decay-rate values in this study. There were 82 decay rate values for SARS-CoV-2 (from 24 publications), 22 decay rate values for SARS-CoV (from 7 publications), 35 decay rate values for MERS-CoV (from 3 publications), 42 decay rate values for influenza virus (from 13 publications), and 16 decay rate values for RSV (from 1 publication). The identified decay rates were classified based on the virus and surface type.

The selected literature used in this study is indicated in Table S1. Across all the 197 values collected, decay rate values ranged between −1.69 and 1.46 $\log_{10}$ (h^{-1}) for all surfaces and aerosol (Fig. 2). The decay rate values of SARS-CoV-2 varied between −1.49 and 1.20 $\log_{10}$ (h^{-1}) on all surfaces and aerosols. The decay rate values of SARS-CoV varied between −1.26 and 1.39 $\log_{10}$ (h^{-1}) on all surfaces and aerosols. The decay rate values of MERS-CoV varied between −1.19 and 0.74 $\log_{10}$ (h^{-1}). The decay rate values of the influenza virus varied between −1.69 and 1.46 $\log_{10}$ (h^{-1}). RSV on aerosols decay rate varied between −0.33 and 0.34 $\log_{10}$ (h^{-1}).

Higher decay rate values were observed for RSV, SARS-CoV-2, and influenza virus than for MERS-CoV and SARS-CoV. Among the decay rate values collected for influenza viruses, there were articles related to the decay rate values of influenza A (H1N1, H7N1, and H3N1), influenza B, avian influenza (H9N2 and H6N1), and bovine influenza viruses. Based on our inclusion criteria, a limited number of articles were identified for each type of influenza virus. According to the International Committee on Virus Taxonomy, influenza A, influenza B, avian influenza, and bovine influenza are classified under the *Orthomyxoviridae* family. Therefore, in this study, the calculated decay rate values for influenza A, influenza B, avian influenza, and bovine influenza were classified into "influenza viruses."

Among the identified decay rate values, the authors used different

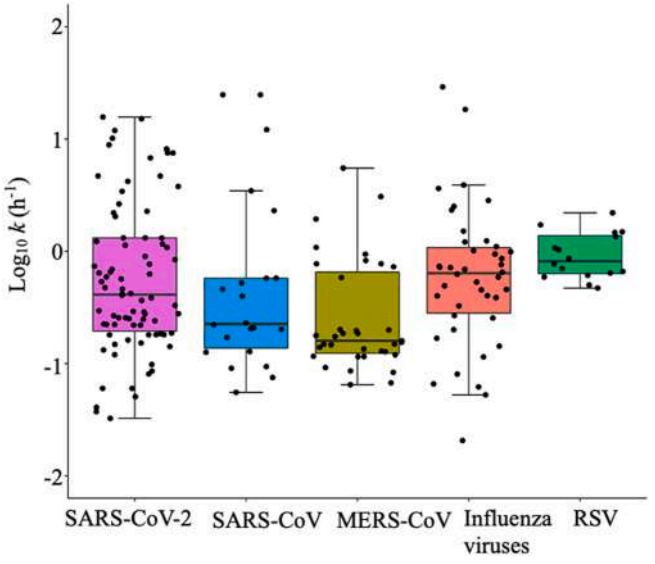

Fig. 2. $\log_{10}$ decay rates of different respiratory viruses from aerosols and other surfaces.

types of surfaces to determine the viability of viruses. Unfortunately, there were limited articles for each type of reported surface. Therefore, it is difficult to identify the surface-specific decay rate for each surface type used in these studies. Hence, we grouped the surfaces into different categories to use the maximum number of decay rates collected in this study. Plain paper, inkjet paper, inkjet photo paper, paper, magazine, and tissue paper were grouped into one category named "paper." Silver, copper, and metals were grouped into the category named "metals." Tyvek, polypropylene, soft toys, and plastic were grouped as "plastic." Surgical masks, N95 masks, and N100 masks were grouped as "masks." The $1 and $20 banknotes were grouped as "banknotes." Cotton, handkerchief, pajama, fabric, and clothing were grouped as "cloth." Accordingly, for this study, we considered 10 categories of surfaces in total (steel, plastic, cloth, glass, banknote, skin, metal, cardboard, paper, and mask).

Eighty-two decay rate values for SARS-CoV-2 were obtained using the Vero E6 cell lines. In addition to those for aerosols (n = 8), decay rate values were reported for a variety of surfaces, including paper (n = 4), steel (n = 32), plastic (n = 11), cloth (n = 2), glass (n = 8), banknotes (n = 4), skin (n = 3), metal (n = 1), cardboard (n = 2), ceramic (n = 1), and masks (n = 6). Twenty-two decay rate values for SARS-CoV were obtained using the Vero E6 and FRhK-4 cell lines. In addition to those for aerosols (n = 2), decay rate values were reported for metal (n = 2) and plastic (n = 18) surfaces. Forty-two decay rate values for influenza viruses were obtained using the Vero E6, MDCK, EBL, and HBE cell lines. In addition to those for aerosols (n = 32), decay rate values were reported for surfaces, including paper (n = 4), plastic (n = 3), and glass (n = 3). Thirty-five decay rate values for MERS-CoV were obtained using the Vero E6 cell lines. In addition to those for aerosols (n = 5), decay rate values were reported for various surfaces, including steel (n = 10), plastic (n = 7), and metal (n = 13). Sixteen decay rate values for RSV were obtained using the Vero E6 cell line for aerosols. Different culture mediums buffered solutions were used in experiments. Culture mediums were Dulbecco's modified Eagle's medium, Glascow minimum essential medium, tissue culture media, and simulated saliva. Buffered solution was phosphate buffered saline (PBS).

Fig. 2 illustrates the obtained distribution of all the decay rate values for each virus type under different environmental conditions (temperature, humidity, and solar radiation), matrices (aerosol, paper, steel, banknote, mask, skin, cloth, cardboard, plastic, glass, metal), enumeration methods (including $TCID_{50}$/ml and PFU/ml), and cell lines (Vero E6, Madin–Darby Canine Kidney, Human Bronchial Epithelial, and Embryonic Bovine Lung). It is important to note that there is a diverse distribution among the decay rate values for each type of virus and within a virus type.

3.2. Effects of environmental factors on virus decay rate

We represented the distribution of all collected decay rate values of respiratory viruses based on variations in temperature and relative humidity (Fig. 3).

Even within the same temperature/humidity range, there is a significant deviation in the decay rates by up to two orders of magnitude (Fig. 3), probably due to the coupling effect of different experimental conditions (Guo et al., 2021). In this study, we separately analyzed the effects of environmental factors on the coronaviruses SARS-CoV-2, SARS-CoV, and MERS-CoV. The effects of temperature and relative humidity on the decay rate of SARS-CoV-2 are shown in Fig. 4, and those of other coronaviruses (SARS-CoV and MERS-CoV) are shown in Figs. S2 and S3.

Based on the decay rate values collected in this study (Table S4), higher decay rate values for SARS-CoV-2 were reported for papers and aerosols. In contrast, the lowest decay rate values were reported for glass, plastic, and metal. For SARS-CoV, higher decay rate values were reported for glass and plastic, whereas the lowest decay rate values were reported for steel surfaces. For MERS-CoV, higher decay rate values were reported for aerosols, and the lowest decay rate values were reported for plastics and steel. In addition, higher decay rate values were reported for steel and paper for influenza viruses, whereas the lowest decay rate values were reported for aerosols and glass surfaces. For RSV, based on the identified decay rates for the aerosols in this study, decay rate values ranged between $-0.63\ h^{-1}$ and $0.34\ h^{-1}$ within a temperature range of 20.5–23.5 °C and a relative humidity range of 20–90%.

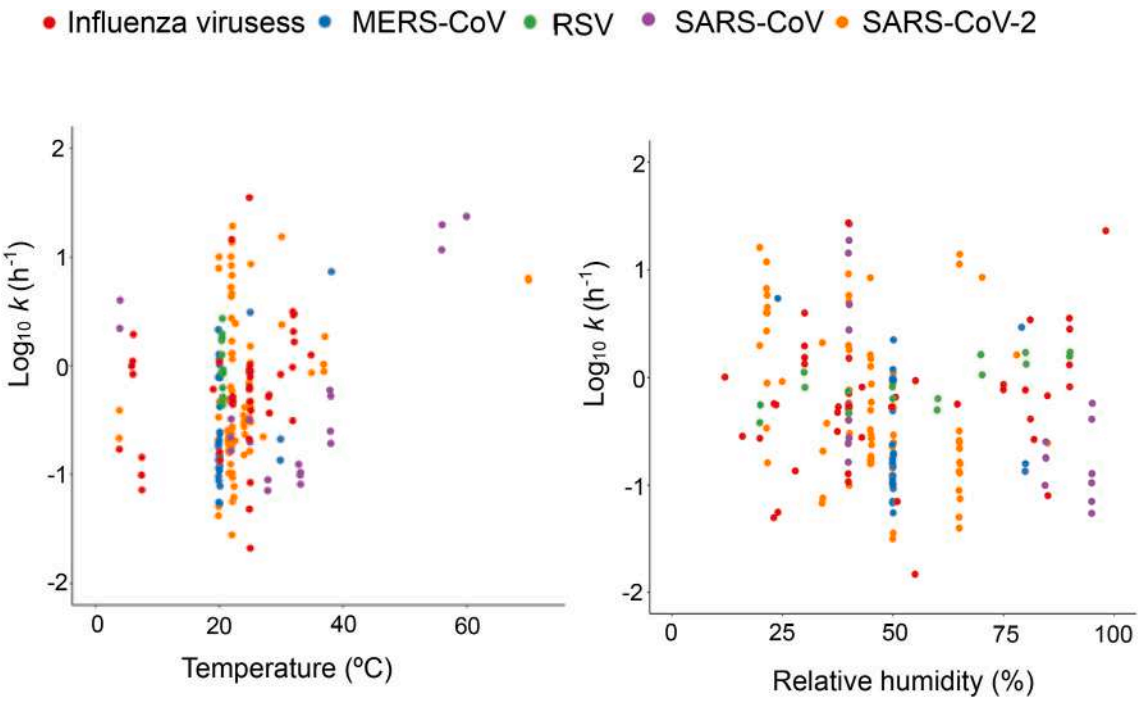

Fig. 3. Distribution of $\log_{10}$ viral decay rates based on environmental factors. (a) Temperature and (b) relative humidity.

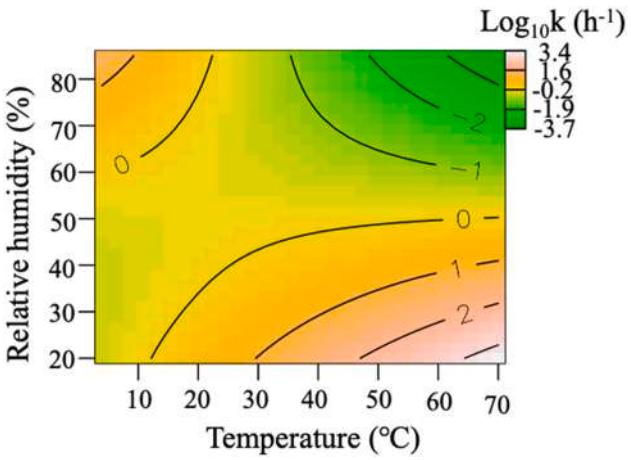

Fig. 4. Effects of environmental factors on the $\log_{10}$ decay rate values of SARS-CoV-2. Rate; contour plot illustrating the effects of relative humidity and temperature on the decay rate.

3.3. Bayesian regression model and ridge regression model estimation

We developed decay rate estimation models using Bayesian regression and ridge regression models. VIF values for the variables for each virus are presented in Table S3. AIC values of decay rates are presented in Table S2. Mean and standard deviation of the values are presented in Table S4. The observed and estimated values obtained using the Bayesian regression model are presented in Fig. 5, and the observed and estimated values obtained using the ridge regression are presented in Fig. 6. The model performance criteria values for the ridge regression and Bayesian regression models are presented in Table 1. R^2 values of the train and test data to check whether the model over-fitted data or not are presented in Table S11. Based on the model performance criteria values for SARS-CoV-2 and influenza viruses, the Bayesian regression model was the better estimation model; meanwhile, the ridge regression model could be considered the better estimation model for SARS-CoV and MERS-CoV. The coefficients of the best estimation models are shown in Fig. 7. Influenza viruses were not included, as there were no available decay rate values for a wide range of temperature and humidity coefficient values. Predicted decay rate values from the better estimation model and the relevant environmental conditions are presented in Table S5, S6 and S7 for SARS-CoV-2, SARS-CoV and MERS-CoV

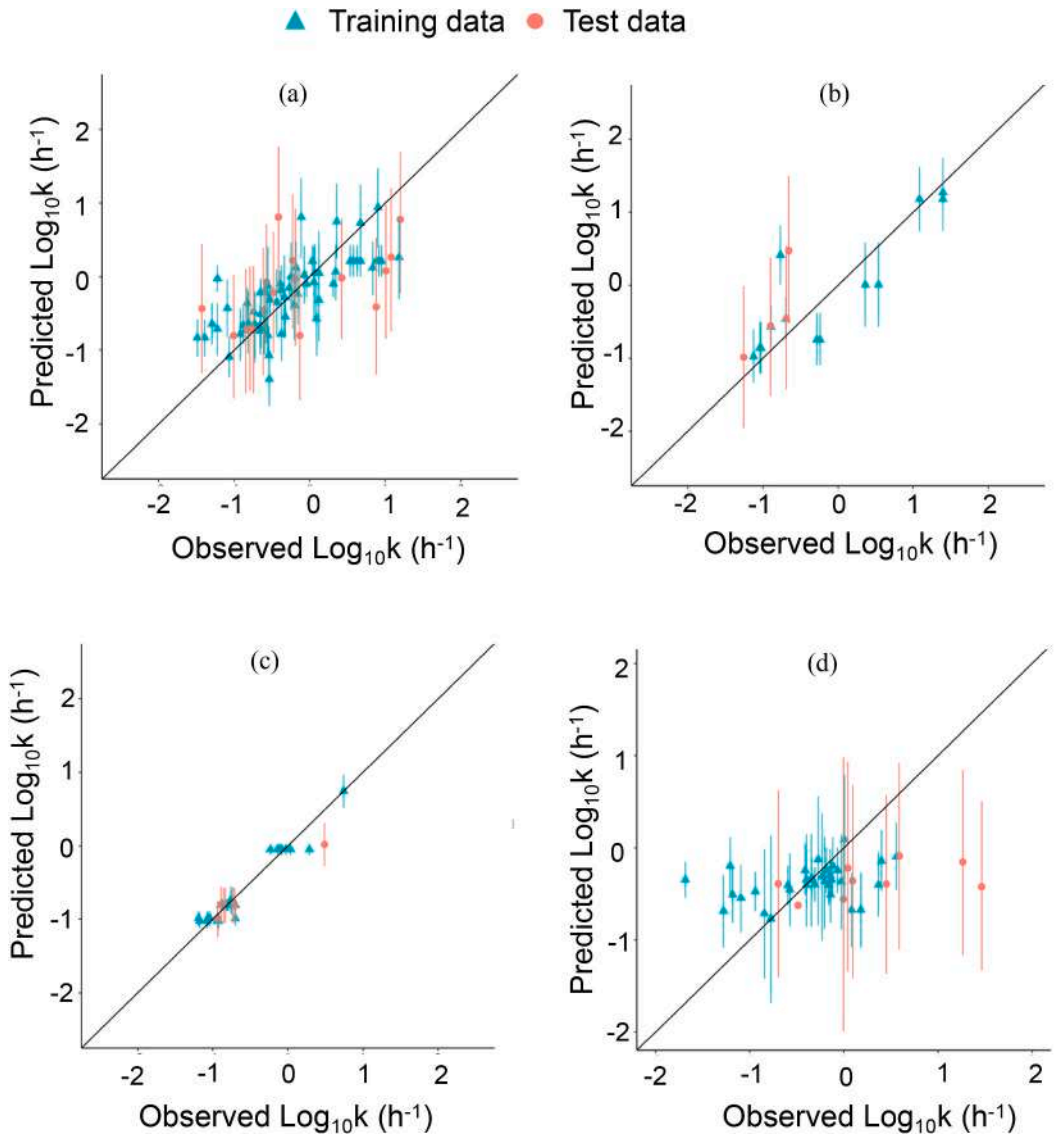

Fig. 5. Comparison of observed and predicted data using the Bayesian regression model. (a) SARS-CoV-2, (b) SARS-CoV, (c) MERS-CoV, and (d) influenza viruses.

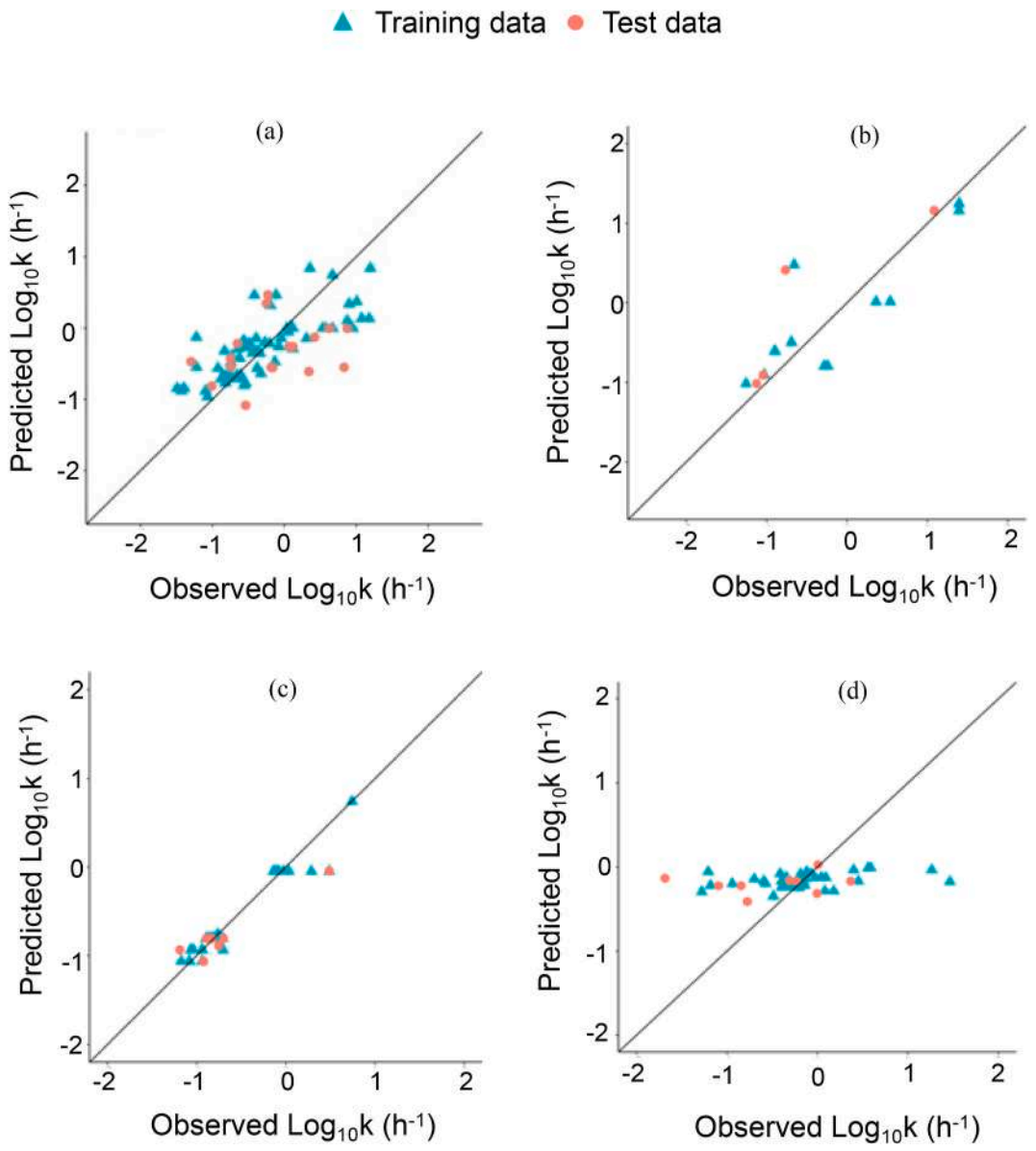

Fig. 6. Comparison of observed and predicted data using the ridge regression model. (a) SARS-CoV-2, (b) SARS-CoV, (c) MERS-CoV, and (d) influenza viruses.

Table 1
Comparison of goodness of fit between the ridge regression and Bayesian regression model based on RMSE, MAE and AUC.

Virus type	RMSE	MAE	AUC
Bayesian regression model			
SARS-CoV-2	0.41	0.31	1
SARS-CoV	0.40	0.37	1
MERS-CoV	0.12	0.08	0.5
Influenza viruses	0.50	0.35	1
Ridge regression model			
SARS-CoV-2	0.45	0.34	1
SARS-CoV	0.35	0.34	0.5
MERS-CoV	0.12	0.08	1
Influenza viruses	0.57	0.40	1

Bold virus type represents the better estimation model. Abbreviations: SAR-CoV-2: sever acute respiratory syndrome coronavirus 2; SAR-CoV: severe acute respiratory syndrome coronavirus; MERS-CoV: Middle East respiratory syndrome coronavirus; RMSE: Root mean square error, MAE: mean absolute error, AUC: area under the curve.

respectively.

We found that the decay rates were lower at lower temperatures (Fig. 7). The temperature in the available dataset ranged between 4 °C and 70 °C. Therefore, we assumed temperatures between 4 °C and 35 °C as a lower temperature range in the simulation. Using better estimation models, we simulated the decay rate values of the selected surfaces under different environmental conditions (Fig. 8). In addition, we estimated the virus decay rates during distinct seasons of the year, assuming two seasons—summer and winter (Fig. 9). We used 20 °C and 40% RH for summer and 28 °C and 70% RH for winter, assuming the indoor conditions.

According to the simulation (Fig. 8) log $_{10}$ decay rate of SARS-CoV-2 ranged from −0.345 to 0.104 h^{-1}, -0.839 to 0.197 h^{-1}, -0.484 to 0.095 h^{-1}, -0.698 to 0.212 h^{-1}, and -0.706 to −0.265 h^{-1} for aerosol, steel, masks, cloth, and plastic respectively. For SARS-CoV log $_{10}$ decay rates ranged from −0.911 to 0.911 h^{-1} for plastic. For MERS-CoV log $_{10}$ decay rates ranged from 0.122 to 0.512 h^{-1}, -0.678 to −0.735 h^{-1}, -0.543 h^{-1} to −0.605 h^{-1}, and -0.345 to −0.786 h^{-1} for steel, aerosol, plastic, and metal respectively (Table S9). The coefficients of the better estimation model are presented in Table S8. Estimated decay rate values for the

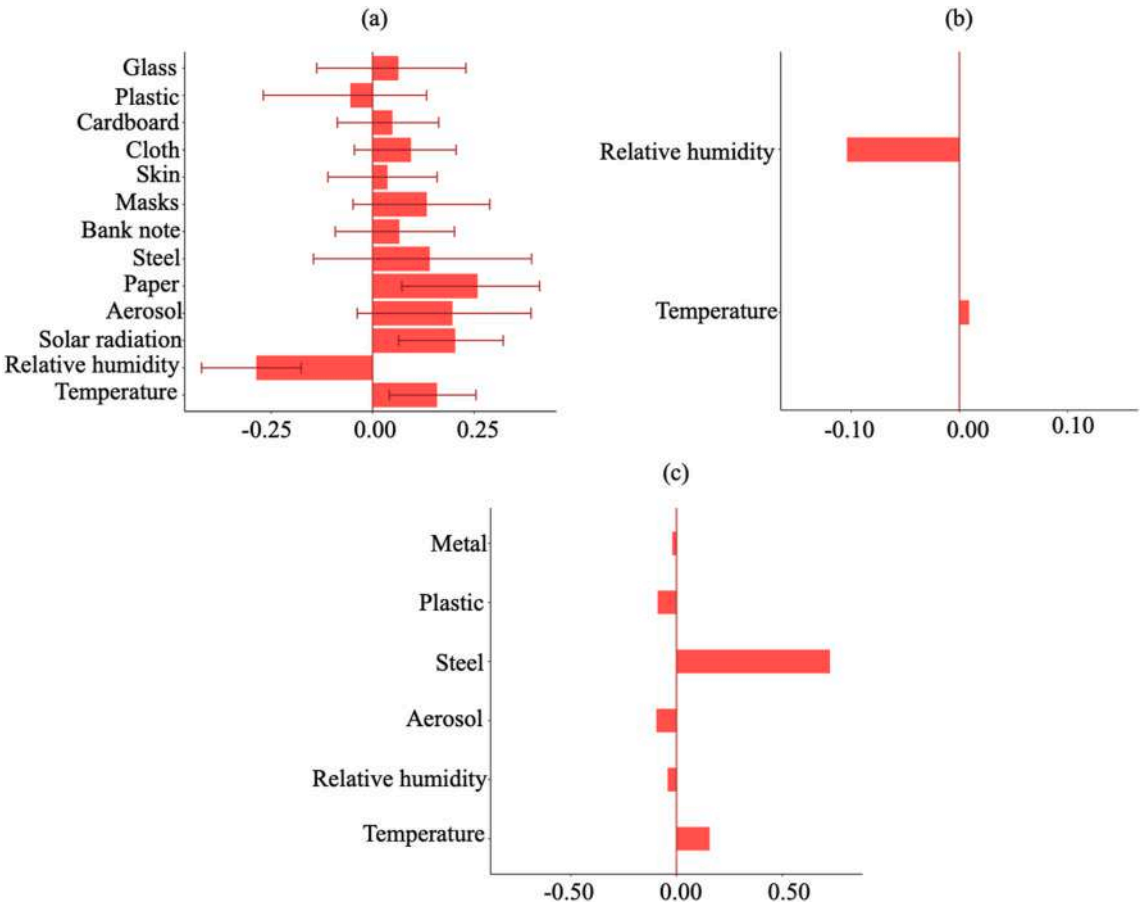

Fig. 7. Coefficient estimates from better estimation models with 2.5 and 95 percentiles (error bars) and posterior medians (bars). (a) SARS-CoV-2, (b) SARS-CoV, and (c) MERS-CoV.

seasons are presented in Table S10.

It is important to highlight that the simulated decay rates on each surface in summer were higher than those in winter. For aerosols, there was an approximately 1.7-fold difference in the decay rate of SARS-CoV-2 and MERS-CoV during summer and winter. For porous surfaces, including papers, there was an approximately 1.6-fold difference in the decay rate of SARS-CoV-2. For non-porous surfaces, including plastics, there was an approximately 1.7-fold difference in the decay rate of SARS-CoV-2, a 3.7-fold difference in that of SARS-CoV, and a 1.9-fold difference in the decay rate of MERS-CoV. The decay rate values for plastics were lowest for both SARS-CoV-2 and MERS-CoV.

4. Discussion

Viruses undergo rapid inactivation under conditions such as high temperatures and high relative humidity (Biryukov et al., 2020b, 2021); mechanisms of viral inactivation include thermal denaturation of proteins and nucleic acids (Marr et al., 2019). However, several studies have also indicated partial inactivation of viruses in aerosols and droplets at high relative humidity levels (Lin and Marr 2020; Yang and Marr 2011). This indicates a non-linear relationship between the decay rate values and environmental factors.

There has been a debate over the use of relative or absolute humidity to determine virus viability. Absolute humidity is the mass concentration that defines the amount of water vapor per air volume. In contrast, relative humidity is the water vapor pressure/concentration ratio to the saturation vapor pressure/concentration (Marr et al., 2019). A few studies have argued absolute humidity to be a more important predictor of viability of viruses than relative humidity (McDevitt et al., 2010; Shaman and Kohn 2009). However, most studies have concluded that relative humidity is important in determining virus viability (Hemmes et al. 1962; Marr et al., 2019). Marr et al., (2019) reevaluated the viability of influenza virus in aerosols and its transmission in animal models and concluded that the combination of relative humidity and temperature is equally valid as absolute humidity as a predictor. Hence, in our study, we included relative humidity to develop an estimation model for virus decay rate values. Relative humidity is an extrinsic factor that affects virus viability in two ways—(1) it can control the evaporation kinetics and change the solute concentration in the droplet, droplet size, and, physical fate; and (2) increasing the solute concentration and frequent exposure to higher solute concentrations can increase the virus inactivation (Lin and Marr 2020; Marr et al., 2019).

The type of deposition solution used in decay experiments tends to influence the activity of viruses, regardless of the environmental conditions. Dulbecco's modified Eagle's medium, when used as a deposition solution, caused a greater inactivation of bacteriophage phi6 than that achieved with PBS under the same relative humidity and temperature range; this highlights the importance of using the right type of deposition solution for enveloped viruses (Rockey et al., 2020). Additionally, salt and protein contents in the medium could further affect viral inactivation (Rockey et al., 2020; Yang et al. 2012); proteins affect the resistance of viruses to drying and hence influence the persistence of the viruses in the environment (Pastorino et al., 2020), whereas high salt concentrations affect the persistence of viruses owing to evaporation effects (Yang et al., 2012). Interactions between proteins and salts at different relative humidity values further complicate virus inactivation predictions (Yang et al., 2012). Yang et al. investigated the influenza virus decay rate with salt concentrations in different media. At 60% relative humidity, influenza virus decay rates increased linearly in the droplets in DMEM with NaCl concentrations up to 420 gL^{-1}; similarly, in

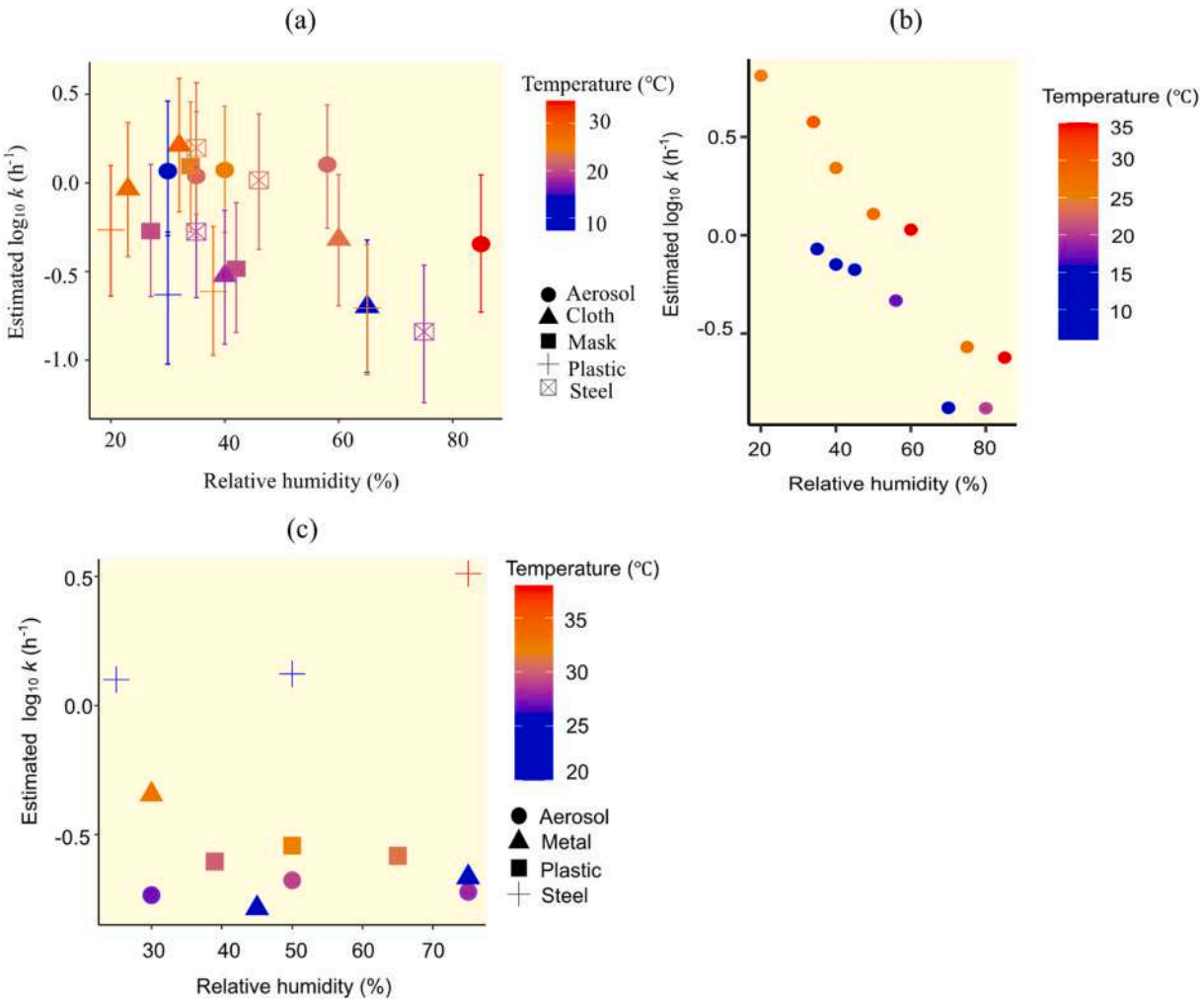

Fig. 8. Simulation of viral decay rates under different environmental conditions using better predictive model on select surfaces and aerosols from a randomly generated dataset. For (a) SARS-CoV-2 using Bayesian regression model. (b) SARS-CoV using ridge regression model for plastic surfaces. (c) MERS-CoV using ridge regression model.

the PBS medium, the decay rate increased linearly with NaCl concentrations up to 25–510 gL^{-1}. Yang et al. concluded that increasing salt concentration increases virus inactivation, and interactions among proteins, salts, and the virus can mitigate the adverse effects of high salt concentrations. However, the salt and protein contents of PBS and DMEM did not significantly affect SARS-CoV-2 inactivation (Rockey et al., 2020). Future work is necessary to test the effects of the ingredients in the culture medium on the virus decay rate under different humidity and elevated temperatures.

Sunlight has a significant effect on the decay rates of viruses. Simulated sunlight inactivates SARS-CoV-2 in aerosols with half-lives shorter than 6 min and a 90% inactivation within 20 min (Rothamer et al., 2020; Schuit et al., 2020). The decay rates of viruses exposed to solar radiation depend on the type (UVA, UVB, and UVC) and intensity (Rothamer et al., 2020; Sutton et al., 2013; Wood et al., 2010). A significant reduction in viral activity under high intensity solar radiation has been reported; UVC light at 254 nm causes rapid inactivation of SARS-CoV on surfaces (Darnell et al., 2004), while influenza virus (H7N1) decay rates increase with solar radiation (UVB and UVC), which causes a 1 $\log_{10}$ titer reduction within 69 min (Sutton et al., 2013).

Casanova et al. (2010) determined the effects of air temperature and humidity on coronavirus (SARS-CoV) survival on surfaces. To overcome the challenges associated with pathogenic viruses, two surrogate viruses, mouse hepatitis virus and transmissible gastroenteritis virus, were used in the study. At 4 °C, viruses persisted for 28 d on stainless steel and inactivation was faster at 20 °C than at 4 °C at all the tested humidity levels (Casanova et al., 2010). Furthermore, Casanova et al., 2010 reported that the relationship between relative humidity and inactivation was not monotonic and that a greater survival effect is exerted at low (20%) and high relative humidity (80%) levels than that at moderate humidity (50%) (Casanova et al., 2010). A similar relationship was observed in our collected decay rate values for SARS-CoV (Fig. S2). Pyankov et al. (2018) reported that the decay rate of MERS-CoV was higher under hot and dry climatic conditions (38 °C and 24% RH), with only 4.7% remaining infectious for over 60 min after aerosolization, while a survival rate of 63.5% was reported at 25 °C and 79% RH (Pyankov et al., 2018). Humidity affects both influenza virus transmissions and survival (Yang et al., 2012). Hemmes et al., (1962) and Harper, 1961 found higher decay rates at both medium and high relative humidities (Harper 1961; Hemmes et al., 1962). However, Schaffer et al., 1976 found moderate inactivation at higher temperatures and higher inactivation rates at medium temperature (Schaffer et al. 1976). The viability of influenza virus in aerosols and droplets is poorly understood (Yang et al., 2012). Based on previous studies on SARS-CoV-2, lower inactivation rate values were reported at low temperatures; however, sensitivity to heat occurs at a high temperature of approximately 70 °C (Aboubakr et al. 2021; Meyerowitz et al., 2021). Similarly, in our study, compared to those at low temperatures (0–40 °C), high decay rates were reported at higher temperatures (75–100 °C).

In addition to temperature, relative humidity, and solar radiation, we

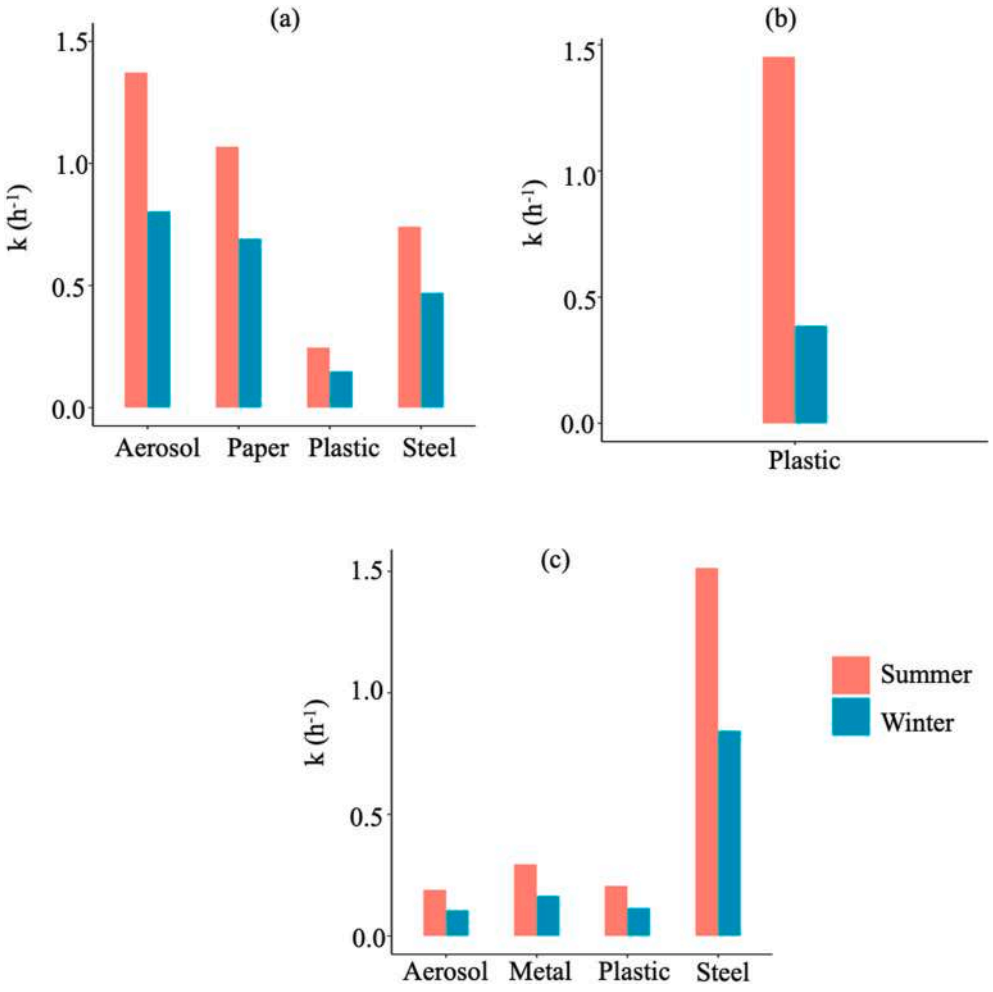

Fig. 9. Simulation of viral decay rates under different environmental conditions for selected surfaces: summer and winter. For (a) SARS-CoV-2 using the Bayesian regression model 50% credible interval of the predicted decay rate values used in the graph; (b) SARS-CoV using the ridge regression model; (c) MERS-CoV using the ridge regression model. The temperature and relative humidity used for the simulations were 20 °C and 40% RH for summer, and 28 °C and 70% RH for winter, respectively.

considered other important factors affecting the virus decay rate, which include particle size, the porosity of the object surface, water absorption degree, surface tension, aggregation state of the droplet on the surface, water absorption degree, surface tension, aggregation state of the droplet on the surface, and type of virus (Guo et al., 2021). However, there is limited information available related to the factors listed above. We highly recommend considering these factors in future research, which will help to determine the decay rate of viruses. Furthermore, the volume of the virus-containing droplet significantly influences virus decay, and rapid virus inactivation results in a lower droplet volume (Guo et al., 2021). There can be differences in the initial volume of the virus-containing droplets titrated in the experiments in the literature. This may have caused significant differences in the experimental data reported (Guo et al., 2021). Guo et al., (2021) analyzed the relationship between the virus decay rate and titration volume and concluded that there was a greater risk of transmission with a larger droplet volume (Guo et al., 2021). Previous literature includes decay rate values on surfaces on different experimental methods with scattered results, making comparisons difficult because of biased information (Guo et al., 2021). Guo et al. (2021) further evaluated the effect of the volume of viral suspensions on the decay rates of viruses used in previous studies. In future research, it is recommended to focus more on the droplet volume used on the surfaces in the viability experiments to avoid experimental bias.

SARS-CoV-2 exhibited lower decay rates on plastic, glass, banknotes, steel, and skin than on surfaces such as paper and masks. The decay rates on porous surfaces (paper and masks) were higher than those on non-porous surfaces (plastic and glass) within a shorter time interval (data not shown). Similarly, for MERS-CoV, lower decay rate values were found in metal and plastic, which are non-porous surfaces. In contrast, for SARS-CoV, higher decay rates were found in plastics, and lower decay rates were found in metals. This may be because our dataset for SARS-CoV included decay rate values at higher temperatures. However, the decay rate values for steel were higher for SARS-CoV-2 and MERS-CoV than for the other non-porous surfaces (data not shown). In future research, we recommend determining the virus decay rate on porous and non-porous surfaces under different environmental conditions. This will help elucidate precise decay rates based on the surface type.

When comparing the decay rates of SARS-CoV-2 based on surface type during summer, there is an approximately 5-fold difference in aerosols and plastics, which is higher than the seasonal difference. For MERS-CoV, there is an approximately 5-fold difference between metals and steel. Based on the strength of our simulation, we recommend that it is vital to reinforce the control measures based on the surface type compared to the seasonal difference. It is essential to disinfect surfaces frequently, such as plastics and metals. For the surfaces with higher decay rates, it is crucial to focus on the source to adopt control measures, such as wearing masks, promoting cough etiquettes, and social distancing. Proper ventilation will reduce the impact of aerosol transmissions by aerosols. The regular implementation of the control measures is recommended to control the transmission of virus.

There are several limitations of this study. First, a few data points are available for viruses and surface types separately for model validation. Limited studies are available on the decay rate estimation of viruses for a shorter time interval on different surfaces. The higher number of data

points improves the model validation and accuracy of the prediction. Thus, we recommend determining virus decay rates in aerosols and surfaces for a shorter time interval in future research. In addition, virus inactivation mechanisms in aerosols and surfaces: porous and non-porous, are different. With the lack of data, we considered both aerosols and surfaces together. In future research, these models can be developed separately for aerosols and surfaces: porous and non-porous, based on the inactivation mechanisms. Virus decay rates are affected by the salts in the buffer solution and culture medium. Therefore, we recommend considering culture medium, buffer solution, and salts in the buffer as variables in the model to predict virus decay. In this study majority of the selected studies used the VeroE6 cell line. Few studies used FRhK-4, MDCK, EBL, and HBE cell lines. Since differences in the cell line can affect virus decay, conducting virus decay rate experiments in different cell lines in addition to diverse environmental conditions is essential.

Surface types in the models were decided based on data availability. Therefore, we recommend using different surface types for decay rate estimations in the model. However, the surfaces used in this study can be used as a basis for the decay rates on other surfaces not included in the model. For instance: the decay rate of paper reflects the higher decay rates of viruses on porous surfaces, which provides an understanding of other porous surface types not provided in this study.

Our study would serve as a basis for decision-makers in determining effective non-pharmaceutical interventions against virus transmission based on the surface type and environmental conditions. Additionally, we identified data gaps in the studies on viral inactivation of surfaces under environmental conditions and the importance of their inactivation in indoor environments for effective control of virus transmission.

5. Conclusions

In this study, we developed models to estimate the decay rate of respiratory viruses in aerosols and on various environmental surfaces using Bayesian regression and ridge regression models. Simulated decay rates demonstrated that decay rates during summer were higher than those during winter. However, the variations in decay rates among different surfaces were greater than seasonal differences in decay rates. According to our simulations, it is vital to reinforce control measures based on surface types rather than seasonal differences. In addition, we recommend determining decay rates of viruses on diverse types of porous and non-porous surfaces under different environmental conditions: temperature, humidity, and solar radiation, and experimental conditions: culture medium, buffer solution, and salts in the buffer in future research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114187.

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Effects of different laying periods on airborne bacterial diversity and antibiotic resistance genes in layer hen houses

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ABSTRACT

Poultry farms are a complex environment for close contact between humans and animals. Accumulating evidence has indicated that pathogens and drug resistance genes in chicken houses may pose a serious threat to public health and economic concerns. However, insufficient knowledge of the indoor aerosol microbiome and resistome profiles of layer hen houses hampers the understanding of their health effects. Environmental surveillance of antibiotic resistance may contribute to a better understanding and management of the human exposure risk of bioaerosols under the environmental conditions of chicken houses. In addition, the chicken house has a long operation cycle, and the bacterial diversity and antibiotic resistance genes of aerosols in different periods may be different. In this study, air samples were collected from 18 chicken houses on three farms, including the early laying period (EL), peak laying period (PL), and late laying period (LL). 16S rRNA gene sequencing and metagenomics were used to study the composition of the bacteria and resistome in aerosols of layer hen houses and the results showed that they varied with laying period. The highest alpha diversity of bacteria was observed in PL bioaerosols. The dominant bacterial phyla included Firmicutes, Bacteroidetes and Proteobacteria. Three potential pathogenic bacterial genera (*Bacteroides*, *Corynebacterium* and *Fusobacterium*) were found. The most abundant ARG type was aminoglycosides in all laying periods. In total, 22 possible ARG host genera were detected. ARG subtypes and abundance were both higher in LL. Network analysis also showed higher co-occurrence patterns between the bacteria and resistome in bioaerosols. The laying period plays an important role in the bacterial community and resistome in layer house aerosols.

1. Introduction

China is the largest egg producer, accounting for approximately 40% of the global share in 2020, and egg production continues to grow worldwide (Dai et al., 2022). Many frequent animal feeding operations may produce high concentrations of bioaerosols, which are generally defined as aerosolized particles containing microorganisms (Heederik et al., 2007; Jiang et al., 2022; Millner, 2009; Viegas et al., 2020; Wang et al., 2019). Antibiotics are often used to prevent animal diseases and improve the performance of layers (Bushen et al., 2021). Increasing antibiotic abuse may contribute to the evolution of antibiotic-resistant bacteria, which may significantly reduce the effects of antibiotics and cause an increased number of deaths each year due to aerosol pollution (Castro-Vargas et al., 2020; Gao, 2018; Zeineldin et al., 2019). Exposure can occur through inhalation or skin contact with this bioaerosol (Hoppin et al., 2014; Paton et al., 2015). Some studies have shown that agricultural workers and residents living near farms have a higher risk of respiratory diseases and bacterial infections (Audi et al., 2017; Hedelin et al., 2016; Viegas et al., 2013). The allergic immune reaction of broiler workers was higher than that of nonagricultural workers (Gautam et al.,

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2018). Specific microbial composition and accumulation of antibiotic resistance genes in the aerosols of poultry farms were two key factors that induced high exposure risks for agricultural workers. On the one hand, with the rapid development of the breeding industry, the development and spread of ARGs in breeding systems have received increasing attention. The levels of ARGs in the feces of chickens are higher than those of pig and cattle farms, and ARG contamination in the feces of layer hens is more severe than that of broilers (Gu et al., 2020; Hubbard et al., 2020). On the other hand, *Bacteroides*, *Corynebacterium* and *Fusobacterium*, which are widely distributed inside poultry farms, have been identified as risk pathogens (Brennan and Garrett, 2019; Hoefer et al., 2021b; Hong et al., 2012; Zafar and Saier, 2021). More importantly, the chicken house has a long operation cycle, and the bacterial diversity and antibiotic resistance genes in different periods may be different. Therefore, it is very important to understand the microbial composition and resistance gene contamination during different laying periods. The egg production of layer hens varies greatly during the laying period, which can be divided into three periods, including the early laying (EL) period, peak laying period (PL) and late laying (LL) period (Zhu et al., 2021). Although antibiotic use is not a sustainable strategy, farmers in some areas still use several antibiotics in the EL period to reduce the immune stress that may occur after repeated vaccination. Antibiotic use is reduced during PL periods, while it is increased again in LL periods due to the rapid decline in egg production caused by problems such as chronic inflammation. These antibiotics often included beta-lactams, tetracyclines, macrolides, and aminoglycosides. Farms that overuse antibiotics are more likely to have problems with resistant bacteria than those with normal usage, and resistant bacteria can persist in the farm environment even after selection pressures have dissipated(Xu et al., 2020).

It has been reported that the main source of microbial pollution in the air of closed livestock and poultry houses is feces (Chien et al., 2011; Clark et al., 1983; Cormier et al., 1990; Donham et al., 1986; Nehme et al., 2008). Until now, most studies of antibiotic resistance in chicken farming have focused mainly on feces (Qiu et al., 2021; Sergeant et al., 2014). Information on antibiotic resistance in the air of layer hen farming systems is still very limited. At the same time, there are also few studies on the microbial composition and potential zoonotic pathogens in the environment of layer houses during different laying periods (Zhu et al., 2021). Indoor air in layer houses is an important reservoir of ARGs (Wychodnik et al., 2020). There is an urgent need for risk assessment of microorganisms and antibiotics in the layer hen house environment to distinguish the environmental impacts of antibiotic use at different periods of eventual egg production.

In this study, bacterial community and resistance genomes in bioaerosols from layer houses at different laying periods were analyzed to obtain taxonomic and compositional changes, as well as their co-occurrence networks in different ecological niches. This can extend our understanding of the link between bioaerosol microbes and ARGs, the key driving forces shaping resistance genes in the air, and facilitate targeted interventions to avoid antibiotic residues and improve egg safety in layer hens.

2. Materials and methods

2.1. Layer hen farms

This study was carried out in three representative commercial layer hen farms in Hebei Province in the winter of 2019 that belong to the same company, and the data were analyzed anonymously. There were three types of layer hens on each farm: EL period (17–30 weeks), PL period (31–50 weeks), and LL period (50–70 weeks). Two samples were drawn from each house type per farm. The samples were taken consecutively but on different days in December. During our visits, none of the farms were dusted, and no disease outbreaks were reported. These farms cover an average area of about 60000 m^2, with 18 chicken houses. Each of the selected layer hen houses covers an average area of about 1020 m^2, with six workers and about 50000 layers. In addition, these workers were not wearing any personal protective equipment during our sampling period. In these chicken farms, Tetracyclines, Sulfonamides, Macrolides, and Aminoglycosides are the commonly-used antibiotics for veterinary purposes. Unfortunately, there is no specific record of the antibiotics used at different layer periods by the farms. Temperature and humidity were controlled with pipe heating and mechanical ventilation, and layers were raised in cages for egg production with numbers ranging from 50000 to 55000.

2.2. Sample collection and preparation

A total of 18 bioaerosol samples were obtained, as shown in Table S1. Each farm has 3 types of stables: 2 EL houses, 2 PL houses and 2 LL houses. Sample data for all farms are given in the table. Collection of air samples was performed according to our previously published method with slight modification (Cui et al., 2022). Briefly, a high-volume air sampler (HH02-LS120 of Beijing Huarui Hean Technology Co., Ltd., China) equipped with a 19.8 cm × 23.8 cm quartz fiber membrane (Cat No. 7204, Tissuquartz 2500 QAT-UP; PALL, NY, U.S.) was placed in the middle of each chicken house corridor at a height of 1.5 m above ground away from heating or cooling equipment to collect aerosol samples. For each sample, the device was run continuously for 12 h at a flow rate of 1000 L/min. Before sampling, the sampler was sterilized with 75% alcohol, and all filter membranes were sterilized by baking in a muffle furnace at 500 °C for 48 h. Samples were immediately transferred to the laboratory in ice boxes after collection. The membranes were stored at −80 °C before use.

2.3. DNA extraction

DNA extraction was performed as described in our previous publications (Cui et al., 2022; Guo et al., 2018). In brief, each membrane was cut into an average of 8 sections with sterile surgical scissors, with a weight error of ±1 mg per section(Gao et al., 2017). One-eighth of the membrane was cut into small pieces and placed in an Eppendorf tube preset to 1 mL ultrapure water (ST876, Yaji, Shanghai, China) and sterile steel beads. Samples were homogenized using a homogenizer to elute particles and then centrifuged (at 25000 g for 10 min at 4 °C). DNA was extracted using the MO-Bio PowerSoil DNA Isolation Kit (Carlsbad, CA, USA.). All DNA template concentrations were quantified by Q5000 UV/Vis spectrophotometry (Quawell, USA). “Blank” samples (sterilized water) were extracted to control for methodological contamination (Salter et al., 2014).

2.4. 16S rRNA amplicon and metagenomic sequencing

The V3/V4 region of the 16S rRNA gene was targeted for amplification using the previously published universal primers 515F and 806R (515F: 5′-TGTGCCAGCMGCCGCGGTAA-3′; 806R: 5′GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2012). Each sample was amplified in triplicate using the PCR conditions described previously (Yang et al., 2018). Samples were verified by 2% gel electrophoresis, and the target bands were excised and purified with the Qiagen QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). Samples were sent to Novogene Co., Ltd. (Beijing, China) for sequencing application on the Illumina MiSeqPE250 platform.

The raw 16S rRNA gene sequence was processed with Mothur software to retain high-quality reads (V1.36). Uparse software (Uparse v7.0.1001, http://www.drive5.com/uparse/) was used to cluster the sequences with 97% identity, which were clustered into operational taxonomic units (OTUs)(Li et al., 2020). OTUs were assigned to rRNA sequences of the SILVA (SSU132) 16S rRNA using an 80% confidence threshold. The DNA extracted for 16S rRNA was also used for metagenomic sequencing on the Illumina HiSeq 4000 platform.

2.5. Calculation of ARG abundance

ARG abundance was determined using ARGs-OAP (Yang et al., 2016). In brief, potential ARG readings and 16S rRNA genes were identified, and ARG-like readings were identified and annotated using BLASTX by applying CARD, the Comprehensive Antibiotic Resistance Database (Li et al., 2015; Yang et al., 2013). The abundance of ARG readings in the samples was calculated and normalized by the number of 16S rRNA genes, defined as relative abundance. ARG abundance was expressed as ARG copies per copy of the 16S rRNA gene (Xiong et al., 2018). ARG types and subtypes are counted automatically using the custom script package described earlier (Yang et al., 2013).

2.6. Data analysis

Alpha diversity was calculated using QIIME 2 (Quantitative Insights Into Microbial Ecology) (Bolyen et al., 2019). All statistical analyses were performed using Graph Pad Prism 9 (Graph Pad Software, USA.). A P value < 0.05 was considered statistically significant. The nonparametric Kruskal–Wallis test was used for statistical comparison. The composition differences of bacterial communities and ARGs in the air of layer hen houses during different periods were analyzed using the Bray–Curtis similarity index and visualized using nonmetric positions scaling (NMDS). The ARG and genus networks were analyzed based on Spearman's correlation matrix. The graph was visualized with Gephi software (Gephi Consortium, USA) to generate a network diagram (V 0.9.2).

2.7. Sequence submission

All sequence data obtained have been submitted to NCBI BioProject under accession numbers PRJNA875241 and PRJNA875383.

3. Results

3.1. Diversity of aerosol bacteria at different laying periods

An average of 88,559 raw reads were obtained from each sample. After quality control, 62,253 valid data points were obtained, and the quality control efficiency was 70.30%. Sequences were clustered into 23,501 OTUs at a 97% identity threshold (mean = 1306, Table S2). As shown in Fig. 1 and Fig. S1, the observed diversity was greatest in the PL period. The observed species, Shannon and ACE indexes of samples from the PL period were higher than those of samples from the EL or LL period ($p < 0.01$) (Table S2). NMDS analysis of the microbial OTU relative abundance data shown in Fig. 1D revealed clear separation of the samples from different periods ($p < 0.05$), and samples from the same period clustered more closely together.

3.2. Composition of abundant airborne bacterial phyla

The dominant phyla (abundance >1%) of all samples are shown in Fig. 2. The most abundant bacterial phyla were Firmicutes (46.00%–68.01%), followed by Bacteroidetes (7.50%–23.68%), Proteobacteria (2.04%–15.93%), Cyanobacteria (1.17%–14.67%), Fusobacteria (3.60%–6.62%) and Actinobacteria (1.25%–2.83%) (Fig. 2A, Table S3). The relative abundance of Firmicutes was significantly higher in the PL period than in the EL and LL periods ($p < 0.01$ Fig. S2A). The relative abundance of Bacteroidetes was significantly higher in LL than EL ($p < 0.05$, Fig. S2B). The relative abundance of Proteobacteria detected in EL was significantly higher than that in PL and LL ($p < 0.01$, Fig. S2C).

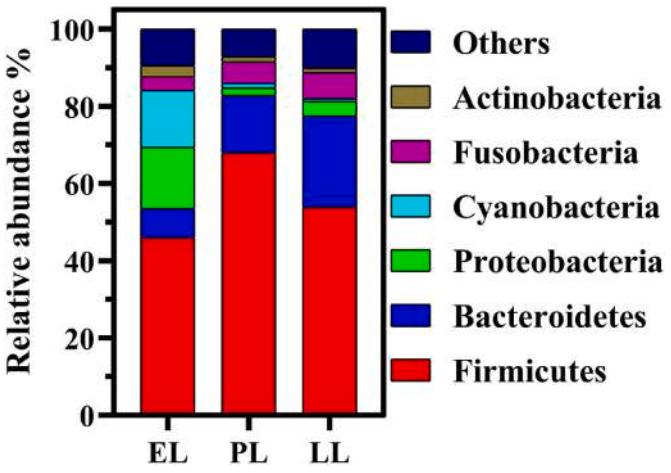

Fig. 2. Compositions of microbial communities at the phylum level among the three laying periods. Composition and relative abundance of different phyla.

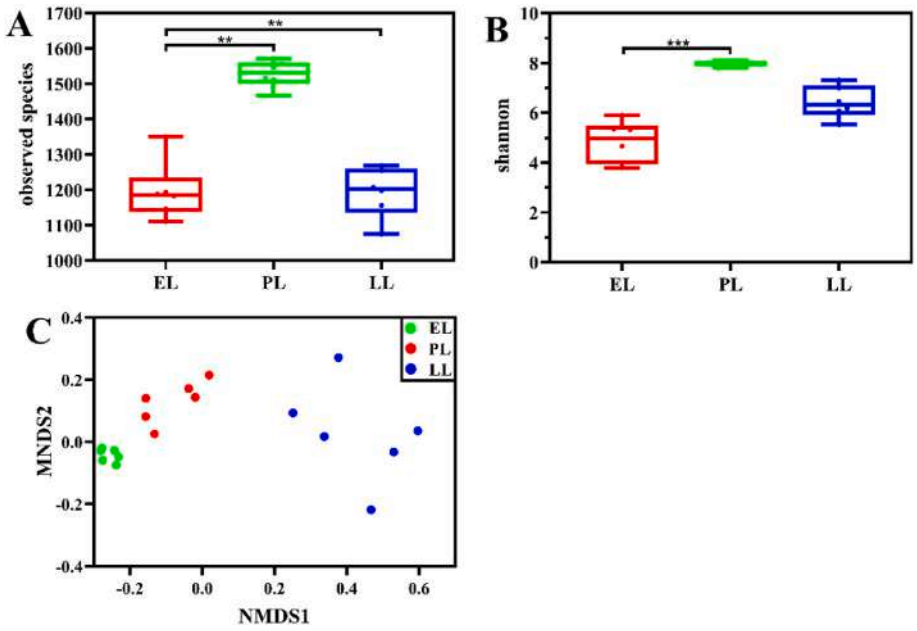

Fig. 1. Diversity analysis of microbial community profiles in air samples of three laying periods. (A) Observed species for each laying period; (B) Shannon index for each laying period; (C) nonmetric multidimensional scaling (NMDS; Bray–Curtis distance) plot based on OTU abundances demonstrating the differences in microbial community composition of air samples among the laying periods. Wilcoxon rank sum test: **($p < 0.01$) and ***($p < 0.001$). Abbreviations: EL, early laying period; PL, peak laying period; LL, late laying period.

3.3. Composition of abundant airborne bacterial genera

The dominant genera (average abundance >1%) within each period are shown in Fig. 3A. There were 6, 7 and 10 dominant genera in the EL, PL and LL periods, respectively (Table S4). *Lactobacillus* was the main genus in EL (55.96%) and PL (63.19%), while *Bacteroides* was the most abundant genus in LL (28.32%) (Fig. 3A). According to the list of human pathogens provided by the Ministry of Health of China (MOHC)(MOHC, 2006; Yan et al., 2021), three potential pathogen genera posing potential risks to humans (*Bacteroides*, *Corynebacterium* and *Fusobacterium*) were identified. The abundances of *Bacteroides*, *Corynebacterium* and *Fusobacterium* in LL buildings were significantly higher than those in EL buildings ($p < 0.01$). The abundance of *Bacteroides* and *Fusobacterium* in LL building was significantly higher than that in PL building ($p < 0.05$, Fig. 3B). There were also significant changes in nonpathogenic bacteria during different periods. The abundances of *Lactobacillus* and *Facklamia* at the EL period were significantly higher than those in PL ($p < 0.01$ Fig. 3B). The abundance of *Comamonas* in the LL and PL periods was significantly higher than that in EL ($p < 0.05$, Fig. 3C). The abundance of *Jeotgalicoccus* in PL was significantly higher than that in LL ($p < 0.01$, Fig. 3C). The abundance of *Stenotrophomonas* in the LL period was significantly higher than that in the EL ($p < 0.01$) and PL ($p < 0.05$) periods (Fig. 3C).

3.4. Airborne ARGs in layer houses

The total number and relative abundance of ARG subtypes were the lowest in the PL period and were significantly lower than those in the LL house ($p < 0.01$, Fig. 4A and B). There was a total of 18 ARG types in all samples, and 14 were shared across all laying periods.

Compared with LL, EL showed a higher abundance of aminoglycosides ($p < 0.05$). EL showed a higher abundance of beta-lactam, fosfomycin, tetracycline, and vancomycin ($p < 0.05$, Fig. 4C) and a lower abundance of multidrug and polymyxin ($p < 0.05$, Fig. 4C) than PL. In addition, PL showed a lower abundance of beta-lactam and macrolide-lincosamide-streptogramin (MLS) compared with LL ($p < 0.05$, Fig. 4C), while Chloramphenicol, Polymyxin and Sulfonamide ($p < 0.01$, Fig. 4C) were significantly higher than LL. The abundance of tetW and tetQ in the PL period was significantly higher than that in the LL period. The abundance of ermF in the EL period was significantly higher than that in the LL period. The abundance of APH (3″)-I in the EL period was significantly lower than that in the LL period ($p < 0.05$ Fig. S3). In addition, NMDS analysis was used to investigate the similarities between different periods based on the abundance of ARG subtypes. The ARG groups in the samples were clustered by different types of chicken houses, and there were significant differences ($p < 0.05$ Fig. 5).

3.5. Network co-occurrence analysis of ARGs and bacterial genera

Network analysis based on Spearman's rank correlations was applied to investigate the relationships between ARG subtypes and taxa. Firstly, $r > 0.8$ and $p < 0.01$ was selected, but there were too many related bacteria genera, which made it difficult to present and visualize. To narrow the research scope, $r > 0.9$ and $p < 0.001$ were selected for analysis. The co-occurrence network contained a total of 89 nodes (22 genera and 67 ARG subtypes) and 636 edges (Fig. 6). The co-occurrence relationships between ARGs and genera identified 22 bacterial genera as possible hosts of the 67 ARGs of eight types (Table S5). *Arcobacter*,

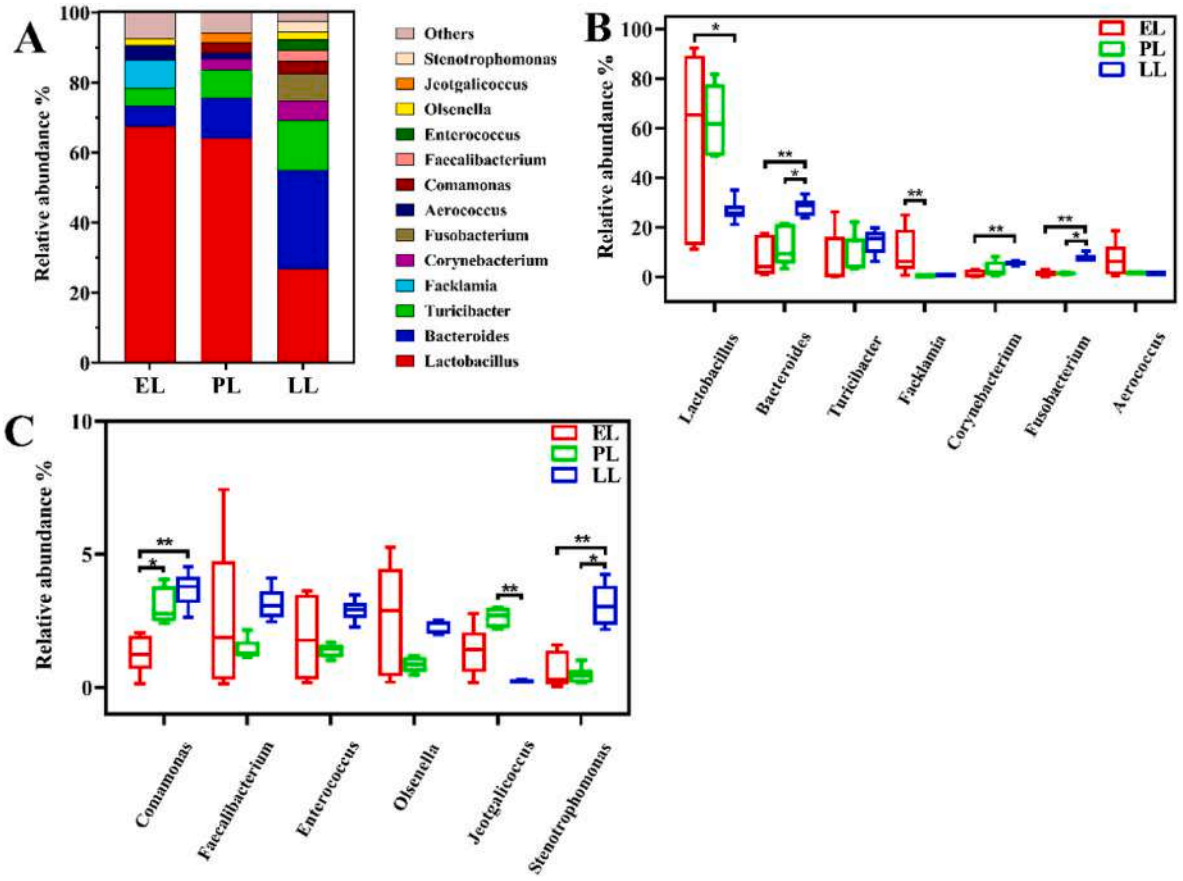

Fig. 3. Compositions of microbial communities at the genus level among the three laying periods. (A) Composition and relative abundance of dominant genera (average abundance >1.0%); (B and C) Comparison of the relative abundance of dominant genera. Wilcoxon rank sum test: *($p < 0.05$) and **($p < 0.01$). Abbreviations: EL, early laying period; PL, peak laying period; LL, late laying period.

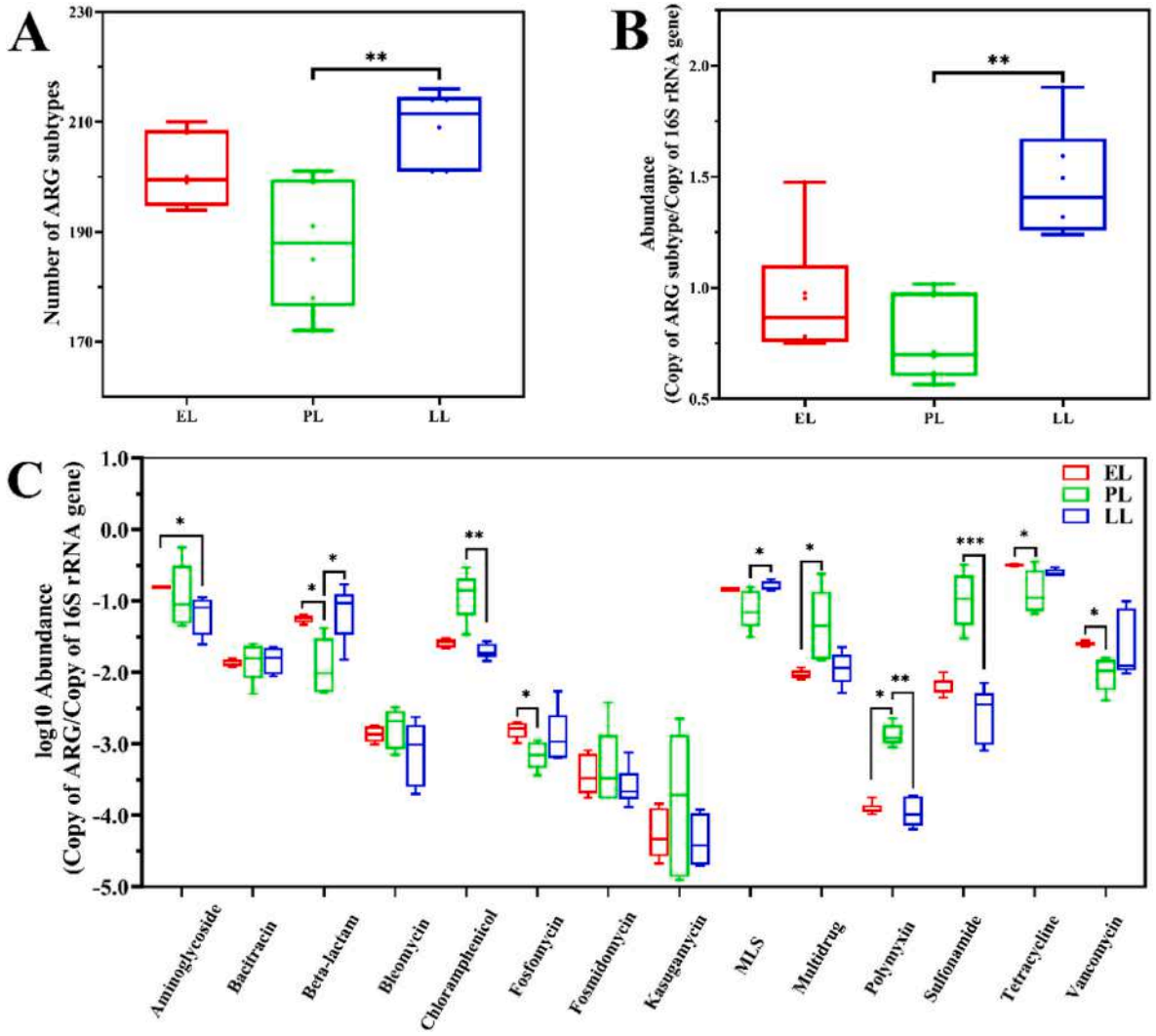

Fig. 4. The number and relative abundances of ARGs detected in different air samples of the three laying periods. (A) Comparison of the total number of ARG subtypes. (B) Comparison of total ARG subtype relative abundance. (C) Comparison of total ARG relative abundance for different antibiotics. Wilcoxon rank sum test: *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$). Abbreviations: EL, early laying period; PL, peak laying period; LL, late laying period. MLS: macrolide-lincosamide-streptogramin.

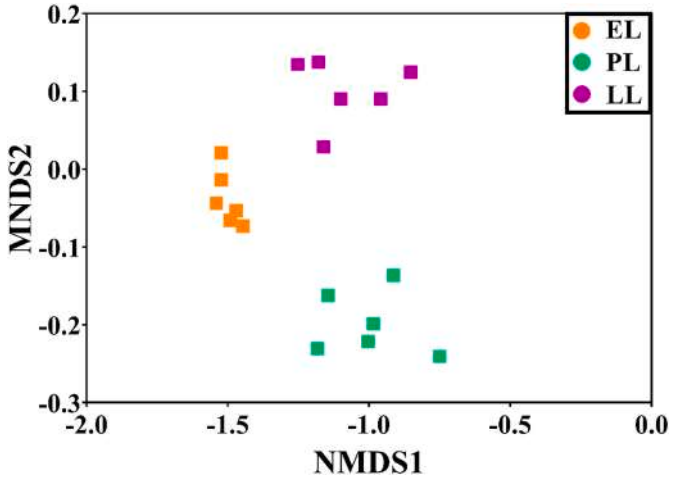

Fig. 5. Nonmetric multidimensional scaling (NMDS) plot of air samples using Bray–Curtis distance matrices based on ARG subtype abundances showing differences in ARG composition. Abbreviations: EL, early laying period; PL, peak laying period; LL, late laying period.

Giesbergeria, and *Helcococcus* were potential hosts for most ARGs, including tetracycline (tet32、tet39、tetH、tetS、tetX、tetX2、tetX3 and tetZ), MLS (erm(TR)、ermX、lnuB、lsa and macB) and aminoglycoside (aac(6′)-I、aac(6′)- II、adeJ、ant(2″)-I、ant(9)-I and aph(3′)-I) (Fig. 6). The genera *Arcobacter*, *Giesbergeria*, *Helcococcus*, *AcetoAnaerobium*, *Acidovorax*, *Anaerobium*, *Peptoniphilus*, *Chryseobacterium*, *Trueperella*, *Arcobacter*, *Neofamilia*, *Paludibacter*, *Simplicispira* and *HalAnaerobium* carried more than 10 ARG types, *Jeotgalibaca*, *Akkermansia*, *Lysinibacillus*, *Lagierella* and *Dysgonomonas* carried 3–9 ARG types, while the genera *Oscillibacter*, *Facklamia*, and *Kocuria* were found to be relevant to only one ARG type (Fig. 6, Table S5). There were obvious co-occurrence patterns within or across ARG types. The network consists of 67 nodes and 323 edges (Fig. S4, Table S6). Among them, beta-lactam (OXA-21), tetracycline (tetX, tetX3, tetH), chloramphenicol (cmlA), vancomycin (vatB), MLS (erm(TR), ereA, macB, lsa), aminoglycoside (aac(6′)-II, aad(9), ant(2″)-I) and multidrug (mexB) resistance genes were associated with more than 10 ARG subtypes. For example, OXA-21 associates with aac(6′)-II, aac(6′)-I, adeJ ant(2″)-I, ant(9)-I, aph(3′)-I, catB, catQ, cmlA, ermX, lnuB, lsa, mexB, OXA-209, tet39, tetG, tetH, tetX3 and tetZ.

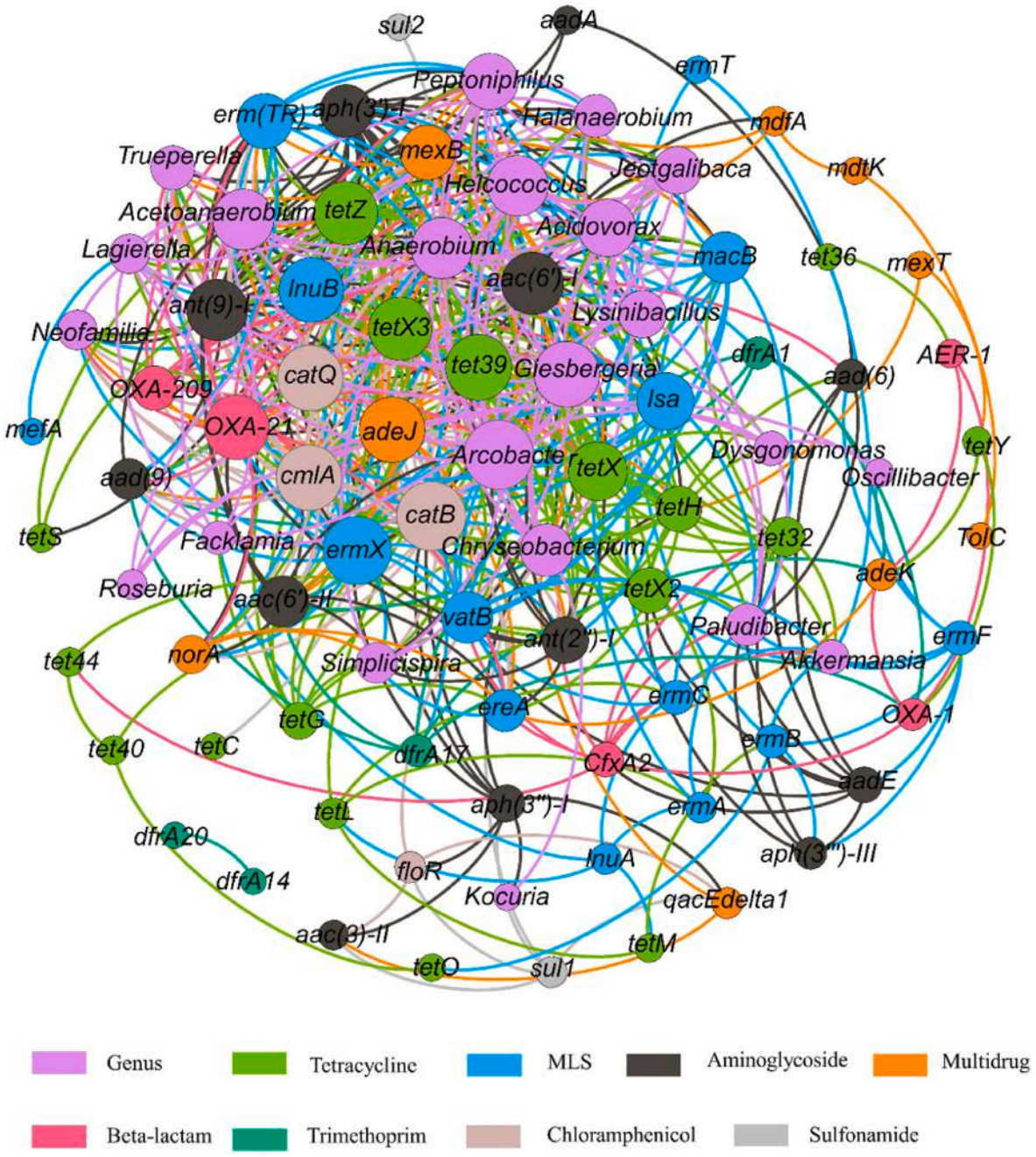

Fig. 6. Network analysis revealing the co-occurrence patterns between ARGs and bacterial genera. A connection represents a strong ($r > 0.9$) and significant ($P < 0.001$) correlation. The nodes were colored according to ARG types and genus. MLS: macrolide-lincosamide-streptogramin.

4. Discussion

In this study, bacterial diversity and resistome profiles were investigated in the air of 18 chicken houses at different laying periods and revealed that they are characterized by unique features. In addition, it was found that the resistome profiles in the air were closely related to the bacteria, and potential pathogenic bacteria and possible hosts of ARG were also identified. This study provides new insights into airborne bacterial microbiota and drug resistance in chicken houses during different laying periods.

4.1. Relationship between airborne bacteria and laying period

In this study, airborne bacteria from the same laying period showed similar characteristics, which might be attributed to the fact that these chicken farms belonged to the same company with the same management (Joat et al., 2021). The difference in bioaerosol samples in different laying periods might be influenced by the laying period. The bacterial diversity in the house air during the PL period was significantly higher than that in the EL period, which may be due to the changes in the physiological and endocrine structure of layer hens after the layer peak supporting the increase in egg production (Dai et al., 2022), leading to the increase in intestinal microbial diversity to improve nutrient utilization and lipid metabolism rate (Videnska et al., 2014). Bacillus subtilis strain DSM 29784 could modulate the cecal microbiome, concentration of short-chain fatty acids, and apparent retention of dietary components in shaver white chickens during grower, developer, and laying periods. The relative abundance of Firmicutes increased from EL to PL, similar to

previous studies, because Firmicutes could provide more nutrients to peak-layer hens through the production of SCFA from complex glycans (Gibiino et al., 2018). In the EL, the high abundance of Proteobacteria in the gut of layer hens was associated with the promotion of immune system development (Oakley et al., 2014). In the PL period, Bacteroidetes replaced Proteobacteria as the second dominant phylum. *Bacteroides* could promote the absorption of carbohydrates in layer hens and provide more energy for the intestinal development of layer hens (Dai et al., 2020; Polansky et al., 2016). This could be explained by the fact that in the PL period, the composition of the intestinal flora was stable, while in the LL period, the relative abundance of Firmicutes decreased and that of Bacteroidetes increased. These changes might reduce the pH of the intestinal chamber, inhibit the colonization of pathogenic bacteria and improve the utilization rate of nutrients to adapt to the new physiological requirements (Polansky et al., 2016). These results were similar to those found when the gut microbiota of layer hens changes during growth (Dai et al., 2022).

This pattern associated with the period of egg production has also been found in bacterial genera associated with potentially airborne pathogens. For example, the relative abundances of *Bacteroides*, *Corynebacterium* and *Fusobacterium* all increased significantly over time. *Bacteroides* could play a dual role according to their position in the host. Usually, *Bacteroides* are beneficial in the intestinal tract, but when they appear in other parts, they have the chance to be pathogenic (Zafar and Saier, 2021). Some *Bacteroides* can induce infectious pathogens in the formation of abscesses (Wexler, 2007). Some species of *Corynebacterium* are opportunistic commensals in the environment and can cause upper respiratory diseases such as laryngitis, nasopharyngitis or tonsillitis (Hoefer et al., 2021a). *Fusobacterium* is an opportunistic pathogen that colonizes the oral mucosa and can cause bacteremia and various rapidly progressive infections (Brennan and Garrett, 2019).

Previous studies have examined changes in the relative abundance of genera associated with potential airborne pathogens in layer hens and broilers (Hong et al., 2012). However, no study has addressed the association of potential airborne pathogens in layer hens with different periods of egg production. This might explain the different respiratory symptoms affecting layer hen and broiler workers (Just et al., 2013). In this study, *Lactobacillus*, *Facklamia*, *Comamonas*, *Jeotgalicoccus*, and *Stenotrophomonas* were the dominant bacterial genera in the bioaerosols of chicken houses and were usually considered relatively harmless (Yang et al., 2018). This study provided valuable information for assessing inhalation exposure and possible human health effects of bioaerosols in layer hens at different periods of egg production.

4.2. Relationship between the air resistance group and laying period

Previous studies on ARGs in chicken houses have shown that chicken manure is one of the major sources of aerosol resistance in chicken houses (Mbareche et al., 2019; Yang et al., 2021). The resistance gene types in the chicken house bioaerosol microbiota in this study were resistant to almost all major antibiotic classes commonly used for clinical and agricultural purposes. According to the previous study, aminoglycosides, tetracycline and MLS resistance genes were the most abundant types of ARGs in all laying periods, and they were also the main ARGs in poultry feces (Wen et al., 2019). The abundance of resistance genes was associated with antibiotic use during feeding (Zhang et al., 2017). Our study showed that the abundances of the ARGs chloramphenicol, multidrug, polymyxin and sulfonamide in the PL period were higher than those in the EL or LL period. This suggests that higher microbial diversity may facilitate horizontal gene transfer in air samples during the PL period. A high abundance of aminoglycoside, fosfomycin, tetracycline and vancomycin genes was observed in the EL period bioaerosols in this study. This might be related to the use of many antibiotics in the growth period of layer hens. Previous studies have reported high levels of aminoglycoside, fosfomycin, tetracycline and vancomycin, which are used to promote growth, in the feces of layer hens in the EL periods (Rivera-Gomis et al., 2021; Zhu et al., 2021). In this study, more than 172 subtypes of ARGs were found, and the abundance of ARGs varied greatly in different laying periods. The order from high to low was LL, EL, and PL. This is consistent with previous studies on ARGs in chicken manure samples at different growth periods and further validates that ARGs in the air may be derived from feces (Zhu et al., 2021). Generally, chicken farms will use many antibiotics before opening and reduce antibiotics during the laying period, resulting in a higher relative abundance in the EL period than in the PL period. In addition, the need to control diseases associated with intensive farming in the LL periods of egg production is also aimed at improving productivity, and large amounts of antibiotics continue to be used (Page and Gautier, 2012; Rivera-Gomis et al., 2021). In addition, the different distribution of ARG subtypes in the air indicated that the laying periods may be the key factor affecting the resistome of bioaerosol in the layer house.

4.3. Relationship between microbiome and resistome profiles

In total, 67 ARG subtypes belonging to 7 ARG types were detected, and 22 bacterial genera were estimated to be potential hosts by network analysis. Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria were the dominant phyla carrying ARGs. Interestingly, they were also the main dominant phyla of airborne bacteria. This suggested that the identified bacterial hosts were under constant antibiotic selection pressure, as the relative abundance of these phyla was found to be different at different laying periods. *Arcobacter*, *Giesbergeria*, *Helcococcus*, *AcetoAnaerobium*, *Acidovorax*, *Anaerobium*, *Chryseobacterium*, *Peptoniphilus*, *Trueperella* and *Neofamilia* carry multiple ARGs, including resistance genes of aminoglycoside, beta-lactam, chloramphenicol, MLS, multidrug and tetracycline. Several possible ARG hosts have been identified in previous studies. For example, *Arcobacter*, known for its pathogenicity, is reported to be an emerging zoonotic foodborne pathogen (Patyal et al., 2011). *Arcobacter* carries several ARG subtypes, such as aminoglycoside, chloramphenicol, MLS, and tetracycline, which is consistent with previous studies (Ferreira et al., 2013; Jia et al., 2017; Lu et al., 2015). These results help to understand the possible host relationship between bacteria and ARGs and provide precise dosing strategies for the treatment of resistant pathogens. However, further study is needed to obtain a more complete picture of antibiotic resistance in complex ecosystems (Gupta et al., 2021; Yu et al., 2020). Significant co-occurrence associations were observed between the internal types of ARGs, such as aminoglycosides, and the external types, such as MLS and chloramphenicol, which was consistent with previously reported results (Gupta et al., 2021; Yang et al., 2021). Overall, the bacterial bioaerosols of chicken houses were found to be an important factor regulating their resistance profiles.

5. Conclusions

In conclusion, this study analyzed the characteristics of airborne bacteria in commercial layer hen houses during different laying periods, providing new insights into the dynamics of bacteria and the resistome, and identified potential ARG host bacteria. The airborne microbiome and resistome varied greatly between different laying periods. Our results indicated that bioaerosols in layer houses might serve as potential reservoirs for bacterial pathogens and ARG hosts. There is a need for further study on the interactions between airborne bacteria and the resistome in different laying periods. Given the evidence of possible resistance to the large number of antibiotics used in poultry production, we recommended reducing the use of antibiotics. In addition, routine surveillance of some potential ARG host bacteria was also recommended to monitor their pathogenicity and ability to transmit antibiotic resistance to the environment.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

All experiments were approved by the relevant regulatory agency of the Animal Ethical Committee of Changchun Veterinary Research Institute (document number: SCXK201906097).

Author contributions

ZG, ZW and JL designed the project. HC, CZ, KZ, JP and YK performed the experiments. Data were analyzed by ZC, YZ, YC, CL, SD and LZ. Manuscript drafted by CZ and HC. ZG and JL revised the manuscript. All authors contributed to the study and approved the final version.

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Declaration of competing interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114173.

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Exposure of Swedish adolescents to elements, persistent organic pollutants (POPs), and rapidly excreted substances – The Riksmaten adolescents 2016-17 national survey

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ABSTRACT

Adolescence is a period of significant physiological changes, and likely a sensitive window to chemical exposure. Few nation-wide population-based studies of chemical body burdens in adolescents have been published. In the national dietary survey Riksmaten Adolescents (RMA) 2016–17, over 13 chemical substance groups, including elements, chlorinated/brominated/fluorinated persistent organic pollutants (POPs) were analysed in blood, and in urine metabolites of phthalates/phthalate alternatives, phosphorous flame retardants, polycyclic aromatic hydrocarbons (PAHs), and pesticides, along with bisphenols and biocide/preservative/antioxidant/UV filter substances (N = 1082, ages 11–21). The aim was to characterize the body burdens in a representative population of adolescents in Sweden, and to compare results with human biomonitoring guidance values (HBM-GVs). Cluster analyses and Spearman's rank order correlations suggested that concentrations of substances with known common exposure sources and similar toxicokinetics formed obvious clusters and showed moderate to very strong correlations ($r \geq 0.4$). No clusters were formed between substances from different matrices. Geometric mean (GM) concentrations of the substances were generally less than 3-fold different from those observed among adolescents in NHANES (USA 2015–16) and GerES V (Germany 2014–17). Notable exceptions were brominated diphenyl ethers (PBDEs) with >20-fold lower GM concentrations, and the biocide triclosan and ultraviolet (UV) filter benzophenone-3 with >15-fold lower mean concentrations in RMA compared to NHANES. Exceedance of the most conservative HBM-GVs were observed for aluminium (Al, 26% of subjects), perfluorooctanesulfonic acid (PFOS, 19%), perfluorooctanoic acid (PFOA, 12%), lead (Pb, 12%), MBP (dibutyl phthalate metabolite, 4.8%), hexachlorobenzene (HCB, 3.1%) and 3-phenoxybenzoic acid (PBA, pyrethroid metabolite, 2.2%). Males showed a higher proportion of exceedances than females for Pb, HCB and PFOS; otherwise no gender-related differences in exceedances were observed. A higher proportion of males than females had a Hazard Index (HI) of substances with liver and kidney toxicity and neurotoxicity >1. Industrialized countries with similarly high standards of living, with some exceptions, show comparable average body burdens of a variety of toxic chemicals among adolescents from the general population. The exceedances of HBM-GVs and HIs strongly suggests that further efforts to limit chemical exposure are warranted.

1. Introduction

Chemicals are used in almost every aspect of society and are found ubiquitously in human exposure media, including food, drinking water, air, dust, and consumer products. There are currently over 26,000 unique substances registered with the European Chemicals Agency, of which approximately 1000 are considered potential endocrine

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Abbreviations

Al	aluminium
BBOEP	bis(2-butoxyethyl) phosphate
BBzP	butylbenzyl phthalate
BHA	3-tert-butyl-4-hydroxyanisole
BMDL1%	benchmark dose of 1% risk
BPS	bisphenol S
BPA	bisphenol A
BP3	benzophenone-3
Cd	cadmium
Co	cobalt
Cr	chromium
br-PFOS	branched perfluorooctanesulfonic acid
CNS	central nervous system
cx-MiDP	mono-carboxy-isononyl phthalate
cx-MiNP	mono-(4-methyl-7-carboxyheptyl) phthalate
cx-MINCH	cyclohexane-1,2-dicarboxylate-mono (7-carboxylate-4-methyl)heptylester
DBP	dibutyl phosphate
p,p'-DDE	1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene
p,p'- DDT	dichlorodiphenyltrichloroethane
DEP	diethyl phthalate
DEHP	diethylhexyl phthalate
DiDP	diiso-decyl phthalate
DiNCH	diisononyl-cyclohexane-1,2-dicarboxylate
DiNP	diiso-nonyl phthalate
DPHP	dipropylheptyl phthalate
EFSA	The European Food Safety Authority
GC-MS/MS	gas chromatography – triple quadrupole mass spectrometry
GM	geometric mean
HBM-GVs	human biomonitoring guidance values
HCB	hexachlorobenzene
HCH	hexachlorocyclohexane
Hg	mercury
HI	Hazard Index
HR	Hazard Ratio
ICP-MS	inductively coupled plasma mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
L-PFOS	linear perfluorooctanesulfonic acid
L-PFHxS	linear perfluorohexanesulfonic acid
L-PFOA	linear perfluorooctanoic acid
MEHP	mono(2-ethylhexyl) phthalate
MEP	monoethyl phthalate
Mn	manganese
MBP	monobutyl phthalate
Ni	nickel
OCPs	organochlorine pesticides/metabolites
OH-MiNP	mono-(4-methyl-7-hydroxyoctyl) phthalate
OH-MINCH	cyclohexane-1,2-dicarboxylate-mono-(7-hydroxy-4-methyl)octyl ester
OH-MPHP	6-hydroxy monopropylheptyl phthalate
oxo-MiNP	mono-(4-methyl-7-oxooctyl) phthalate
PAHs	polycyclic aromatic hydrocarbons
PBDEs	polybrominated diphenyl ethers
PCBs	polychlorinated biphenyls
Pb	lead
PFDA	perfluorodecanoic acid
PFNA	perfluorononanoic acid
PFUnDA	perfluoroundecanoic acid
PFAS	per- and polyfluoroalkyl substances
PFOA	perfluorooctanoic acid
PFOS	perfluorooctanesulfonic acid
POPs	persistent organic pollutants
RMA	Riksmaten Adolescents
Se	selenium
SFA	Swedish Food Agency
TPP	triphenyl phosphate
TBEP	tri(2-butoxyethyl) phosphate
TCS	triclosan
TCP	trichloropyridinol
UBA	German Environment Agency
UPLC	ultra performance liquid chromatograph
US	United States
UV	ultraviolet
1-HP	1-hydroxy-pyrene
2-OH-PH	2-hydroxy-phenanthrene
3-PBA	3-phenoxybenzoic acid
4,4-BPF	bisphenol F
5-cx-MEPP	mono-(2-ethyl-5-carboxypentyl) phthalate
5-OH-MEHP	mono-(2-ethyl-5-hydroxyhexyl) phthalate
5-oxo-MEHP	mono-(2-ethyl-5-oxohexyl) phthalate

disrupting compounds (ECHA, 2022; Street et al., 2018). Furthermore, approximately 1000 new chemicals are registered with the United States Environmental Protection Agency per year (EPA, 2022), often with unknown or poorly understood safety profiles. Many chemicals are known to interfere with the human hormonal system and have previously been associated with a wide range of negative health consequences including neurologic, reproductive and other physiological effects (Braun, 2017; Wan et al., 2021; Yilmaz et al., 2020). Thus, considering both the number and effects of chemicals to which humans are exposed, understanding the variation of chemical mixture exposures within human populations (i.e., the chemical 'exposome') is of great importance.

Most studies examining human exposure to chemicals focus on single compound groups, such as elements, per- and polyfluoroalkyl substances (PFAS) and polychlorinated biphenyls (PCBs) (Marques et al., 2021; Preston et al., 2020; Roth et al., 2021), with few looking at the wider part of the chemical exposome. In reality, human exposure to chemicals is a complex process which involves multiple substances and pathways. Combination toxicity may occur when multiple chemicals occurring at sufficiently high concentrations act on a common endpoint to elicit an effect (Kortenkamp, 2014). In general, such mixtures may elicit a combination effect by either 'concentration addition' or 'independent action', although synergistic or antagonistic effects are also possible, albeit less frequently observed in experimental studies (Martin et al., 2021). 'Concentration addition' occurs when a toxicity threshold is exceeded by the sum concentration of multiple chemicals with the same mechanism/mode of action. In comparison, 'independent action' may occur when chemicals with different modes-of-action reach their individual toxicity thresholds on the same endpoint, thus eliciting a toxic effect (Kortenkamp, 2014). Concentration addition presents a unique challenge to risk assessors because individual toxic compounds may not be found in sufficiently high concentrations in study populations to pass observable health-risk thresholds, but may exceed such thresholds in combination with other chemicals sharing the same mechanism/mode of action. Further layers of complexity arise whereby chemical substances with independent modes of action can still share the same adverse health effects (Christiansen et al., 2020).

Biomonitoring of chemical body burdens in blood and urine provides valuable information about cumulative exposure at the time of sampling. Adolescence is likely a sensitive developmental window to

chemical exposure due to the significant physiological changes that occur. Nevertheless, few nation-wide studies have been published on adolescent exposure to a broad range of chemicals. The few well-known examples include NHANES in the United States (US) and GerES V in Germany (GerES, 2021; NHANES, 2022). Additionally, FLEHS IV in Belgium (Schoeters et al., 2022) represents another similar (albeit regional) study on the chemical exposome of adolescents. These studies, to the best of our knowledge, have not fully investigated the correlations between exposure of adolescents to the measured substances/substance groups. Knowledge of such correlations, which may be due to common exposure sources and/or similarities in toxicokinetics, are crucial when interpreting associations between health outcomes and body burdens of chemical mixtures. The previous studies have compared the observed concentrations of measured chemicals with human biomonitoring guidance values (HBM-GVs), in order to identify the chemicals with concern for adolescent health. However, they have not investigated possible gender and age differences in this context, which may be important for health development during this period of rapid physiological change. Moreover, to the best of our knowledge, no such study has attempted to investigate possible combination toxicity risks with substances with common target organs.

The overall aim of the present study is to characterize the body burdens of over 60 different substances across 13 chemical substance groups (elements (e.g., heavy metals), chlorinated, brominated, and fluorinated persistent organic pollutants (POPs), phthalates, phthalate alternatives, bisphenols, phosphorous flame retardants, polyaromatic hydrocarbons (PAH), pesticides, biocides/preservatives and UV-filters) in Swedish adolescents. To address this goal, we compared measured concentrations in whole blood, serum and urine to those reported in similar studies from the United States, Germany and the Flanders region of Belgium. We also examined correlations among individual substances, in an attempt to identify shared sources of exposure. Finally, we compared measured concentrations to published HBM-GVs and determined Hazard Indices in an effort to assess cumulative health risk (Kortenkamp and Faust, 2010).

2. Method

2.1. Study group and design

The study population was a sub-sample of Riksmaten Adolescents 2016–17 (RMA), a cross-sectional national population-based study of dietary habits and chemical exposure of Swedish adolescents, conducted by the Swedish Food Agency (SFA). Details about the recruitment process, study design and data collection are described elsewhere (Moraeus et al., 2018). Briefly, 619 schools were invited, being representative of the entire country across the 5th (median age, range: 11 years, 10–13), 8th (14 years, 11–15) and 11th (17 years, 16–21) school grades. Of these schools, 259 were invited to participate in the biomonitoring part, with 62 school classes across 57 unique schools opting-in (Moraeus et al., 2018) (Fig. S1, supplement). In total, 1305 students, out of 2377 invited, accepted to be sampled and 1082 had sufficient biomonitoring data to be included in our analysis (Table 1 & Table S1, Supplement). The study design included online-based questionnaires (RiksmatenFlexQ) addressing background factors such as age and gender (Moraeus et al., 2018).

2.2. Ethics

Ethical approval was obtained from the Regional Ethical Review Board in Uppsala (No 2015/190). Participants in grades 8 and 11 gave written informed consent to participate in the study, whilst legal guardians gave consent for 5th graders.

Table 1
Gender differences in substance concentrations among RMA participants.

Substances[a]		Geometric mean (95% CI)			p-value[b]
Abbreviation	Unit/ Matrix	Male (N = 476)	Female (N = 606)	Total (N = 1082)	
Cr	µg/L whole blood	0.58 (0.57–0.60)	0.55 (0.54–0.57)	0.57 (0.56–0.58)	0.092
Mn	µg/L whole blood	10.1 (9.67–10.4)	11.0 (10.7–11.2)	10.6 (10.3–10.8)	<0.001
Co	µg/L whole blood	0.11 (0.10–0.11)	0.14 (0.14–0.15)	0.13 (0.12–0.13)	<0.001
Ni	µg/L whole blood	0.62 (0.61–0.64)	0.62 (0.61–0.64)	0.62 (0.61–0.63)	0.466
Se	µg/L whole blood	94.7 (91.8–97.7)	95.0 (93.9–96.1)	94.8 (93.4–96.3)	0.141
Cd	µg/L whole blood	0.11 (0.11–0.12)	0.13 (0.13–0.14)	0.12 (0.12–0.13)	<0.001
Hg	µg/L whole blood	0.75 (0.69–0.81)	0.60 (0.56–0.64)	0.66 (0.63–0.69)	<0.001
Pb	µg/L whole blood	8.17 (7.77–8.58)	6.72 (6.49–6.97)	7.32 (7.11–7.55)	<0.001
HCB	pg/mL serum	47.3 (45.8–48.9)	38.5 (37.3–39.8)	42.2 (41.2–43.2)	<0.001
p,p'-DDE	pg/mL serum	124 (115–133)	99.7 (93.2–107)	110 (104–115)	<0.001
PCB-118	pg/mL serum	7.01 (6.67–7.36)	6.41 (6.14–6.70)	6.67 (6.45–6.89)	0.006
PCB-138	pg/mL serum	30.3 (28.6–32.2)	23.3 (22.1–24.6)	26.2 (25.2–27.2)	<0.001
PCB-153	pg/mL serum	49.0 (46.1–52.1)	36.9 (34.9–39.0)	41.8 (40.0–43.6)	<0.001
PCB-170	pg/mL serum	13.7 (12.8–14.7)	9.86 (9.24–10.5)	11.4 (10.9–12.0)	<0.001
PCB-180	pg/mL serum	27.7 (25.7–29.8)	19.7 (18.5–21.1)	22.9 (21.8–24.1)	<0.001
PCB-187	pg/mL serum	6.14 (5.71–6.61)	4.49 (4.20–4.80)	5.15 (4.90–5.41)	<0.001
L-PFOA	ng/g serum	1.26 (1.21–1.31)	1.17 (1.13–1.22)	1.21 (1.18–1.25)	0.001
PFNA	ng/g serum	0.39 (0.37–0.41)	0.34 (0.32–0.35)	0.36 (0.35–0.37)	<0.001
PFDA	ng/g serum	0.15 (0.14–0.16)	0.15 (0.14–0.16)	0.15 (0.14–0.16)	0.219
L-PFHxS	ng/g serum	0.55 (0.50–0.61)	0.39 (0.36–0.42)	0.45 (0.43–0.48)	<0.001
L-PFOS	ng/g serum	2.43 (2.29–2.59)	1.92 (1.82–2.03)	2.13 (2.05–2.22)	<0.001
br-PFOS	ng/g serum	1.12 (1.05–1.19)	0.85 (0.80–0.90)	0.96 (0.92–1.00)	<0.001
MEP	ng/mL urine	31.5 (28.8–34.6)	51.0 (46.4–56.2)	41.3 (38.6–44.2)	<0.001
MBP	ng/mL urine	38.9 (36.5–41.3)	43.7 (41.6–45.9)	41.5 (39.9–43.1)	0.001
MBzP	ng/mL urine	6.33 (5.73–6.99)	7.61 (6.98–8.30)	7.02 (6.57–7.49)	0.006
MEHP	ng/mL urine	1.61 (1.52–1.71)	1.82 (1.72–1.93)	1.73 (1.66–1.80)	0.005
5OH-MEHP	ng/mL urine	7.81 (7.35–8.29)	8.19 (7.69–8.73)	8.02 (7.67–8.38)	0.230
5oxo-MEHP	ng/mL urine	5.98 (5.62–6.37)	6.69 (6.31–7.10)	6.37 (6.10–6.65)	0.033
5cx-MEPP	ng/mL urine	6.80 (6.39–7.24)	7.69 (7.25–8.16)	7.29 (6.98–7.61)	0.010
2cx-MEHP	ng/mL urine	2.05 (1.92–2.18)	2.18 (2.06–2.32)	2.12 (2.03–2.22)	0.120
OH-MiNP	ng/mL urine	4.23 (3.89–4.61)	4.93 (4.52–5.38)	4.61 (4.33–4.91)	0.042
oxo-MiNP	ng/mL urine	1.94 (1.79–2.09)	2.34 (2.16–2.54)	2.15 (2.04–2.28)	0.001

(continued on next page)

Table 1 (*continued*)

Substances[a]		Geometric mean (95% CI)			p-value[b]
Abbreviation	Unit/ Matrix	Male (N = 476)	Female (N = 606)	Total (N = 1082)	
cx-MiNP	ng/mL urine	6.34 (5.84–6.88)	7.89 (7.27–8.57)	7.17 (6.76–7.60)	0.001
cx-MiDP	ng/mL urine	0.36 (0.34–0.39)	0.44 (0.42–0.47)	0.41 (0.39–0.43)	<0.001
OH-MPHP	ng/mL urine	1.07 (1.00–1.15)	1.16 (1.08–1.24)	1.12 (1.07–1.18)	0.189
oxo-MiNCH	ng/mL urine	1.20 (1.07–1.33)	1.36 (1.24–1.50)	1.29 (1.20–1.38)	0.032
cx-MINCH	ng/mL urine	0.88 (0.78–0.98)	1.01 (0.92–1.12)	0.95 (0.88–1.03)	0.034
OH-MINCH	ng/mL urine	0.92 (0.82–1.03)	0.99 (0.89–1.10)	0.96 (0.89–1.03)	0.321
DPP	ng/mL urine	1.82 (1.71–1.93)	2.13 (2.01–2.25)	1.99 (1.91–2.07)	0.001
DBP	ng/mL urine	0.13 (0.12–0.14)	0.17 (0.15–0.18)	0.15 (0.14–0.16)	<0.001
BPA	ng/mL urine	0.87 (0.80–0.95)	0.88 (0.81–0.95)	0.88 (0.83–0.93)	0.536
BPS	ng/mL urine	0.12 (0.11–0.13)	0.16 (0.15–0.18)	0.14 (0.14–0.15)	<0.001
4,4BPF	ng/mL urine	0.09 (0.08–0.11)	0.11 (0.10–0.13)	0.10 (0.09–0.11)	0.026
2-OH-PH	ng/mL urine	0.16 (0.15–0.17)	0.17 (0.16–0.19)	0.17 (0.16–0.18)	0.160
TCP	ng/mL urine	1.24 (1.16–1.31)	1.35 (1.28–1.43)	1.30 (1.25–1.35)	0.021
3-PBA	ng/mL urine	0.27 (0.25–0.29)	0.29 (0.28–0.31)	0.28 (0.27–0.30)	0.029
TCS	ng/mL urine	0.25 (0.23–0.29)	0.33 (0.30–0.37)	0.30 (0.27–0.32)	0.002
BHA	ng/mL urine	0.27 (0.21–0.36)	1.33 (1.05–1.69)	0.66 (0.55–0.80)	<0.001
BP3	ng/mL urine	0.64 (0.56–0.73)	1.56 (1.35–1.81)	1.05 (0.95–1.17)	<0.001

[a] Substances with over 50% of samples < LOQ or LOD are not included in this table. For the purpose of calculating geometric means and 95% confidence intervals (CI), determined concentrations < LOQ or < LOD were used and concentrations determined to be 0 were converted to the lowest concentration above 0 divided by sqrt(2) (see section Handling of data below LOQ/LOD). When LOD was available PFAS concentrations < LOD were converted to LOD/sqrt(2), else concentrations < LOQ were converted to LOQ/sqrt(2).

[b] Wilcoxon rank sum test on comparison of concentrations by gender. p-values <0.05 indicate significant difference of concentration between genders.

2.3. Sample collection

One single-spot non-fasting blood and urine sample, along with anthropometric data, were collected from each participant by trained staff during school visits on weekdays throughout 2016 (September)-2017 (May). Blood samples (10 ml) for serum were collected in Vacutainer serum tubes with coagulation activator (Becton Dickinson, article # 367896, Sweden), centrifuged at 1500×*g* for 10 min and then aliquoted to cryotubes. Whole blood samples (4 ml) were collected in lithium-heparin Vacuette tubes (Greiner Bio-one, article# 454056, Germany). Urine samples were collected in acetone-rinsed paper cups by the individual participants during the school visit, and these samples were then aliquoted to polypropylene tubes. All aforementioned samples were promptly frozen at −20 °C at the site of collection and shipped frozen to the SFA where they were stored at −80 °C until being sent to external laboratories for analysis. None of the samples analysed in this study had known disruptions in the cold chain.

2.4. Sample analysis

Full names and abbreviations of the analysed substances, CAS numbers, and their main uses are provided in Table S2 (Supplement). Decisions on inclusion of substances were made by experts from the SFA at the time of study design and were based on toxic properties of the substances and presence in food and/or drinking water. Some essential/suspected essential elements were also included based on suspected interactions with non-essential toxic elements in the body (chromium (Cr), manganese (Mn), selenium (Se), cobalt (Co)) (Table S2, supplement).

2.5. Elements

Samples were analysed for Cr, Mn, Co, nickel (Ni), Se, cadmium (Cd), mercury (Hg), lead (Pb), and aluminium (Al) by the Department of Laboratory Medicine, Lund University, Lund, Sweden (Table S2, Supplement). Concentrations of Pb, Cd and Hg in RMA have been published previously (Almerud et al., 2021). Sample analyses were performed in duplicate. In short, the samples were treated as previously described (Barany et al., 1997). The concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS; iCAP Q, Thermo Fisher Scientific, Bremen, GmbH) equipped with collision cell with kinetic energy discrimination and helium as collision gas. The limit of detection (LOD) varied from 0.05 to 5.0 μg/L. Method precision varied from 2.8 to 15% depending on analyte.

2.6. PCBs, organochlorine pesticides/metabolites (OCPs) and polybrominated diphenyl ethers (PBDEs)

The Department of Health Security, National Institute for Health and Welfare, Kuopio, Finland analysed the chlorinated and brominated POPs in serum (Table S2, Supplement) as described previously (Gasull et al., 2019). Some results from the analyses have been published previously (Zamaratskaia et al., 2022). In brief, concentrations were measured using gas chromatography – triple quadrupole mass spectrometry (GC-MS/MS). The instrument used was an Agilent 7010 GC-MS/MS system (Wilmington, DE, U.S.), GC column DB5MS UI (J&W Scientific, 20m, ID 0.18 mm, 0.18 μm). Limits of quantification (LOQ) ranged from 5 pg/mL for PCB congeners and trans-nonachlor to 40 pg/mL for 1, 1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE). Two blank samples and two control samples (NIST SRM 1958) were included in each batch of samples. Measured concentrations of chlorinated and brominated POPs in SRM1958 were 80–105% of the certified/reference concentrations. The coefficient of variation (CV%) from SRM 1958 (n = 18) was <3.6% for all compounds.

2.7. PFAS

Serum samples were analysed for PFASs by the Department of Environmental Science, Stockholm University, Sweden (Table S2, Supplement). Details of the extraction and instrumental analysis are described in Nyström et al. (2022). Briefly, samples were fortified with a suite of isotopically-labelled internal standards and then extracted twice with acetonitrile in an ultrasonic bath, followed by clean-up with acidified graphitized carbon. The cleaned-up extract was diluted and fortified with volumetric standards and thereafter stored at −20 °C prior to analysis. Instrumental analysis was carried out on a Acquity ultra performance liquid chromatograph coupled to a Waters Xevo TQS triple quadrupole mass spectrometer operated in negative electrospray ionisation, multiple reaction monitoring mode. Quantification was based on isotope dilution or an internal standard approach (see Nyström et al. (2022) for details). LOQs are summarized in Table S3 (Supplement). In the present study only data on selected perfluoroalkyl acids: linear perfluorooctanoic acid (L-PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), linear perfluorohexanesulfonic acid (L-PFHxS), branched and linear perfluorooctanesulfonic acid (br-PFOS, L-PFOS), were used due to the low detection frequency of other PFASs analysed in RMA (Nyström et al., 2022).

2.8. Substances measured in urine

Analyses were performed at the Department of Laboratory Medicine, Lund University, Sweden, as previously described (Alhamdow et al., 2017; Cequier et al., 2014; Liljedahl et al., 2021) with some modifications. In brief, the samples were analysed on a liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS; QTRAP 5500, AB Sciex, Framingham, MA, USA). Two urinary quality control samples were included in the analysis, one authentic and one spiked. The CV% calculated from QC samples included in all sample batches (N = 34) as a between-run precision, did not exceed 20% for almost all compounds with the exception of mono-carboxy-isononyl phthalate (cx-MiDP, 27% at concentration of 0.6 ng/mL), bisphenol S (BPS, 25% at concentration of 0.8 ng/mL), dibutyl phosphate (DBP, 41% at concentration of 0.1 ng/mL) and 3-tert-butyl-4-hydroxyanisole (BHA, 21% at concentration of 0.8 ng/mL). The analyses of bisphenol A (BPA), 1-hydroxy-pyrene (1-HP), benzophenone-3 (BP3), triclosan (TCS), 3-phenoxybezoic acid (3-PBA), trichloropyridinol (TCP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP), mono-(2-ethyl-5-oxohexyl) phthalate (5-oxo--MEHP), is part of G-EQUAS inter-laboratory control program (University of Erlangen-Nuremberg, Germany). The laboratory also participates in the HBM4EU QA/QC program, and has qualified as HBM4EU laboratory for the analysis of: BPA, BPS, BPF, 1-HP, monobenzyl phthalate (MBzP), mono-(2-ethylhexyl) phthalate (MEHP), 5-OH-MEHP, 5-oxo--MEHP, mono-(2-ethyl-5-carboxypentyl) phthalate (5-cx-MEPP), mono-(4-methyl-7-hydroxyoctyl) phthalate (OH-MiNP), mono-(4-methyl-7-oxooctyl) phthalate (oxo-MiNP), mono-(4-methyl-7-carboxyheptyl) phthalate (cx-MiNP), cyclohexane-1,2-dicarboxylate-mono (7-carboxylate-4-methyl)heptylester (cx-MINCH), cyclohexane-1,2-dicarboxy late-mono-(7-hydroxy-4-methyl)octyl ester (OH-MINCH). Urine concentrations were adjusted for individual differences in urine density in the RMA population as described by (Carnerup et al., 2006). LODs are summarized in Table S4 (Supplement).

2.9. Handling of data below LOQ/LOD

The number of samples with concentrations below LOQ/LOD for each substance are presented in Tables S3 and S4 (Supplement). Except for PFASs, concentrations below LOQ or LOD were handled as previously described (Darnerud et al., 2015; Gyllenhammar et al., 2017; Svensson et al., 2021). Briefly, concentrations above 'background noise' in the analyses were determined to the concentration estimated in the analytical run. When a concentration was estimated not to be above 'background noise' in the chemical analyses, the concentration was set to zero (AMC, 2001). In the case of PFAS, concentrations below LOQ but above LOD were used when available, while concentrations below LOD were set to zero. Concentrations determined to be below LOD or LOQ are more uncertain and should be regarded as semi-quantitative. Nevertheless, the use of these determined concentrations in statistical analyses, as opposed to using substituted or imputed data, is generally considered to result in less statistical bias (AMC, 2001; Bergstrand and Karlsson, 2009). Fig. S2 (Supplement) provides examples of Al, PCB-183 and bis(2-butoxyethyl) phosphate (BBOEP) showing the change in concentration distribution of the substances after replacement of concentrations < LOQ or LOD with determined concentrations instead of fixed concentrations at LOQ or LOD/$\sqrt{2}$. Nevertheless, it is important to be aware of that use of data with a large proportion of determined concentrations below LOQ or LOD will add uncertainty in the interpretation of the results. For GM calculations, concentrations determined to be zero were by necessity further converted to the lowest determined concentrations above zero divided by $\sqrt{2}$. In these cases, PFAS concentrations below LOQ or LOD were set to LOQ or LOD/$\sqrt{2}$.

2.10. Statistical analysis

Analysis was performed using *R* (ver: 4.0.2). In the cluster analysis, the 'varclus' function from the 'hmisc' package (ver: 4.4–0) was used to visualise the clustering of the chemical substances using spearman correlation coefficients with complete linkage. Correlation analyses of relationships between substance concentrations were assessed using Spearman Rank Correlation coefficients. By using results from the cluster analysis in combination with the correlation analysis it is possible to get a more comprehensive description of the relationships between individual substances. It is usually not probable that the cluster analysis identifies clusters including all substances that are similarly correlated to one another. In this case, the correlation analysis provides information about correlations between substances separated in different clusters. In both the cluster and the correlation analysis, substances with more than 70% of concentrations above zero were included in the analyses (Tables S3 and S4, supplement), with the full range of values used for calculations. The strength of the correlations was assessed using definitions proposed by (Schober et al., 2018), where a correlation coefficient of 0.00–0.39 is classified as negligible to weak, 0.40–0.69 is moderate, 0.70–0.89 is strong, and 0.90–1.00 is a very strong correlation. Wilcoxon rank sum and signed rank test ($\alpha = 0.05$) was used to analyse if there were gender-related differences in concentrations, and a Kruskal-Wallis rank sum test ($\alpha = 0.05$) to analyse if there are significant concentration differences in at least one of the school grades compared to the others. The Chi-Squared Test was used to test if there were gender- or school grade-related differences in proportions of exceedances of HBM-GVs and HI > 1 (see section 'Comparisons with HBM-GVs and HI assessment' below).

2.11. Study comparison

The literature was searched for nation-wide population-based studies that analysed the larger chemical exposome in both blood and urine amongst adolescents during the same period as RMA. We found two studies matching this criteria, United States NHANES 2015–17 (NHANES, 2022), German GerEs V 2014–17 (GerES, 2021), and additionally the regional Flemish Belgium FLEHS IV 2016–2020 (FLEHS, 2020).

Concentrations for NHANES were mostly reported as weighted GMs. In the case of chlorinated and brominated POPs, concentrations were instead reported as arithmetic means of pooled samples from separate ethnic groups and genders. We therefore included the reported minimum and maximum ethnical group/gender mean concentrations reported in NHANES 2015-17 within our comparison table (Table 2). Values for GerES V were reported as GMs but sometimes included younger children than those included in RMA in the reporting of certain substances. Values for FLEHS IV were reported as GMs for the adolescent study population, ages 14–15. Comparatively, we reported the RMA results as GMs including all ages (ages 10–21) analysed within our study population.

2.12. Comparisons with HBM-GVs and HI assessment

To assess the potential health concerns associated with chemical exposure in Swedish adolescents, HBM-GVs were obtained for the investigated substances from the peer-reviewed literature. We focussed on published values from the German Environment Agency (UBA) and the HBM4EU project (Apel et al., 2020; UBA, 2015) (Table 4). If HBM-GVs were published by both organizations, the HBM4EU value was used. In certain cases when HBM-GVs from these two sources were lacking we used published biomonitoring equivalents (Hays and Aylward, 2009), or published health-based values from other sources that could be used when comparing with our observed concentrations (Table 4). While the search for HBM-GVs was not exhaustive, data were obtained for many of the substances in the present work.

In order to examine possible combination toxicity of measured substances, the HI assessment approach was used (Borg et al., 2013). This approach assumes concentration addition between measured substances

Table 2
Comparison of geometric mean (GM) of substance concentrations in whole blood and serum observed in RMA with those reported for 12–19 years old adolescents from NHANES, USA, 3–17 years old children/adolescents from GerES V, Germany, and 14–15 years old adolescents from the Flemish regions of Belgium (FLEHS IV)[a].

Substances[b]	RMA (2016–2017) GM (N = 1082)	NHANES (2015–2016) Weighted GM[e] (N = 353–565)	GerES (2014–2017) GM (age) (N = 516–2256)		FLEHS IV (2016–2020) GM (N = 428)
Whole blood (μg/L)					
Mn	11	11			9.4
Se	95	190			
Cd	0.12	0.13	<0.12	(12–17)	0.19
Hg	0.66	0.40			
Pb	7.3	4.7	8.4	(12–17)	7.7
Serum (pg/ml)[c]					
HCB	42	24–37000 (NHBF – AHM)	<70	(14–17)	25
beta-HCH	3.1	<4–60 (NHBF - AHM)	<20	(3–17)	3.7
Oxychlordane	0.13	<4–7900 (NHBF - AHM)			4.0
trans-Nonachlor	2.9	9.3–12000 (NHBF – AHM)			2.5
p,p'-DDT	0.16	<4–16000 (NHBF - AHM)	<20	(14–15)	5.9
p,p'-DDE	110	160. – 690000 (NHBF - AHM)	134	(14–17)	135
PCB-74	3.5	<2–51 (NHWF - NHWM)			
PCB-99	3.9	0.2–3800 (MAF - AHM)			
PCB-118	6.7	4.1–5500 (MAF - AHM)	<20	(3–17)	
PCB-138	26	6.2–10000 (MAF - AHM)	45	(14–17)	
PCB-153	42	8.2–13000 (MAF - AHM)	62	(14–17)	
PCB-156	3.8	<0.8–6.2 (NHBF - NHWM)			
PCB-170	11	2.1–2900 (NHBF - AHM)			
PCB-180	23	4.1–7000 (MAF - AHM)	32	(14–17)	
PCB-183	2.1	<0.8–4.1 (NHBF - AM)			
PCB-187	5.2	2.1–2300 (MAF - AHM)			
∑PCB-138, 153, 180	92		140		71
BDE-47	0.78	27–83 (AF - NHBM)			<1
BDE-99	0.29	7.9–20 (AF - MAM)			<1
BDE-153	0.68	16–54 (AF - NHBM)			<1
Serum (ng/g serum)[d]					
L-PFOA	1.2	1.1	1.1	(12–17)	1
PFNA	0.36	0.47	<0.49	(3–17)	0.3
PFUnDA	0.10	<0.10	<0.24	(3–17)	
PFHxS	0.45	0.89	0.35	(12–17)	0.47
L-PFOS[f]	2.1	2	2.4	(12–17)	2.1
br-PFOS	0.96	0.85			

[a] (GerES, 2021; NHANES, 2022; Schoeters et al., 2022).
[b] For the purpose of calculating geometric means in RMA, determined concentrations < LOQ or < LOD were used and concentrations determined to be 0 were converted to the lowest concentration above 0 divided by sqrt(2) (see section Handling of data below LOQ/LOD). When LOD was available, PFAS concentrations < LOD were converted to LOD/sqrt(2), else concentrations < LOQ were converted to LOQ/sqrt(2).
[c] NHANES/GerES/FLEHS; ng/g lipid of chlorinated and brominated POPs converted to pg/ml serum using average lipid content of serum in RMA of 0.33%.
[d] NHANES/GerES/FLEHS; PFAS ng/mL converted to ng/g using the specific gravity of serum (1.0275) (Sunderman and Boerner, 1949).
[e] Chlorinated POPs; arithmetic mean in pooled samples (N = 2–11, each consisting of 8 individuals) from different ethnical groups: NHBF = non-hispanic black females. AHM = all hispanic males. MHWF = non-hispanic white female. NHWM = non-hispanic white male. MAF = mexican american females. AM = asian male. AF=Asian female. MAM = Mexican american male.
[f] GerES & FLEHS linear + branched isomers.

in the assessment of possible cumulative health concerns. A conservative approach was used by grouping substances with the same target organ of critical effects (Table 4), specifically in this present study kidney toxicity, liver toxicity and neurotoxicity. Each participant's Hazard Ratio (HR) of the substance in question was calculated by dividing the measured concentration of the substance and its most conservative HBM-GV. Each individual's HRs were summed together to a HI according to the shared target organ of critical effects (see formula below). A potential concern to human health exists if the HI is > 1 for each target organ. See Table 5 for selected substance HRs included within each HI.

$$\text{Hazard Index} = \frac{C1}{GV1} + \frac{C2}{GV2} + \frac{C3}{GV3} + \ldots\ldots\ldots\frac{Ci}{GVi}$$

C_i: concentration of substance i in serum/whole blood/urine. GV_i: the HBM-GV for substance i (see Table 4). Hazard Index = the sum of the HRs of each substance in an individual with the same target organ of critical effects (see Table 5)

3. Results and discussion

RMA showed ubiquitous exposure of Swedish adolescents to mixtures of many of the studied elements, chlorinated pesticides, PCBs, and PFASs (Table 1 & Table S3, Supplement). Moreover, >50% of the participants had quantifiable concentrations of urinary metabolites of phthalates/phthalate alternatives, as well as metabolites of phosphorous flame retardants, PAHs, and pesticides, and bisphenols, and biocide/preservative/UV filter chemicals (Table 1 and Table S4, Supplement). This is in line with results from similar studies on adolescents from Germany and the USA (GerES, 2021; NHANES, 2022), and the regional study in Belgium (Schoeters et al., 2022).

Many of the studied elements and POPs have half-lives in the human body ranging from months to years (Nordberg et al., 2014; Verner et al., 2009); consequently, concentrations are representative of medium-to long-term cumulative exposure prior to sampling. In contrast, urine substances have short elimination half-lives (i.e., hours to a few days) and are therefore representative of very recent exposure (Perrier et al., 2016). Nevertheless, the almost ubiquitous quantification of these 'short-lived' substances in adolescent urine suggests that exposure

Table 3
Comparison of geometric means (GM) of substance concentrations in urine observed in RMA, with those reported for 12–19 years old adolescents from NHANES and 14–17 years old adolescents from GerES.

Substances	RMA (2016–2017) GM (N = 1082)	NHANES (2015–2016)[a,b] Weighted GM (N = 405)	GerES (2014–2017)[c] GM (N = 516–2256)	FLEHS IV (2016–2020)[d] GM (N = 416)
Urine (ng/mL)				
MEP	41	35	26	38
MBP	42	12	21	20
MBzP	7	6.1	3.1	3
MEHP	1.7	1.2	1.4	1.1[e]
5OH-MEHP	8	5.8	11	6.7
5oxo-MEHP	6.4	3.8	7.6	4.2
5cx-MEPP	7.3	9.4	12	16
OH-MiNP	4.6		6.9	3.9[e]
oxo-MiNP	2.2	2.6	2.8	
cx-MiNP	7.2		5.9	1.7[e]
cx-MiDP	0.41	2.2	0.9	1.2[e]
OH-MPHP	1.1		0.3	
oxo-MiNCH	1.3		0.93	
cx-MINCH	0.95	0.58	1.1	0.98[e]
OH-MINCH	0.96	0.97	2.3	1.2[e]
DPP	2	1.4		
DBP	0.15	0.21		
BPA	0.88	1.2	1.9	1.1
BPS	0.14	0.37		0.13
4,4BPF	0.10	<0.2		0.17
2-OH-PH	0.17	0.06	0.09	
1-HP	0.07	0.16	0.12	
3-PBA	0.28	0.64		
TCS	0.30	5.2	<1	
BP3	1.1	16	<2	

[a] DPP and DBP from 2013 to 2014 instead. 2-OH-PH from 2011 to 2012 instead. 1-HP and 3-PBA from 2013 to 2014 instead.
[b] (NHANES, 2022) Volumes II & III, updated March 2021.
[c] (Murawski et al., 2020; Schwedler et al., 2020a, 2020b; Tschersich et al., 2021).
[d] (Schoeters et al., 2022).
[e] (Bastiaensen et al., 2021).

occurred on a more-or-less daily basis in Sweden during the RMA study period. However, the use of a single-spot urine sample from each participant makes it difficult to estimate individual long-term exposure due to large intra-individual temporal variation in exposure (Perrier et al., 2016). Nonetheless, the RMA results give estimates of the overall exposure situation among adolescents in Sweden 2016–17 (Aylward et al., 2017).

3.1. Correlations between substance concentrations

This is, to the best of our knowledge, one of the most comprehensive studies of relationships between whole blood, serum and urine concentrations of chemicals in a national population study of adolescents. In the cluster analysis, most elements with diverging dietary exposure sources (Livsmedelsverket, 2017), did not form clear cluster groupings and in most cases negligible to weak correlations were observed (Fig. 1 and Fig. S3, Supplement). Moreover, with a few exceptions, correlations between elements and POPs were negligible to weak (Fig. S3, Supplement), most likely due to differences in toxicokinetics (much shorter half-lives of most elements, many POPs being lipid soluble) (Andersen et al., 2021; Nordberg et al., 2022; Verner et al., 2009) and/or exposure sources (mainly plants-based foods vs foods of animal origin) (Livsmedelsverket, 2017). Within-group clusters were observed among the POPs with similar dietary exposure patterns (Krauskopf et al., 2017; Lignell et al., 2011; Livsmedelsverket, 2017; Nyström et al., 2022) and toxicokinetics (Andersen et al., 2021; Verner et al., 2009) (Fig. 1). However, no overlaps were observed between the chlorinated, brominated and fluorinated POPs (Fig. 1). As suspected from the very short half-lives and diverging exposure sources, the urine substances did not cluster together with any of the substances measured in blood (Fig. 1) and correlations were negligible to weak (results not shown). Among substances measured in urine, metabolites of diethylhexyl phthalate (DEHP), diiso-nonyl phthalate (DiNP), and diisononyl-cyclohexane-1, 2-dicarboxylate (DiNCH) clustered separately from one another and displayed moderate to strong correlations within a given cluster (Fig. 1 & Fig. S4, Supplement). Se and Hg, and Co and Cd, formed separate clusters among the elements (Fig. 1). Hypothetically, Se and Hg relationships could be due interactions between these elements in the body (Bárány et al., 2003; Bates et al., 2006; Glynn and Lind, 1995; Ralston and Raymond, 2010). For Co and Cd similar dietary exposure sources (cereals and sugar/sweets) (Livsmedelsverket, 2017) and toxicokinetics (sharing of iron-transporting mechanisms) (Bárány et al., 2005) may have contributed. Hexa-to hepta-chlorinated PCBs clustered separately from tetra-to penta-chlorinated PCBs as well as from the chlorinated pesticides (Fig. 1). The indoor environment may still be a significant exposure source of the latter PCBs (Johansson et al., 2003; Meyer et al., 2013; Wingfors et al., 2006). For HCB, hexachlorocyclohexane (HCH) and p,p'-DDE, fish consumption has been a less important dietary source than for PCBs in Sweden (Livsmedelsverket, 2017; Törnkvist et al., 2011). BDE-47 and -99 formed a cluster separate from BDE-153 (Fig. 1), whereas BDE-153 was moderately correlated with highly chlorinated PCBs (Fig. S3, Supplement), as also observed among nursing women from Sweden (Lignell et al., 2011). The indoor environment appears to be a more significant source of human BDE-47 and BDE-99 exposure than of BDE-153 exposure (Björklund et al., 2012; Fromme et al., 2016). Among young Swedish women, BDE-153 body burdens were significantly related to consumption of fish (as was the case for certain PCBs), whereas no such relationship was found for BDE-47 and -99 (Lignell et al., 2011). As in a previous RMA study using PCA clustering (Nyström et al., 2022), L-PFHxS, L-PFOS and br-PFOS clustered separately from L-PFOA, PFNA, and PFDA (Fig. 1), which was suggested to be due to exposure to the former PFASs from drinking water (Nyström et al., 2022).

Apart from phthalates/phthalate alternatives with common 'mother

Table 4
Estimated fraction (%) of exceedances of toxic substance concentrations among RMA participants (N = 1082) in relation to non-cancer HBM-GVs, gender and school grade differences in exceedances, and critical effects of the HBM-GVs.

Substance (metabolite)	Type of HBM-GV[a]	Matrix	HBM-GV	Participants exceeding HBM-GV (%)	Male/female participants exceeding HBM-GV (%)	Participants in grade 5/8/11 exceeding HBM-GV (%)	Critical effect	Reference for HBM-GV
Se	Biomonitoring equivalents (BE)	Whole blood	400 µg/L	0			Selenosis	Hays et al. (2014)
Cd	BE	Whole blood	1.4 µg/L	0.8			Kidney toxicity	Hays et al. (2008)
Hg	Human biomonitoring I (HBM I) children/adults	Whole Blood	5 µg/L	0.4			Neurotoxicity	Apel et al. (2017)
	HBM II children/adults	Whole blood	15 µg/L	0			Neurotoxicity	Apel et al. (2017)
Pb	BMDL1% developmental neurotoxicity children	Whole blood	12 µg/L	12	17/8.8*	12/13/11	Neurotoxicity	EFSA (2010)
	BMDL1% systolic blood pressure	Whole blood	15 µg/L	7.1	8.4/6.1	7.3/7.4/6.6	Hypertension	EFSA (2010)
	BMDL10% chronic kidney disease	Whole blood	36 µg/L	0.4			Kidney toxicity	EFSA (2010)
Al	Occupational threshold adverse effects	Serum	6.8 µg/L	26	27/25	27/26/25	Neurotoxicity	Riihimäki et al. (2000)
HCB[b]	BE	Serum	80 pg/mL	3.1	5.9/1.2*	2.7/4.4/2.3	Liver toxicity	Aylward et al. (2010)
p,p'-DDE[b]	Safe level developmental effects	Serum	12.8 ng/mL	0			Reproductive toxicity	WHO (2012)
NDL-PCB[c]	HBM I (138 + 153+180x2)	Serum	3.5 ng/mL	0			Neuro-/immunotoxicity	Apel et al. (2017)
	HBM II	Serum	7 ng/mL	0			Neuro-/immunotoxicity	Apel et al. (2017)
PBDE-99[b]	BE	Serum	1.7 ng/mL	0			Neurotoxicity	Krishnan et al. (2011)
PFAS[d]	PFOA + PFNA + PFHxS + PFOS at TWI young women	Serum	6.9 ng/mL	21		34/15/20*	Immunotoxicity	EFSA (2020)
PFOA[d]	HBM I general population	Serum	2 ng/mL	12	12/13	16/6.9/14*	Mixed toxicity	Hölzer et al. (2021)
	HBM II general population	Serum	10 ng/mL	0			Mixed toxicity	Schümann et al. (2021)
	HBM II females child-bearing age	Serum	5 ng/mL	0.3			Developmental toxicity	Schümann et al. (2021)
PFOS[d]	HBM I general population	Serum	5 ng/mL	19	27/14*	25/19/18*	Mixed toxicity	Hölzer et al. (2021)
	HBM II general population	Serum	20 ng/mL	1.7	1.7/1.8	5.5/0.25/0*	Mixed toxicity	Schümann et al. (2021)
	HBM II females child-bearing age	Serum	10 ng/mL	3.6		9.8/1.8/1.4*	Developmental toxicity	Schümann et al. (2021)
DEP	BE (MEP)	Urine	18000 µg/L	0			Body growth	Aylward et al. (2009)
DBP	HBM guidance value (HBM-GV), children (MBP)	Urine	120 µg/L	4.8	5.5/4.5	5.2/4.0/5.8	Reproductive/developmental toxicity	Lange et al. (2021)
	HBM-GV adults/adolescents	Urine	190 µg/L	1.5	1.7/1.3	1.2/1.7/1.4	Reproductive/developmental toxicity	Lange et al. (2021)
BBzP	HBM-GV children (MBzP)	Urine	2000 µg/L	0			Reproductive/developmental toxicity	Lange et al. (2021)
	HBM-GV adults/adolescents	Urine	3000 µg/L	0			Reproductive/developmental toxicity	Lange et al. (2021)
DEHP	HBM-GV children (5-oxo-MEHP+5-OH-MEHP)	Urine	340 µg/L	0.2			Reproductive/developmental toxicity	Lange et al. (2021)
	HBM-GV adults/adolescents	Urine	500 µg/L	0.09			Reproductive/developmental toxicity	Lange et al. (2021)
	HBM-GV children (5cx-MEPP+5-OH-MEHP)	Urine	380 µg/L	0.2			Reproductive/developmental toxicity	Lange et al. (2021)
	HBM GV adults/adolescents	Urine	570 µg/L	0.09			Reproductive/developmental toxicity	Lange et al. (2021)

(*continued on next page*)

Table 4 (*continued*)

Substance (metabolite)	Type of HBM-GV[a]	Matrix	HBM-GV	Participants exceeding HBM-GV (%)	Male/female participants exceeding HBM-GV (%)	Participants in grade 5/8/11 exceeding HBM-GV (%)	Critical effect	Reference for HBM-GV
DPHP	HBM GV adults (OH-MPHP)	Urine	220 μg/L	0			Thyroid toxicity	Lange et al. (2021)
	Children	Urine	140 μg/L	0			Thyroid toxicity	Lange et al. (2021)
DiNP	BE (OH-MiNP + oxo-MiNP + cx-MiNP)	Urine	14700 μg/L	0			Liver toxicity	Hays et al. (2011)
DiNCH	HBM GV children (OH-MINCH + cx-MINCH)	Urine	3000 μg/L	0			Kidney toxicity	Lange et al. (2021)
	Adults/adolescents	Urine	4500 μg/L	0			Kidney toxicity	Lange et al. (2021)
BPA	HMB GV children	Urine	135 μg/L	0			Kidney toxicity	Ougier et al. (2021)
	HMB GV adults	Urine	230 μg/L	0			Kidney toxicity	Ougier et al. (2021)
3-PBA	BE Tier 1	Urine	1.7 μg/L	2.2	2.3/2.5	3.3/1.2/2.9	Neurotoxicity	Aylward et al. (2018)
	BE Tier 2	Urine	87 μg/L	0			Neurotoxicity	Aylward et al. (2018)
TCS	HBM I children	Urine	2000 μg/L	0			Haemato-/spleen toxicity	Apel et al. (2017)
	HBM I adults	Urine	3000 μg/L	0			Haemato-/spleen toxicity	Apel et al. (2017)

[a] HBM I is the threshold for early warning and concern, whereas HBM II is the threshold for an increased risk for adverse health effects.
[b] Recalculated from concentration in blood lipids to concentration in serum using a mean lipid content in RMA serum of 0.33%. *p ≤ 0.05, Chi-square test.
[c] Non-dioxin like.
[d] PFAS ng/g converted to ng/mL using the specific gravity of serum (1.0275) (Sunderman and Boerner, 1949).

Table 5
Hazard index (HI) in RMA (N = 1082) based on the same target organ critical effect.

Target organ[a]	Composition	Total N with hazard index >1 (%)[b]	Median (range) of HI	Median (range)[c] of HI > 1
Liver	HCB, DiNP	35 (3.2)	0.53 (0.15–93)	1.1 (1.01–93)
Kidney	Cd, Pb, DiNCH, BPA	16 (1.5)	0.31 (0.01–4)	1.64 (1.08–4)
Central nervous system	Hg, Pb, Al, NDL[d]-PCB, PBDE-99, 3-PBA	1018 (94)	1.82 (0.58–15)	1.86 (1–15)

[a] Critical effect target for HBM-GVs as seen in Table 4.
[b] HI calculated per target organ critical effect by dividing participants blood/serum/urine concentrations with corresponding HBM-GV (hazard ratio, HR) from Table 4, then summing HR together. Total numbers of those with HI > 1 shown.
[c] Median and range calculated for participants with HI > 1.
[d] Non-dioxin-like.

substances', the dibutyl phthalate (DBP) metabolite monobutyl phthalate (MBP) and the butylbenzyl phthalate (BBzP) metabolite MBzP formed a cluster suggesting common exposure sources (Fig. 1). MBP and MBzP were also moderately correlated with the DEHP metabolites (Fig. S4, Supplement), as correspondingly observed for MBzP among Swedish and Danish children, and for MBP among Flemish adolescents (Bastiaensen et al., 2021; Langer et al., 2014; Larsson et al., 2014). Common exposure from building materials/consumer products may contribute to the observed relations (KEMI, 2015). As among Swedish and Danish children, and Flemish adolescents (Bastiaensen et al., 2021; Langer et al., 2014; Larsson et al., 2014), in RMA only negligible/weak correlations were observed between the metabolite of diethyl phthalate (DEP), monoethyl phthalate (MEP), and the other phthalate metabolites (Fig. S4, Supplement). DEP is currently the main phthalate used in cosmetic products in the EU (KEMI, 2015). DiNP, diiso-decyl phthlate (DiDP) and dipropylheptyl phthalate (DPHP) have been introduced on the European market more recently than the other phthalates (Fréry et al., 2020), and the metabolites of these three phthalates were moderately correlated with each other (Fig. S4, Supplement). DiNP and DPHP metabolites were also moderately correlated with the DEHP metabolites (Fig. S4, Supplement), also observed for DiNP and DEHP metabolites in Flemish adolescents (Bastiaensen et al., 2021). DiNP is a direct alternative to DEHP as a general plasticizer and are used together with both DiNP and DPHP in PVC production (KEMI, 2015). The two PAH metabolites 2-hydroxy-phenanthrene (2-OH-PH) and 1-HP formed a cluster and were moderately correlated (Fig. 1 & Fig. S4, Supplement), as expected due to the common exposure sources (Murawski et al., 2020)

3.2. Comparisons with other studies

Comparisons of RMA results with other studies of adolescents are somewhat uncertain due to differences in study design/populations and analytical methods. Nevertheless, mean concentrations of elements, POPs and urine substances with a few exceptions varied less than 3-fold between RMA against NHANES (USA), GerES (Germany) and FLEHS (Flanders, Belgium) (Tables 2 and 3). The relatively small differences in average exposure of adolescents between the four studies are most probably due to the similarly high standard of living in these populations, in combination with world-wide distribution of chemicals and products. NHANES showed large ethnic differences in mean serum concentrations of chlorinated and brominated POPs, in some cases up to several orders of magnitude (Table 2) (NHANES, 2022). In RMA, mean concentrations of chlorinated POPs were within these ranges or somewhat lower (Table 2). The concentrations of the three PBDEs were in most cases below the LOQ (<20 pg/ml serum) in RMA (Table S3, supplement). However, the determined concentrations below LOQ in RMA strongly suggested considerably lower mean serum PBDE concentrations than in NHANES, with a minimum of a 23-fold difference (BDE-153) (Table 2) (NHANES, 2022). The higher body burdens of PBDE in the US than in Sweden and other European countries is in line with studies of adults (Fromme et al., 2016). Differences in PBDE legislation between the US and Europe most likely contributes to the exposure differences (Horton et al., 2013). The mean concentration of

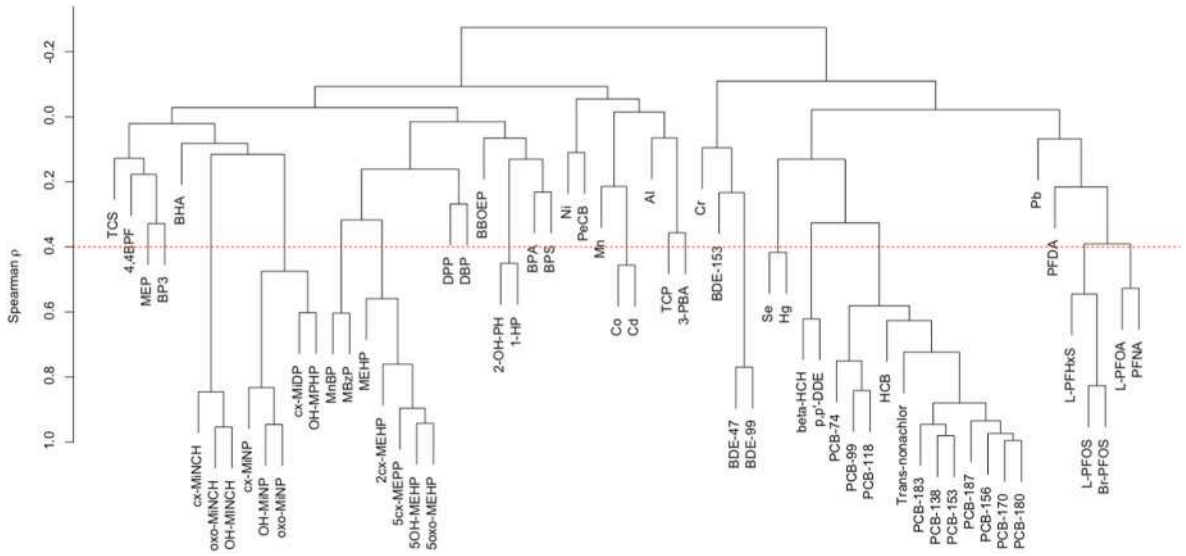

Fig. 1. Cluster analyses of correlations between toxic substance concentrations among adolescents in RMA (N = 1082). Correlation coefficients should be read at where branches separate and not on the names. Figure shows absolute spearman correlation coefficients with both negative and positive correlations. The dotted red line represents the set cut-off for moderate correlation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

dichlorodiphenyltrichloroethane (p,p'- DDT) was reported to be 37-fold higher in FLEHS compared to RMA, but the concentration of the DDT metabolite p,p-DDE did not differ markedly (Table 2). Studies of breast milk suggest similar average p,p'-DDT exposures among women in child-bearing age in Sweden and Belgium around the same time period as the RMA study (Aerts et al., 2019; Kallerman et al., 2021). Further studies are needed to determine the reason behind the large differences in p,p'-DDT concentrations in RMA and FLEHS.

The average concentrations of phthalate metabolites in urine did in many cases not differ more than 3-fold between RMA and the other studies, suggesting similar exposure levels among the adolescents (Table 3). Among the European adolescents this may be due to the common plastics regulation within the EU (CEP et al., 2022; Pereira et al., 2022). Nevertheless, the exceptions to the <3-fold difference were MBP, cx-MiNP, cx-MiDP and 6-hydroxy monopropylheptyl phthalate (OH-MPHP). The mean MBP concentrations in NHANES, GerES and FLEHS were lower than in RMA (Table 3), at most being 3.6-fold lower in NHANES compared with RMA. Similarly, the mean cx-MiNP was also lower in GerES and FLEHS (not reported in NHANES), occurring at a 4.2-fold lower mean concentration in FLEHS. However, the differences in mean concentrations of the other DiNP metabolites were less than 3-fold and RMA did not consistently show the highest concentrations (Table 3). cx-MiDP was lowest in RMA, at most 5.4-fold lower than in NHANES (Table 3), and OH-MPHP was 3.7-fold lower in GerES than in RMA. There was no consistent pattern in the observed differences in mean concentrations of the four phthalate metabolites between RMA and the other three studies, suggesting diverging substance-related exposure sources. The largest differences of concentrations of urine substances were observed for TCS and BP3, being on average about 15-fold higher in NHANES adolescents compared to RMA (Table 3). Although concentrations of these substances were below LOQ in GerES, they were also clearly lower than in NHANES. Differences in the levels of TCS and BP3 in consumer products and also in usage patterns, between Germany and the US were suggested as explanations of divergence in TCS exposure between GerES and NHANES (Tschersich et al., 2021), and is likely also the reason for the large difference between RMA and NHANES.

3.3. Comparisons with HBM-GVs and HI assessment

We surveyed the literature for HBM-GVs and found several from multiple sources (Table 4). There are uncertainties when comparing the different proposed HBM-GV, in part due to variation in the quality of the toxicological database, in procedures used in the development of the different values, safety margins used between point-of-departure of critical effect concentrations and HBM-GVs, and the fact that some of them have not been updated based to the most recent risk assessments.

Moreover, some HBM-GVs listed in Table 4 were specifically developed for distinct sub-populations like children, adolescents, adults or women in childbearing age. UBA, Germany (see Table 4 references) developed HBM I and HBM II values, and in the view of UBA there is no risk of adverse health effects if concentrations of substances are below HBM I. If the concentrations are above HBM I, but below HBM II, efforts should be made to search for potential sources of exposure and to mitigate identified exposure sources. An exceedance of HBM II is by UBA regarded as an increased health risk, and therefore there is a need for acute action to reduce exposure and biomedical advice should also be provided. Biomonitoring Equivalent (BE) values are based on existing health-based guidance values, such as tolerable intakes, and exceedances of BE may be regarded as a health concern (Hays et al., 2007). HBM4EU has developed HBM-GVs that in reality are equivalent to UBA's HBM I values (Apel et al., 2020). The values for Pb, Al, and $PFAS_4$ (Table 4) were based on benchmark concentrations/effect level concentrations in humans without any safety factors (EFSA, 2020; Riihimäki et al., 2000). Moreover, the Al value was based on relationships between occupational exposure and health among aluminium welders (Riihimäki et al., 2000). Despite all these uncertainties, as a conservative measure, exceedances of the HBM-GVs among the RMA participants were regarded as indications of a health concern.

The largest degree of exceedance of HBM I, HBM-GVs for children, and benchmark concentrations/effect levels were observed for Al, $PFAS_4$, PFOS, PFOA and Pb in declining order, all being ≥12% of the adolescent population when looking at the most conservative values (Table 4). Based on the size of the adolescent population in Sweden during 2016 for ages 12, 15 and 18 (N = 324776) (SCB, 2022), the number of adolescents exceeding the HBM-GVs can be estimated to range from 32700 ($PFAS_4$, females only) to 84400 (Al). For HCB, MBP and 3-PBA percent exceedances corresponded to 10000, 16300 and

7100 adolescents, respectively (Table 4). The percentages of exceedances of PFASs are likely overestimated since 5% of the participants were living in areas with a history of PFAS contamination of drinking water (Nyström et al., 2022), which is disproportionally high from a nation-wide population perspective. To the best of our knowledge, the PFAS contamination in some areas of Sweden is a special case not representative for the other substances included in our study. Nevertheless, contribution of unknown local hotspot contamination of other substances to the exceedances of HBM-GVs cannot be excluded. Exceedances were also observed for Cd, Hg, DEP, and DEHP, although to a lesser degree (Table 4). Overall, 54% of the participants exceeded at least one of the HBM-GV, 18% at least two, and 5% at least three. Taken together the results show that a significant fraction of the adolescent population had overall high exposures to several of the toxic substances in relation to the HBM-GVs. When using HBM-GVs for adults and/or adolescents (Pb, DBP, DEHP) or HBM II values (Hg, PFOS) the percentages exceeding the HBM-GVs were less frequent (Table 4). Considering that the concentrations of toxic substances in RMA, GerEs V and FLEHS were comparable (Tables 2 and 3), it is not surprising that GerES and FLEHS also reported exceedances of HBM-GVs of Cd, Pb (FLEHS), Hg (GerES), HCB (FLEHS), PFASs, DBP, DEHP (GerES) and 3-PBA (FLEHS) (Schwedler et al., 2020b; Vogel et al., 2021).

As suggested by the higher GM concentrations of Pb, HCB, and PFOS among males than females in RMA (Table 1), fewer females exceeded the benchmark dose of 1% extra risk (BMDL1%) for neurotoxicity of Pb in children, the BE for HCB, and the HBM I value for PFOS (Table 4). However, observed gender differences in concentrations of the toxic substances (Table 1) did not necessarily result in gender differences in exceedances of HBM-GVs, as observed for PFOA, the DBP metabolite MBP, and 3-PBA (Table 4). Moreover, no gender differences in exceedances of HBM-GVs were observed for Al, and for less conservative HBM-GVs of Pb, PFOS, and MBP. When looking at age-dependent differences in exceedances in relation to observed associations between age and concentrations there were no general trend, except in the case of PFASs that showed a higher degree of exceedances among 5th graders (Table 4), most probably due to a particular subset of the 5th-grade participants (N = 58) having a known high exposure of PFHxS and PFOS from drinking water in Uppsala and Ronneby (Nyström et al., 2022).

Efforts to eliminate environmental pollution of Pb, HCB, PFOA, PFNA, PFHxS and PFOS have resulted in slowly declining temporal trends in different Swedish populations (Gyllenhammar et al., 2021; Lundh et al., 2020; Miaz et al., 2020; Norén et al., 2021; Wennberg et al., 2017). Similar efforts to reduce Cd pollution have not resulted in declining Cd exposures during the last few decades, whilst mixed trends for Hg exposure (no decrease/slow decrease) have been reported (Kippler et al., 2021; Lundh et al., 2020; Wennberg et al., 2017). Taken together, further efforts to reduce exposures to Pb, Hg, Cd, Al, HCB and PFASs in Sweden are clearly needed. A study of young women in Sweden have reported decreasing exposures to DEP, DBP and DEHP over the last few decades, suggesting that the exposure situation also is slowly improving for these rapidly metabolized substances (Gyllenhammar et al., 2017).

In accordance with the RMA findings of exceedances of HBM-GVs of Pb, Hg (methyl-Hg), Al, and PFASs, The European Food Safety Authority (EFSA) concluded that the tolerable intakes of these substances from the diet were exceeded by parts of the EU population at the time of risk assessment (EFSA, 2012a, 2012b, 2010, 2008). Regarding phthalates, EFSA stated that the group tolerable intake of DBP, BBP, DEHP and DiNP (as DEHP equivalents) were not exceeded by EU populations even when considering the worst-case total dietary intake mainly originating from plastic food contact materials (CEP et al., 2019). However, diet is only one of several potential exposure sources of these phthalates, and the contribution of the additional sources most likely contributes to the high concentrations of metabolites observed in a small fraction of the RMA participants (Bastiaensen et al., 2021; Langer et al., 2014; Larsson et al., 2014; Schwedler et al., 2020b). However, considering that RMA only included single-spot urine sample there are still uncertainties about the risks of long-term individual exceedances of the HBM-GVs for these phthalates among adolescents in Sweden.

BPA concentrations in urine did not exceed the published HBM-GV, the GM concentration being 148-fold lower than the HBM-GV for children (Tables 1 and 4). However, in 2021 an EFSA draft opinion on health risks of BPA in foods proposed a considerably lower tolerable intake compared to the pervious assessment in 2015 (EFSA, 2021). It was proposed that populations with both average and high exposures to BPA in all age groups exceed the new tolerable intake. The publication of the final risk assessment will reveal if the exposure of the Swedish adolescent population will be regarded as too high or not from a health risk assessment perspective.

In the cumulative health risk assessment liver toxicity, including HCB and DiNP, showed a median HI (range) of 0.53 (0.15–93), with 3.2% of the participants showing a HI > 1 (Table 5). HCB was the driver of the health concern contributing with 99.9% (median, range: 98.8–100%) to the HI among participants with HI > 1. As with HCB alone, the highest proportion of participants with a health concern HCB was observed among males (5.9%), with a significantly lower proportion among females (1.2%, Chi-square test $p \leq 0.05$). In the case of kidney toxicity, the median HI (range) was estimated to 0.31 (0.006–4.0) and 1.5% of the participants had a HI > 1 (Table 5). Cd contributed 76.3% (3.1–91%) to the HI among participants with HI higher than 1, followed by Pb 23% (7.7–96%). The proportion of males and females with a HI higher than 1 was 1.6% and 1.3%, respectively, with gender difference not being statistically significant. Neurotoxic substances consisting of Hg, Pb, Al, NDL-PCB, PBDE-99 and 3-PBA showed a higher median HI of 1.8 (range: 0.58–15) compared to substances with liver and kidney toxicity, with 94% of the participants showing a health concern (HI > 1). Al contributed the most 39.5% (0–93%) to the HI among participants with HI higher than 1, followed by Pb 33.8% (0–96%). As with liver and kidney toxicity males showed a higher proportion of 96% exceeding HI = 1, whereas the proportion of females was 93% (Chi-square test $p \leq 0.05$). The HI analysis indicates that if the assumption of cumulative additive target organ toxicity effect holds true, significant portions of the adolescent population may be at risk of having too high cumulative exposures to neurotoxic substances. However, granted that the target organ of the central nervous system (CNS) is highly complex and the neurotoxicity of each individual contributing substance may affect different sections or processes of the CNS, the observation should be considered preliminary.

3.4. Strengths and limitations

As previously eluded to, a major strength of this nation-wide population-based study of adolescents is the inclusion of wider groups of toxic substances. There are some minor demographic/life-style differences between the RMA participants and the adolescent population in Sweden, yet participants are still considered as representative of the adolescent population in Sweden with regard to school type/size, and parental education and income (Moraeus et al., 2018). The urine substances have very short half-lives in comparison to the elements and POPs. As a consequence there is intra-individual variation of urine substance concentrations depending on timing of sampling. Other studies have shown that there may be seasonal differences in concentrations for some of the substances in urine (Bastiaensen et al., 2021). Although the possibility of seasonal differences in concentrations were not analysed statistically in the present study, the present results may be regarded as representative for the general adolescent population in Sweden during the sampling period. While aspects of the present work are comparable to prior studies (e.g. NHANES and GerES) some caution is warranted when comparing between studies due to potential differences in study design, analytical methods, and data processing/analyses. Human biomonitoring initiatives of toxic substances has been increasingly moving

towards combination and mixture effects. Yet, most studies still only focus on single substances/substance groups. Inclusion of a wider portion of exposome as in the present study, uncovered patterns of chemical body burdens that are valuable for interpretation of future RMA health studies. In the analyses of urine substances, the precision of cx-MiDP, BPS, DBP and BHA was >20%. This introduced an additional uncertainty in the statistical analyses, and in comparisons with other studies and HBM-GV.

The HBM4EU initiative, as well as UBA, has made steady progress to developing HBM-GVs for the general population (Apel et al., 2020), but nevertheless many toxic substances currently lack established HBM-GVs. We attempted to include the HBM-GVs appropriate for a general adolescent population, but if unavailable we instead used either those developed for children/adults or values proposed for occupational exposure (Al), making comparison of the results uncertain. Moreover, there are uncertainties connected to our limited HI approach of grouping of substances with the same target critical effect organs, and the HI results therefore should be considered as hypothesis generating for future studies of possible combination effects of the included chemicals.

4. Conclusion

Although many biomonitoring studies are available involving a range of individual chemical substances, our study is one of the few attempts at looking at the wider chemical exposome within a single study of adolescents, giving a more holistic and clearer picture of the current state of the chemical body burden in Swedish adolescents. With few exceptions, chemical body burdens in the present work were similar to those observed in adolescents from Germany and the US, showing a similar exposome for adolescents in these economically and industrially developed countries from two different continents. In RMA, the moderate to strong correlations between substances within the same chemical groupings were most likely due to common sources of exposure and/or similar toxicokinetics. However, while the chlorinated, brominated and fluorinated POPs included here share common properties of environmental persistence and bioaccumulation in humans, measured concentrations of the different contaminant classes were rarely correlated. Moreover, no correlations were generally observed between substances measured in different matrices. These observations are important for better interpretation of results in future evaluations of RMA data with regards to possible combination effects. The gender patterns observed, with generally higher average body burdens of neurotoxic Pb, Hg and some POPs in males compared to females, reflected in males having higher proportions of HI $>$ 1 for neurotoxicity also adds important knowledge for future combination effect studies. Our results give support to further efforts to reduce human exposures to toxic chemicals.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114196.

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Impact of diesel exhaust exposure on urinary 1-hydroxypyrene in underground salt and potash workers

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ABSTRACT

Background.

Diesel engine exhaust (DEE) and some of the polycyclic aromatic hydrocarbons (PAH) it contains are carcinogenic to humans (for example benzo(a)pyrene) and can cause lung cancer in workers. The objective of this study was to assess exposures to DEE and its component PAH and the potential associations between these two health hazards in a salt and potash mining population.

Methods.

Between 2017 and 2019, 1003 underground workers (mining n = 801, maintenance n = 202) and 243 aboveground facility workers from two German mines participated. Personal exposure to DEE was assessed in air as elemental carbon for diesel particulate matter (EC-DPM), whereas exposure to PAH was assessed in pre- and post-shift urine samples in terms of 1-hydroxypyrene (1-OHP). Associations between EC-DPM and 1-OHP were studied using linear regression models.

Results.

The highest EC-DPM exposures were measured in mining workers (median 0.06 mg/m^3) followed by workers in the maintenance (0.03 mg/m^3) and facility areas (<0.02 mg/m^3). Exposures above the current German occupational threshold level of 0.05 mg/m^3 were observed in 56%, 17%, and 5% of mining, maintenance and facility workers, respectively. 1-OHP increased statistically significantly across a work shift in underground workers but not in facility workers. Regression analyses revealed an increase of post-shift 1-OHP by almost 80% in mining and 40% in maintenance compared with facility workers. 1-OHP increased with increasing EC-DPM among underground workers. However, internal exposure of 1-OHP mainly remained at levels similar to those of the German general population in more than 90% of the urine samples.

Conclusions.

While exposures to DEE above the current German OEL for EC-DPM are quite common in the studied population of underground salt and potash miners (39.5% overall), urinary concentrations of 1-OHP did not reflect these findings.

1. Introduction

Emissions from diesel engines are complex mixtures of diesel particulate matter (DPM) containing elemental and organic carbon and gases such as carbon and nitrogen oxides, among others (IARC, 2014). Diesel engine exhaust (DEE) also contain polycyclic aromatic hydrocarbons (PAH) and several studies have specifically investigated the PAH content of DEE (Corrêa and Arbilla, 2006; Marr et al., 1999).

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Although PAH profiles in DEE can considerably vary, no significant differences for total PAH concentrations could be detected (Borrás et al., 2009).

DEE is a major contributor to air pollution and global warming (Campbell-Lendrum and Prüss-Ustün, 2019) and has been classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC, 2014). In recent decades, increasingly stringent emission standards have been introduced, especially for road vehicles. Exhaust gas treatment and fuel efficiency have been improved, resulting in lower emissions (Frey, 2018).

DEE poses also an occupational health risk. A review of occupational exposure to DEE found highest exposure concentrations for enclosed underground work sites where heavy equipment is used (e.g. mining and mine maintenance), and lowest when working outdoors or separated from the exposure source (Pronk et al., 2009). A suitable surrogate marker for DEE commonly used for occupational monitoring is elemental carbon in diesel particulate matter (EC-DPM) as it constitutes a large fraction of the particulate mass and can be quantified at low levels (Birch and Cary, 1996). However, external exposure measurements cannot estimate internal workers exposure.

To assess internal exposure to DEE, 1-hydroxypyrene (1-OHP), a metabolite of pyrene, has been commonly used as a surrogate biomarker (Ciarrocca et al., 2014; Hansen et al., 2008; Louro et al., 2022). Reasons include that pyrene has been found in DEE emission. For example, pyrene was the sixth most abundant of the 16 designated high priority PAHs by the US Environmental Protection Agency in a study exploring underground DEE in a Swedish iron ore mine (Gren et al., 2022). In addition, an increase in 1-OHP over the course of a shift was also seen in miners from an Australian goldmine (Du et al., 2019) suggesting that 1-OHP is an appropriate surrogate biomarker to study exposures to DEE and PAH in miners. 1-OHP has also been used in other studies for assessing exposure to PAH thus allowing cross-study evaluation of PAH exposures between different occupational settings rather than settings with DEE exposure alone. For example, the highest 1-OHP concentrations have been previously reported for workers in petrochemical industries (coke-oven workers) and metallurgy workers (Hansen et al., 2008). Less pronounced exposure have been found in firefighters due to their use of high-level personal protective equipment (Hoppe-Jones et al., 2021; Taeger et al., 2023).

Urine concentrations of 1-OHP are also elevated by non-occupational sources of PAH such as tobacco smoking, air pollution, dietary intake, dermal absorption of pharmaceuticals, or contact with contaminated soil (Jongeneelen, 2001). It has been shown that the strongest predictor of urinary 1-OHP excretion in the general population is tobacco smoking (Wilhelm et al., 2008).

To reduce health hazards from DEE in the workplace, an occupational exposure limit (OEL) of 0.05 mg/m^3 for EC-DPM was established in Germany in 2017; a transition period has been granted for underground mining (GESTIS Substance Database). As part of this 5-year transition plan to reduce exposure in salt and potash mining, an epidemiological study was conducted to investigate potential health effects within an 8-h shift. First results on selected cardiovascular, inflammatory and respiratory effects of this study have been published recently (Gamrad-Streubel et al., 2022). The aim of this additional analysis was to determine the exposure to PAH using urinary 1-OHP and to study its relationship with DEE in a salt- and potash-mining population commonly exposed to exhausts from large diesel-powered mining equipment and vehicles that are required for underground mining operations.

2. Material and methods

2.1. Study design and study population

The study population of this analysis comprised 1246 men employed at two salt and potash mining sites in Germany. The rationale, design, and conduct of the underlying cross-sectional study were previously described in detail (Gamrad-Streubel et al., 2022). In brief, all employees working for at least one year in the facility above ground or in the mine underground were eligible. Facility workers who had previously been employed underground or who were regularly occupationally exposed to higher diesel exhaust levels (e.g., by operating small diesel-powered machines) were excluded, as were underground workers who had previously been employed in other mines. According to the workplace risk assessment, neither underground nor surface workers wear respiratory protection in their daily work.

Data were collected on a single 8-h shift between August 2017 and January 2019. Each participant completed a pre-shift survey and was medically examined before and after the shift. Body height and weight were measured before the shift to assess the body mass index (BMI). Smoking status was categorized as current daily smoker, former smoker, and non-smoker (i.e., never and occasional smokers with less than five cigarettes per week).

The study was approved by the Ethics Committee of the Ruhr University Bochum, Germany (Reg. No. 176024). Written informed consent was obtained from all participants.

2.2. Urine sampling and analysis

Urine samples could be collected from 1229 participants before and for 1240 participants after the same shift on any day during the work week. These were stored at −20 °C until analysis. Urinary 1-OHP [µg/L] was evaluated and analysed by high-performance liquid chromatography (HPLC) with fluorescence detection. Urinary creatinine [g/L] was measured to standardize for diuresis. Creatinine-adjusted 1-OHP [µg/g creatinine] was calculated for creatinine levels between 0.3 g/L and 4.0 g/L although only values between 0.3 g/L and 3.0 g/L have been suggested to represent normally hydrated adults (Bader et al., 2020). However, we used an upper value of 4.0 g/L for creatinine adjustment because of the higher muscle mass of the studied miners compared to the general population. In 1224 urine samples before and in 1234 urine samples after the shift, 1-OHP could be determined. Of these, 559 (49%) pre-shift and 473 (38%) post-shift 1-OHP concentrations were below the limit of detection (LOD) of 0.1 µg/L. Due to missing creatinine values or values outside the range of 0.3 and 4.0 g/L for creatinine, 48 pre-shift and 56 post-shift values of creatinine-adjusted 1-OHP could not be determined. Therefore, a total of 1176 pre-shift and 1178 post-shift values of creatinine-related 1-OHP concentrations have been evaluated.

In Germany, the MAK Commission (Permanent Senate Commission of the Deutsche Forschungsgemeinschaft for the Investigation of Health Hazards of Chemical Compounds in the Work Area) established a biological reference value (BAR) of 0.53 µg/L and 0.3 µg 1-OHP/g creatinine (Deutsche Forschungsgemeinschaft, 2022; Klotz, 2021; Wilhelm et al., 2008). The BAR represents the background concentration (95th percentile) of 1-OHP of non-smoking adults in the general population of Germany who are not exposed to PAH at the workplace. Because no BAR exists for current smokers, the 95th percentile of 1-OHP of smoking individuals from the general population was based on the 1998 Environmental Survey in Germany (Becker et al., 2002) and set to 1.03 µg/L and 0.73 µg 1-OHP/g creatinine. We compared these evaluation standards of the non-occupationally exposed general population to the measured concentrations of 1-OHP in salt and potash miners to assess the extent of occupational exposure. Furthermore, Biological Exposure Index (BEI®) levels published by the American Committee of Governmental Industrial Hygienists (ACGIH) exist to evaluate occupational health hazards and risks. In the case of PAH exposures, the BEI® is based on the relationship between urinary 1-OHP and various genotoxic endpoints and should be adjusted to the specific PAH mixture of the workplace. As this has not been determined in salt and potash mining sites, the default value of 2.5 µg/L proposed by the ACGIH has been used for interpreting our results (ACGIH, 2017).

2.3. Ambient measurements

The exposure of DEE was assessed by measuring EC-DPM. Each participant was equipped with personal dust samplers during the entire shift. The respirable dust sampling system PGP FSP2 cyclone with a SG 5100ex battery-driven sampling pump (GSA Messgerätebau, Ratingen, Germany) was used (Gamrad-Streubel et al., 2022). The shift mean values of EC-DPM [mg/m^3] related to an 8-h shift were used as personal exposure index. Sampling rate and measurement duration have a decisive influence on the limit of quantification (LOQ) for EC-DPM, resulting in different values per measurement. At a sampling rate of 2 L/min and an 8-h shift, the LOQ for EC-DPM was 0.025 mg/m^3. EC-DPM could not be determined for 11 subjects and 33% of EC-DPM measurements were below LOQ (n = 406).

2.4. Statistical analysis

Continuous variables to characterize the study population were presented with the arithmetic mean and range. Exposure characteristics were described with median, range, and the number of values below LOD. Boxplots with median, interquartile range (IQR), and whiskers representing the minimum and maximum were used to show the distribution of creatinine-adjusted 1-OHP. Group comparisons were performed by Kruskal-Wallis tests (KWT) or Chi^2 tests, accordingly. Location differences between pre- and post-shift values were tested using signed rank tests (Wilcoxon tests).

We used a maximum likelihood estimation method for multiple imputations of values below LOD. Assuming an equal probability distribution below and above LOD, values below LOD were imputed 100 times at random from a log-normal distribution (Lotz et al., 2013). The results of the imputation analyses were combined using the SAS procedure PROC MIANALYZE.

The monotonic relationships between internal and external exposure were presented by Spearman rank correlation coefficients (r_S) with 95% confidence intervals (95% CI) and p-values. Following a study of miners from a Western Australian gold mine (Du et al., 2019), we captured the association between external exposure and post-shift creatinine-adjusted 1-OHP using linear regression models that included imputed values below LOD. As exposure variable, we included either exposure group (facility, maintenance, mining) or EC-DPM as a continuous variable. In all models, pre-shift creatinine-adjusted 1-OHP was included as an independent variable. In further models, a second adjustment set consisting of age [per 10 years], BMI [kg/m^2], and smoking status (current, non-current) was considered. We used the method of Harel to estimate the coefficient of determination R^2 as a measure of goodness of fit (Harel, 2009).

Statistical analyses were performed using SAS, version 9.4 (SAS Institute Inc., Cary, NC, USA). Figures were prepared using GraphPad Prism, version 9 (GraphPad Software, La Jolla, California, USA).

3. Results

The study population consisted of 243 above-ground facility workers and 1003 exposed underground workers, of whom 202 were in the maintenance group and 801 in the mining group and were stratified by exposure group (Table 1). Mining workers were slightly younger (p_{KWT}<0.001) and duration of employment was slightly shorter (p_{KWT} = 0.005) compared to the other study groups. In addition, current smoking was more prevalent in the mining group.

Facility workers served as reference group due to their low personal exposures to EC-DPM. Accordingly, 82% of the EC-DPM concentrations were below LOD in the facility workers (median < LOD, range < LOD-0.11 mg/m^3), followed by maintenance (49.5% <LOD, median 0.025 mg/m^3, range < LOD-0.17 mg/m^3) and mining (13.7% <LOD, median 0.06 mg/m^3, range < LOD-0.34 mg/m^3) (Table 2). Accordingly, the German OEL was exceeded least frequently in the facility (5%) and more frequently underground in maintenance (17%) and mining (56%) (p^2_{Chi}<0.001). Overall, 488 of the workers studied were above the OEL for EC-DPM (39.5%).

Internal exposure measured by 1-OHP also differed between groups. Volume-adjusted urinary 1-OHP [µg/L] was below LOD in more than 70% of facility workers (reference group), with little difference between pre- and post-shift concentrations. Among underground workers, 1-OHP concentrations below LOD were less frequent, especially after the shift, with median exposures increasing. In example, post-shift 1-OHP was below LOD in 208 miners (26%) only and their median level was 0.3 µg/L. Miners also had the highest creatinine-adjusted 1-OHP concentrations (pre-shift 0.10 µg/g creatinine, post-shift 0.16 µg/g creatinine).

Fig. 1 presents the creatinine-adjusted 1-OHP concentrations before and after the shift per exposure group and stratified by smoking status. Among underground workers, internal exposure increased statistically significantly during the shift. Among facility workers, an increase of creatinine-adjusted 1-OHP was observed only in current smokers who, unlike underground workers, were allowed to smoke during shift breaks. Evaluation standards (BAR levels) for 1-OHP were partially exceeded. For creatinine-adjusted values, exceedances were most common among workers in mining (current smokers, 5.2%; non-smokers, 9.4%), whereas the corresponding frequencies were lower among maintenance workers (3.8% and 1.5%, respectively) and facility workers (3.2% and 1.8%, respectively) (Table S1).

Table 1
Characteristics of the study population of 1246 men.

	Above-ground facility				Underground							
	Reference group (n = 243)				Maintenance (n = 202)				Mining (n = 801)			
	N	Mean	Min	Max	N	Mean	Min	Max	N	Mean	Min	Max
Age [years]	243	41.4	21	65	202	40.4	19	64	801	38.4	19	63
Body mass index [kg/m^2]	243	28.5	18.0	43.0	202	27.8	19.7	44.4	801	28.0	19.6	44.3
Smoking (N, %)												
Non[a]	110 (45.3)				101 (50.0)				342 (42.7)			
Former	64 (26.3)				45 (22.3)				177 (22.1)			
Current	69 (28.4)				55 (27.2)				277 (34.6)			
Missing	0				1 (0.5)				5 (0.6)			
Duration of employment [years]	243	18.0	1.1	46.8	202	16.5	1.6	42.3	801	14.9	0.5	41.6
Hiring date [MM/YYYY]	243	07/2000	03/1971	10/2017	202	11/2001	08/1976	08/2016	801	06/2003	09/1976	02/2018

[a] Non-smokers include never smokers and occasional smokers (<5 cigarettes per week).

Table 2
Exposure characteristics.

	Above-ground facility				Underground							
	Reference group (n = 243)				Maintenance (n = 202)				Mining (n = 801)			
	N	N $_{<LOD}$ (%)	Median	Min-Max	N	N $_{<LOD}$ (%)	Median	Min-Max	N	N $_{<LOD}$ (%)	Median	Min-Max
EC-DPM [mg/m^3]	241	198 (82.2)	<0.02	<0.010–0.11	200	99 (49.5)	0.03	<0.02–0.17	794	109 (13.7)	0.06	<0.02–0.34
Urinary 1-OHP [µg/L]												
Pre-shift	241	170 (70.5)	<0.1	<0.1–3.6	200	102 (51.0)	<0.1	<0.1–1.2	783	327 (41.8)	0.2	<0.1–2.5
Post-shift	242	177 (73.1)	<0.1	<0.1–3.9	201	88 (43.8)	0.1	<0.1–2.1	791	208 (26.3)	0.3	<0.1–3.8
Urinary creatinine [g/L]												
Pre-shift	241	0 (0)	1.59	0.12–4.44	199	0 (0)	1.64	0.19–4.18	786	0 (0)	1.61	0.98–6.74
Post-shift	243	0 (0)	1.35	0.15–3.87	202	0 (0)	1.54	0.14–4.86	794	0 (0)	1.68	0.61–5.54
Urinary 1-OHP [µg/g creatinine][a]												
Pre-shift	231	–	0.05	0.00–2.23	193	–	0.07	0.00–1.85	752	–	0.10	0.00–3.11
Post-shift	230	–	0.05	0.00–2.57	188	–	0.11	0.00–0.92	760	–	0.16	0.00–4.32

1-OHP 1-hydroxypyrene; *EC-DPM* Diesel particulate matter measured as elemental carbon; *LOD* Limit of detection.
[a] Multiple imputed 1-OHP [µg/L] if below LOD and calculation of 1-OHP [µg/g creatinine] only for creatinine concentrations in the range between 0.3 g/L and 4 g/L.

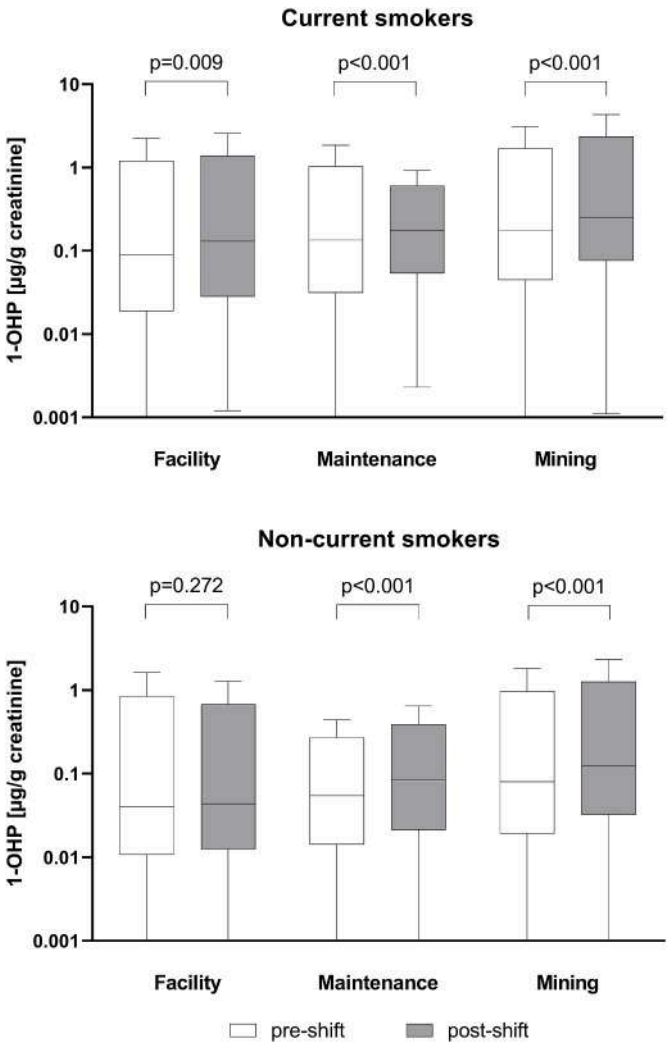

Fig. 1. Boxplots representing median, first and third quartiles, minimum and maximum of imputed creatinine-adjusted 1-hydroxypyrene (1-OHP) stratified by smoking status and exposure group. Location differences between pre-shift (white boxes) and post-shift (gray boxes) concentrations were determined with signed rank tests.

Occupational data for 674 out of the 683 participants of one study site were available and a total of 41 different occupations were reported by those workers. At the facility, one in three participants reported working as a plant operator. The most common occupations among underground workers were large equipment operators (34%), locksmiths (16%), electricians (8%), foremen (7%), and hewers (especially exploration (n = 29) and blasting hewers (n = 8), 7%). Hewers are miners operating cutting and drilling machines underground to loosen minerals (salt and potash) at the road front of the mine. Operators of large diesel engine powered wheel loaders transport the loosened raw material to the next tipping point, where the salt rocks are crushed and carried on conveyor belts to the shaft. Both also operate machines that drill blast holes in the rock, which are then filled with explosives by blasting hewers. Hewers and large equipment operators are higher exposed to DEE compared to other miners since it is difficult to provide a high ventilation rate at the underground road front of the mine (dead end). Large equipment operators and blasting hewers were most likely to be above the OEL (82% and 88%, respectively) as well as above the BAR level for 1-OHP exposure after shifts (16% and 25%, respectively). A total of five participants (all current smokers) were above the BEI® for 1-OHP after the shift. These were four miners (two large equipment operators, one exploration hewer, one miner with no occupational information) and one facility worker without further occupational information.

Spearman correlations of internal (1-OHP) and external (EC-DPM) exposure demonstrated lower correlations of EC-DPM with pre-shift 1-OHP concentrations (creatinine-adjusted 1-OHP: r_S 0.17, 95% CI 0.10–0.23) than with post-shift 1-OHP concentrations (r_S 0.30, 95% CI 0.24–0.36; Table S2). Among mining workers, correlations of EC-DPM and post-shift 1-OHP differed only slightly between current smokers and non-current smokers. However, among maintenance workers, current smokers had slightly higher correlations than their non-current smoking colleagues, indicating a possible effect of smoking in the less exposed underground group compared to mining. As about 80% of EC-DPM were below LOD in the facility, only data of mining and maintenance are presented (Table S2). Nonetheless, there was an association between exceedance of OEL and post-shift BAR in current smokers in the facility and non-current smokers in mining (Table S3).

Positive associations between post-shift creatinine-adjusted 1-OHP and external exposure assessed by either exposure group or EC-DPM were observed after adjusting for pre-shift creatinine-adjusted 1-OHP and persisted when additionally controlling for age, BMI, and smoking status in the statistical models. Compared with facility workers, post-shift creatinine-adjusted 1-OHP was increased by almost 80% in mining workers and 40% in maintenance workers (Table 3). Similarly, there was a statistically significant increase in post-shift creatinine-adjusted 1-OHP with increasing EC-DPM among underground workers (Table 4). Pre-shift creatinine-adjusted 1-OHP concentrations and smoking status had a similarly strong effect on post-shift concentrations. Current smokers had about 50% increased post-shift creatinine-adjusted 1-OHP

Table 3
Linear regression modelling of post-shift creatinine-adjusted 1-hydroxypyrene (1-OHP) concentrations as a function of exposure group.

	Adjustment set 1				Adjustment set 2			
	exp(est)	95%CI		p	exp(est)	95%CI		p
Intercept	0.27	0.22	0.34		0.23	0.14	0.36	
Pre-shift 1-OHP [ln µg/g creatinine]	1.68	1.57	1.80	<0.001	1.57	1.47	1.69	<0.001
Maintenance (ref: Facility)	1.37	1.15	1.65	0.001	1.38	1.16	1.65	<0.001
Mining (ref: Facility)	1.76	1.52	2.03	<0.001	1.76	1.52	2.03	<0.001
Age [per 10 years]					0.99	0.95	1.05	0.843
Body mass index [kg/m^2]					1.00	0.98	1.01	0.533
Current smokers (ref: non-current smokers)					1.58	1.41	1.78	<0.001
R^2	**0.38**	**0.32**	**0.43**		**0.41**	**0.36**	**0.46**	

CI confidence interval.

Table 4
Linear regression models of the association between post-shift creatinine-adjusted 1-hydroxypyrene (1-OHP) and elemental carbon in underground workers.

	Adjustment set 1				Adjustment set 2			
	exp(est)	95%CI		p	exp(est)	95%CI		p
Intercept	0.97	0.73	1.30		0.76	0.45	1.27	
Pre-shift 1-OHP [ln µg/g creatinine]	1.64	1.52	1.77	<0.001	1.54	1.42	1.67	<0.001
EC-DPM [ln mg/m^3 imputed]	1.31	1.20	1.42	<0.001	1.31	1.20	1.42	<0.001
Age [per 10 years]					1.02	0.96	1.07	0.565
Body mass index [kg/m^2]					1.00	0.98	1.01	0.568
Current smokers (ref: non-current smokers)					1.53	1.35	1.74	<0.001
R^2	**0.35**	**0.28**	**0.41**		**0.38**	**0.32**	**0.45**	

CI confidence interval; *EC-DPM* Diesel particulate matter measured as elemental carbon.

concentrations compared with non-current smokers. Linear mixed regression modelling of creatinine-adjusted 1-OHP concentrations at both time points, accounting for the dual measurement per subject, yielded similar risk estimates (maintenance exp(est) = 1.40, 95% CI 1.16–1.70; mining 2.10, 1.81–2.43; EC-DPM 1.37, 1.24–1.50). However, performance was slightly poorer when compared to the presented linear models in Table 3 (R^2 0.12 and 0.41, respectively) and Table 4 (R^2 0.15 and 0.38, respectively).

Due to different EC-DPM exposures at the two study sites (site A mining: median = 0.07 mg/m^3, range < LOD-0.32 mg/m^3; site B mining: 0.04 mg/m^3, <LOD-0.34), we also stratified our analyses. As expected, the linear regression models showed a stronger influence of current smoking on post-shift creatinine-adjusted 1-OHP at the site with lower EC-DPM exposure compared to the site with higher EC-DPM exposure (Table S4).

4. Discussion

The present study aimed to investigate exposure to PAH in terms of 1-OHP in urine and its associations with EC-DPM in workplace air among 801 miners, 202 underground maintenance workers, and 243 workers from above-ground facilities of a German salt and potash mining company. The concentration of 1-OHP increased statistically significantly across an 8-h shift in underground workers but not in surface workers. Although the current German OEL for EC-DPM of 0.05 mg/m^3 was exceeded by almost half of the underground workers, the 1-OHP concentrations were predominantly within the range of the general population that is not occupationally exposed to PAH. This is consistent with the general observation that the workers studied had no clinically relevant indicators of acute cardiovascular, inflammatory, immunologic, or respiratory effects assessed with biomarkers before and after the shift. Most biomarker measurements were within their respective reference ranges, and only a few (thrombocytes, neutrophils, myeloperoxidase, tumor necrosis factor α, immunoglobulin E, fractional exhaled nitric oxide) showed statistically significant post-versus pre-shift differences. However, these differences were independent of exposure group (Gamrad-Streubel et al., 2022).

Consistent with an earlier study (Pronk et al., 2009), miners were exposed to higher concentrations of EC-DPM than underground maintenance workers. The exposure was related to the different amounts of diesel exhaust and the different ventilation conditions in the different areas of the mines. The workplace and specific activity also had an impact on personal exposure. As previously shown in another study at a potash mine in the US (Stanevich et al., 1997), we also observed the most frequent OEL exceedances among large equipment operators with a median EC-DPM exposure of 0.087 mg/m^3. However, this exposure is far less than the average personal exposure to total EC of 0.453 mg/m^3 among ramcar operators that have been observed in a US potash mine and mill (Stanevich et al., 1997). It should be noted that, according to the authors, EC was generated almost exclusively by diesel fuel combustion.

In general, the median EC-DPM exposure of underground workers in our study was quite low (0.048 mg/m^3). A recent study of underground gold miners from Western Australia reported a similar median exposure of 0.056 mg/m^3 (Du et al., 2020). However, earlier studies reported mean exposures in underground mining up to ten times higher (Pronk et al., 2009). The current workplace exposure was up to 53% lower than the exposure observed in the 1990s and early 2000s at the same salt and potash mine. (Dahmann et al., 2007; Lotz et al., 2008). The decrease in exposure within the past 20–30 years could be attributed to increasing efforts to improve diesel engine technology and to switch from standard diesel fuel to renewable fuel in order to reduce DEE in the mining industry. Furthermore, good ventilation is important for reducing underground exposure. However, in the mines studied here, it has been shown that an increase in the ventilation rate is not feasible due to limited cross-sections and, correspondingly, low flow speeds in the shafts (Dahmann et al., 2007).

1-OHP is the most commonly used biomarker in Europe to assess occupational exposure to PAHs (Louro et al., 2022) and has also previously been used as a biomarker to determine exposure to DEE in miners (Du et al., 2019; Scheepers et al., 2002; Seidel et al., 2002). Median results for creatinine-adjusted 1-OHP of facility workers in the present study (pre and post-shift 0.05 µg/g creatinine) were even slightly lower than the median level (0.10 µg/g creatinine) observed in urine samples of the general German population (Becker et al., 2002). Furthermore, the examined non-current smokers from the reference group exceeded

the evaluation standards in less than 2% before and after the shift (Table S1). Similarly, less than 2% of maintenance workers exceeded the BAR levels and thus were also well within the range of the general population. In contrast, post-shift creatinine-adjusted 1-OHP was above the evaluation standard in 46 non-smokers from the mine (9.4%) thus indicating very low exposures to PAH in miners. The median value of all miners was 0.13 μg/g creatinine and therefore comparable to the results of a recent study among Australian gold miners (0.16 μg/g creatinine) (Du et al., 2019). In contrast, much higher concentrations of urinary 1-OHP were found among workers in other industries (Hansen et al., 2008; Louro et al., 2022). For example, studies conducted in Germany (Gündel et al., 2000; Marczynski et al., 2009; Pesch et al., 2011; Strunk et al., 2002) among mastic asphalt workers (0.44 μg/g creatinine), coke oven workers (3.6–19.7 μg/g creatinine depending on the specific workplace), refractory workers (8.4 μg/g creatinine), graphite electrode workers (9.7 μg/g creatinine), converter workers (13.5 μg/g creatinine), and workers producing refractory materials (11.1 μg/g creatinine) were substantially higher. Hence, the concentration of PAH in DEE cannot be considered high.

Urinary 1-OHP is not a specific indicator of internal dose of diesel exhaust as PAH are not only found in DEE but also in tobacco smoke or emissions from other incomplete combustion of organic sources. Thus, internal exposure to PAH measured by 1-OHP is influenced by individual behavioural patterns such as cooking, diet, or smoking in addition to occupational exposure (Hansen et al., 2008). This study also shows statistically significantly higher 1-OHP concentrations for smokers compared to non-smokers, analogous to a previous study (Du et al., 2019). In addition, exceedances of the evaluation standards for creatinine-adjusted 1-OHP were predominantly seen in mining. Not surprisingly, non-current smokers were most affected based on the fact that an important life-style and confounding factor (i.e., smoking) is actually missing in non-smoking workers thus work-related exposures are far easier to track on non-smoking rather than smoking workers.

Due to the presence of nitrogen oxides in diesel and their interaction with PAH, nitrated PAH compounds (nitro-PAH) are also formed during combustion in diesel engines (IARC, 2014). It has been suggested that metabolites of nitro-PAH are more suitable for exposure assessment of DEE, with 1-nitropyrene (1-NP) being a major nitro-PAH and a major constituent of DEE that is partially metabolized to 1-aminopyrene (1-AP) and excreted in urine (Bamford et al., 2003; Riley et al., 2018; Toriba et al., 2007). Some studies among workers exposed to DEE showed statistically significant correlations between 1-NP and urinary 1-AP (Ochirpurev et al., 2022; Wadikar et al., 2021) Furthermore, a study among Australian gold miners also concluded that 1-AP is a more robust and specific biomarker of DEE compared to 1-OHP (Du et al., 2019). However, although 1-AP seems promising for assessing exposures to DEE in future, 1-AP measurements in urine are much less established yet. Most importantly, compared to 1-OHP, simple and robust analytical methods including international round robin programs to guarantee quality-controlled results are missing for 1-AP.

The strengths of the present study are its sample size of more than 1000 underground miners and a high participation rate of 60% among underground workers. In addition, personal exposure to DEE was measured with personal monitors instead of stationary exposure measurements thus allowing a more reliable exposure assessment. However, there are also a few limitations of this study. First, the single-day cross-sectional study design may have caused exposure misclassification if the exposures on the study day were not representative of usual exposure concentrations. Second, due to the study design, the measured 1-OHP concentrations can also not be fully compared with those from other studies where exposure was assessed after the last shift of a working week, as recommended for the BEI®. Third, the interpretation of exposure by job title should take into account the low numbers in some cases, especially among blasting hewers (n = 8).

5. Conclusions

In the two investigated study sites of a salt and potash mining company, the current German OEL for EC-DPM was exceeded, particularly in mining. However, the urine concentrations of 1-OHP do not reflect the expected internal DEE exposure of workers based on the results of the EC-DPM air sampling. Although 1-OHP concentrations increased during a work shift among underground workers, suggesting that this biomarker reflects short-term DEE exposure, the concentrations still remained in the range of the general German population. If at all, individual exceedances occurred predominantly in mining, with non-current smokers being particularly affected. Except for blasting hewers and large equipment operators, workers in salt and potash mining in Germany are unlikely to experience elevated urinary 1-OHP concentrations at current workplace exposure levels of DEE compared to the general population according to our results.

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Declaration of competing interest

Swaantje Casjens, Savo Neumann, Dirk Pallapies, Jürgen Bünger, Heiko U. Käfferlein, Thomas Behrens, Thomas Brüning, and Dirk Taeger, as staff of the Institute for Prevention and Occupational Medicine (IPA), were employed until the end of 2021 at the German Social Accident Insurance Institution for the Raw Materials and Chemical Industry (BG RCI), a public body. IPA is an independent research institute of the Ruhr University Bochum. Jörg Giesen and Volker Neumann, as staff of the Institute for the Research on Hazardous Substances (IGF), Bochum, Germany, are also employed at BG RCI. IGF is an independent research institute. Katrin Rühle, Lisa Gamrad-Streubel, Lisa-Marie Haase, Katharina K. Rudolph, and Thomas Birk are staff of Ramboll, a consultancy commissioned by the BG RCI to conduct the study. All authors are independent of the sponsors in all aspects of this research including study design, collection, analysis and interpretation of data, and the right to publish. The views expressed in this paper are those of the authors and not necessarily those of the sponsors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ijheh.2023.114190.

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Investigation of polychlorinated biphenyls in breast milk from two regions in Bulgaria

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ABSTRACT

Human breast milk is an optimally balanced infant food and a suitable tool for assessing the burden of humans with lipophilic persistent organic pollutants. The aim of this study was to investigate the accumulation profile of polychlorinated biphenyls in breast milk of women living in Bulgaria and to assess the health risk to infants.

Breast milk samples were obtained from 72 healthy primiparae and multiparae mothers, living in two regions in northeastern Bulgaria – Varna region and Dobrich region, in the period October 2019–July 2021. Important information for the study, such as age, body mass, smoking and dietary habits, was collected through a questionnaire. Fifteen congeners of PCBs, including six indicator congeners, were determined by capillary gas chromatography system with mass spectrometry detection.

The lipid content of the tested samples was in the range from 0.5% to 6.7%, with average value 3.25%. The six indicator PCBs in human milk samples formed up to 89% of the total PCBs levels. The most abundant congener was PCB 153, followed by PCB 138 and PCB 180. Five of the 15 PCB congeners (77, 126, 128, 156, 169) were not detected in any of the milk samples. The arithmetic mean PCB levels in milk samples from Varna (32.7 ng/g lw) were found higher than PCB levels in breast milk of mothers from Dobrich (22.5 ng/g lw). The highest PCB levels were found in milk samples from primiparae mothers in 36–40 age group (for both regions). Infant exposure to PCBs present in human milk was estimated using toxic equivalents (TEQ). The health risk to infants was assessed and was compared to the tolerable daily intake (TDI).

Positive correlation was found between the arithmetic mean PCBs levels and two important factors – the age and body mass index of the primiparae group. The mean values of the analyzed PCB congeners in breast milk samples from multiparae were lower than in those from primiparae mothers. The regional differences in PCB concentrations were small, suggesting similar exposures in the studied regions. The levels of PCBs in breast milk were found lower than levels from studies in other European countries. Statistical data does not show any association between PCB levels in milk and dietary habits. The results showed that infants are not at risk of any adverse effects caused by PCBs through breast milk.

1. Introduction

Polychlorinated biphenyls (PCBs) are a class of industrial chemicals that were mass-produced globally and in several Europe countries (Germany, France, Italy, Czech Republic) from the late 1920s to 1985, even after their ban in the end of 1970s (Cerná et al., 2012; Grimm et al., 2015; Komprda et al., 2019).

PCBs are lipophilic, organic compounds from the persistent organic pollutants (POPs) group with the high potential for bioaccumulation and long-distance transfer (WHO Europe, 2003; Alharbi et al., 2018). Due to their environmental and biological persistence, low levels of PCBs are still found in wildlife and humans (Ashraf, M., 2017; Gyllenhammar et al., 2021). Pollutants tend to reach raised concentrations in organisms from higher trophic levels, including humans, due to biomagnification through aquatic and terrestrial food chains (Stancheva et al., 2017; Metcalfe et al., 2022). More than 90% of the total daily human exposure to PCBs is made up of intake from fat-rich food of animal origin (Massart et al., 2008; Sun et al., 2022).

PCBs have low acute toxicity but may pose a health risk in case of chronic human exposure (ATSDR, 2000; ATSDR, 2011). Experimental data indicate that exposure to low levels of PCBs may be associated with chronic non-lethal effects such as endocrine disruption, immune

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dysfunction, neurological disorders, liver injury, diabetes, cardiovascular problems and carcinogenicity (World Health Organization, 2009; Fernández-Cruz et al., 2017; Guo et al., 2019).

Human biomonitoring is a suitable tool for assessing human exposure to PCBs (Zietz et al., 2008). Biomonitoring data directly reflect the total body burden taking into account all routes of exposure, as well as the interindividual variability in exposure levels, metabolism and excretion rates (WHO, 2015). Biomonitoring involves measurements of biomarkers in biological fluids, such as blood, urine, saliva, breast milk and sweat. Human breast milk analysis is a non-invasive method for assessing the actual exposure of the mothers and has an advantage over any other type of human sample due to its high lipid content (Angerer et al., 2007). However, the determination of PCBs in breast milk has its limitations. Samples can be obtained only during lactation and only from lactating women, which excludes other groups of the population. (Brajenović et al., 2018)

The six indicator PCBs (IUPAC № 28, 52, 101, 138, 153 and 180) have been selected by the European Food Safety Authority (EFSA) as the major congeners which are present in various food matrices in high concentrations (EFSA, 2005). Low chlorinated congeners, such as PCB 28, 52 and 101 can metabolize by the human body faster than highly chlorinated PCBs due their half-life of almost 5 years (El-Shahawi et al., 2010; Helou et al., 2019). Highly chlorinated PCB congeners, containing more than five chlorine atoms (PCB 138, 153, 170, 180), tend to bioaccumulate in adipose tissue, human serum lipids and breast milk (Grimm et al., 2015) and can persist for 10–47 years (ATSDR, 2000).

Since 1987, the World Health organization (WHO) and the United Nations Environment Programme (UNEP) have conducted seven global surveys of POPs in human milk (van den Berg et al., 2017). Bulgaria was included in the 3rd Round of WHO coordinated Exposure Study on the POP levels in breast milk. In the third survey (2000–2003) pooled milk samples from three groups of Bulgarian mothers were analyzed for PCBs and organochlorine pesticides. The levels of indicator PCBs measured in milk samples from Bulgaria (2003), reported by van den Berg et al. (2017), were among the lowest in the European countries. There are limited data on PCB levels in breast milk of mothers from Bulgaria in the last twenty years.

The aim of the study was to examine the accumulation profile of polychlorinated biphenyls in breast milk of women, living in two regions in north-eastern Bulgaria, and to assess its relation to individual characteristics.

2. Material and methods

2.1. Study design

The study participants were selected from two regions: Varna (a port city with developed industry) and Dobrich (an agricultural region). Inclusion criteria for the participant mothers: 1) age 25–40 years, 2) breastfeeding period 30–40 days after childbirth; 3) resident in Varna or Dobrich for ≥10 years; 4) informed consent signed. The study protocol was based on the requirements of the WHO coordinated surveys on POPs in human milk (WHO, 2007). The recruitment of participants was done in four medical centers with pregnancy schools. Participants received information about the study and instructions for collecting and storing breast milk samples. Data on age, parity, pre-pregnancy weight, height, smoking and dietary habits were obtained from a validated questionnaire. The interview to fill in the questionnaire for collecting personal characteristics was conducted face-to-face by a qualified person.

The study protocol, the informed consent form and the questionnaire were approved by the Commission for Scientific Research Ethics at Medical University – Varna, Bulgaria (protocol № 85/26.07.2019).

2.2. Sampling and sample preparation

The present study was based on the voluntary participation of donors. Human milk samples were obtained from healthy mothers living in two regions in northeastern Bulgaria – Varna region and Dobrich region, in the period October 2019–July 2021. The recruitment period was extended due to Covid restrictions. In the Varna and Dobrich regions, 47 and 25 individual milk samples were collected, respectively. Participating mothers collected milk samples at home manually or using a manual breast milk pump. Milk samples (≥50 ml) were sampled in sterile containers after and/or during nursing. After sampling, the containers were stored in the refrigerator (+4 °C) for maximum 72 h and then in the laboratory at −20 °C until analysis.

2.3. Chemical analysis

Preparation of milk samples was performed by applying analytical protocol based on EN ISO 1528–1996 the European standardized methods with some modifications. The milk samples were defrosted, then slowly warmed up to 36–37 °C and carefully homogenized. Ten grams of each individual milk sample were weighted in a glass centrifuge tube (50 mL) and the sample was spiked with internal standards (PCB 30 and PCB 204, Dr. Ehrenstorfer Laboratory). Three-step extraction with organic solvents was applied to extract lipids and polychlorinated biphenyls from breast milk. The mix of solvents applied includes hexane/acetone in ratio 1:0 v/v (5 mL), 2:1 v/v (9 mL), 1:1 v/v (8 mL), respectively. After every extraction step, the sample was vortexed for 3 min, then centrifuged by 2500 rpm for 10 min. The hexane layers were collected, and evaporated to near dryness in a rotary vacuum evaporator. The lipid content of the milk samples was determined graphimetrically. The lipid extract was cleaned-up on a multilayer glass column filled with anhydrous sodium sulfate, 2 g of neutral silica (60–230 mesh), 2 g acid silica and 1 g neutral silica from bottom to top. The elution of PCB congeners were performed with 10 mL n-hexane and 20 mL mix n-hexane/dichloromethane in the ratio 9:1 (v/v). The collected eluates were concentrated by a rotary vacuum evaporator to near dryness and resolved in 0.5 mL hexane.

The instrumental determination of the individual PCB congeners was carried out by gas chromatograph GC FOCUS using POLARIS Q Ion Trap mass spectrometer (Thermo Electron Corporation, USA) and equipped with an autosampler AI 3000. The experimental parameters of mass spectrometer were the following: Ion source and Transfer line temperatures 220 °C and 250 °C, respectively; splitless Injector temperature 250 °C; experimental temperature program – 60 °C for 1 min, then 30 °C/min to 180 °C, 2 °C/min to 260 °C, 30 °C/min to 290 °C with a final hold for 2.0 min. Splitless injections of 1 μl were performed using a TR-5ms capillary column coated with cross-linked 5% phenyl methyl siloxane with a length of 30 m, 0.25 mm ID and a film thickness of 0.25 μm. The flow rate of helium as carrier gas was 1 ml/min.

All solvents (acetone, dichloromethane, hexane), reagents and chromatography silica gel (200–300 mesh) used for sample preparation and analysis were HPLC grade from Sigma-Aldrich (St. Louis, MO, USA), USA. Ultra-pure water came from a Milli-Q, IQ Water Purification System. Pure reference standard solution of 15 PCBs (PCB Mix 20 - Dr. Ehrenstorfer Laboratory, Augsburg, Germany) was used for instrument calibration, recovery and quantification of compounds.

Fifteen PCB congeners (IUPAC № 28 + 31, 52, 77, 101, 105, 118, 126, 128, 138, 153, 156, 169, 170 and 180) were measured in the purified extracts from milk samples.

The concentrations of the individual "dioxin-like" PCBs (dl-PCB, UPAC No 77, 105, 118, 156, 126, 169) in milk samples are multiplied by their respective toxic equivalency factors (TEF) according to WHO (2005) and subsequently summed to give total concentration of dioxin-like compounds expressed in TEQs (Van den Berg et al. 2006).

2.4. Quality control

The quality control was performed by analysis of certified reference material: BCR450 (PCBs in milk) – Institute for Reference Materials and

Measurements, European commission. The recovery of PCB congeners in the reference material was in the range of 82.6–98.6% (See Supplementary material). Each series of samples included a procedure blank (Milli-Q water). The limits of detection (LOD) varied for individual PCB congeners from 0.2 to 0.7 ng/g lipid weight (lw).

The limit of detection (LOD) were estimated based on the low concentration of the analytes in matrix samples as 3 times the standard deviation and LOQ is the analyte concentration corresponding to ten times standard deviation. LOD for individual PCBs: 0.2 (PCB 28, 77, 118, 153, 105, 138, 126, 128, 180, 169, 170), 0.4 (PCB 52, 101), 0.7 (PCB 156) ng/g lipid weight. Limit of quantification (LOQ) were calculated from 0.66 to 2.3 ng/g.

2.4.1. Statistical methods

Levene's homogeneity test and the Kolmogorov–Smirnov normality test of the data were applied for analysis of variance. The results showed a normal distribution of the data. The statistical differences between mean values of the data was evaluated by a Student t-test and a significance level of $p < 0.05$ was used. All statistical calculations were made by SPSS V19.0 software package for Windows (SPSS Inc., Chicago, IL, USA). The values under LOD were given as LOD/2 in the calculation of mean concentrations.

3. Results and discussion

3.1. Characteristics and dietary habits of the participants

A total of 72 individual human breast milk samples were collected from two regions in northeastern Bulgaria (47 from Varna region and 25 from Dobrich region). The personal characteristics, residence and dietary habits of women are presented in Table 1. The participants were between 25 and 40 years old, with an average age of 31.7 years (32.5 years for the women from Varna; 30.3 years for the women from Dobrich). These values are higher than those in the representative data for both regions in Bulgaria (Bulgarian National Statistical Institute database, 2021). Authors of similar studies in other European countries (Aerts et al., 2019; Polder et al., 2009; Zietz et al., 2008) reported lower mean mothers' age.

Table 1
Personal characteristics of the participants in the study.

Subject	Characteristics	% of all participants	Varna region	Dobrich region
			n (mean)	n (mean)
Age (years)	25–30	45.8	17 (28.2)	16 (27.1)
	31–35	30.6	18 (33.3)	4 (33.5)
	36–40	23.6	12 (37.4)	5 (38.2)
BMI, kg/m^2	<18.5 (underweight)	15.2	6 (17.3)	5 (18.0)
	18.5–24.9 (normal)	69.4	34 (21.1)	16 (20.8)
	25.0–30.0 (overweight)	9.8	4 (26.5)	3 (25.9)
	>30.0 (obese)	5.6	3 (33.2)	1 (31.1)
			n (%)	**n (%)**
Parity	Primiparae	59.7	25 (53.2)	18 (72.0)
	Multiparae	40.3	22 (46.8)	7 (28.0)
Diet: Fish	None	8.4	2 (4.3)	4 (16.0)
	1 x/month	18.0	8 (17.0)	5 (20.0)
	2 x/month	18.0	10 (21.3)	3 (12.0)
	1 x/week	48.6	24 (51.0)	11 (44.0)
	2 x/week	7.0	3 (6.4)	2 (8.0)
Meat	None	–	–	–
	1 x/week	7.0	1 (2.1)	4 (16.0)
	2 - 4 x/week	59.7	31 (66.0)	12 (48.0)
	>5 x/week	33.3	15 (31.9)	9 (36.0)
Smoking	Yes	29.2	11 (23.4)	10 (40.0)
	No	70.8	36 (76.6)	15 (60.0)

n (%) – number (and %) of participants in the category from the study region.

The results showed a mean pre-pregnancy body mass index (BMI) of the mothers 21.7 kg/m^2 with insignificant difference between the two study regions (21.9 kg/m^2 for Varna and 21.3 kg/m^2 for the women from Dobrich). The mean values of BMI of Bulgarian mothers were lower than the BMI of participants in other European studies on breast milk (Aerts et al., 2019; Polder et al., 2009; Zietz et al., 2008). Almost 70% from all donors were with normal BMI (ranging between 18.6 and 24.4). The underweight women were 15% (mean BMI 17.6 kg/m^2) and 15% were overweight or obese (mean BMI 28.6 kg/m^2).

In our study were included 43 first-time mothers (primiparae) - 60%, and 29 multiparae mothers (second or third child delivery). Mean age of primiparae mothers was 30 years (range 25–40 years) and their body mass index (BMI) was 21.2 kg/m^2. Multiparae mothers' age and BMI were 34 years and 22.4 kg/m^2 respectively. Non-smokers were 77% of all donors from Varna and 60% of all donors from Dobrich. All participants in the present study had a mixed type of diet including fish and meat. Only 8% stated that they do not eat fish and fish products. A small group of mothers (7%) consumed fish according to the recommendations (2 x/week), and 18% consumed fish once a month. Most of the women (59.7%) consumed meat 2 to 4 times a week.

The mean lipid content of the human milk samples was 3.2% (ranging from 0.5 to 6.7%) and no regional differences were observed.

3.2. PCBs levels in human milk samples

The concentrations of fifteen polychlorinated biphenyls (reported as Sum PCBs), including six indicator congeners (I-PCBs) were measured in 72 individual milk samples (Table 2).

The di-ortho congener PCB 153 showed the highest mean concentrations, followed by PCB 138, 180 and 118. A similar distribution pattern of PCB congeners in human milk was reported by several authors in Norway, Slovakia, the Netherlands, Sweden and Croatia (Polder et al., 2009; Čechová et al., 2017; Glynn et al., 2011; Gyllenhammar et al., 2021; Klinčić et al., 2016). The congener PCB 153 was present in all milk samples in both study regions. PCB 138 was found in 97% of the breast milk samples. The most predominant congeners (PCB 153, 138, 118, 180) formed 90% (Dobrich region) and 97% (Varna region) of the total PCB levels in the studied human milk samples.

The mean concentrations of PCB 153, 138, 180 and 118 in the mothers' milk from Varna were found higher than in the milk samples from Dobrich. Five of the analyzed PCB congeners (77, 126, 128, 156 and 169) were detected in concentrations below the limit of detection (LOD) in all breast milk samples from Varna region. In the milk samples from Dobrich region, PCB congeners 28, 52, 77, 105, 126, 128, 156 and 169 were detected below LOD.

The mean PCB levels in milk samples from Varna (32.7 ng/g lw) were found higher than PCB levels in breast milk of mothers from Dobrich (22.5 ng/g lw) and were formed mainly by the sum of the six indicator PCB congeners (28.6 ng/g lw for Varna and 19.7 ng/g lw for Dobrich, respectively) - Fig. 1. These results can be explained by greater potential exposure to PCBs of mothers who live in Varna (region with high industrial and port activities), compared to mothers from Dobrich (rural area). The percentage of detected dl-PCBs in milk samples from Varna and Dobrich was 11.3% (PCB 105, 118) and 8.4% (PCB 118), respectively.

Our results showed that the exposure of mothers is lower than the WHO survey data in the region of Sofia, 2003, demonstrated (sum of the six I-PCBs – 42 ng/g milk fat) (van den Berg et al., 2017).

The mean levels of PCBs found in breast milk samples in our study were in the same range or lower than those found in human milk in other European countries (Čechová et al., 2017; Glynn et al., 2011; Gyllenhammar et al., 2021; Klinčić et al., 2016; Polder et al., 2009; Zietz et al., 2008). Čechová et al., 2017 reported the highest levels of the six indicator PCBs in breast milk from Slovakia (144 ng/g milk fat), followed by Norway (62.02 ng/g milk fat) and the Netherlands (39.09 ng/g milk fat) – Table 3. The sum of the three predominant congeners (PCB 138, 153

Table 2
Polychlorinated biphenyls concentrations (ng/g lipid weight) in the human milk samples collected from Varna and Dobrich regions, Bulgaria.

Compound	Varna region (N = 47)					Dobrich region (N = 25)				
	% of positive samples		ng/g lipid weight			% of positive samples		ng/g lipid weight		
	>LOD	≥LOQ	Mean[a]	95th percentile	Range[b]	>LOD	≥LOQ	Mean[a]	95th percentile	Range[b]
PCB 28* + 31	19	17	0.43	2.16	0.62–4.93	0	0	< LOD	–	–
PCB 52*	4	2	0.22	0.20	0.49–1.00	0	0	< LOD	–	–
PCB 101*	12	10	0.34	1.51	0.66–2.00	40	32	1.87	5.39	2.33–12.79
PCB 77	0	0	< LOD	–	–	0	0	< LOD	–	–
PCB 118	89	89	3.56	7.62	0.69–12.60	64	60	1.87	6.79	1.20–7.13
PCB 153*	100	100	16.41	35.03	3.17–38.98	100	100	10.37	32.38	1.32–34.37
PCB 105	6	4	0.15	0.19	0.31–1.47	0	0	< LOD	–	–
PCB 138*	98	98	7.16	16.48	0.87–20.94	96	96	4.28	9.29	0.54–11.05
PCB 126	0	0	< LOD	–	–	0	0	< LOD	–	–
PCB 128	0	0	< LOD	–	–	0	0	< LOD	–	–
PCB 156	0	0	< LOD	–	–	0	0	< LOD	–	–
PCB 180*	90	90	4.07	8.46	0.77–16.39	80	80	3.17	10.77	1.39–12.36
PCB 169	0	0	< LOD	–	–	0	0	< LOD	–	–
PCB 170	14	10	0.37	2.19	0.21–3.73	20	16	0.53	2.82	1.08–3.91
Sum PCBs			32.74	66.22	4.07–72.99			22.49	59.71	2.76–64.42
WHO-TEQ pg/g lipid weight			1.47	3.10	0.22–5.96			1.02	2.92	0.15–3.34

*Indicator PCB.
[a] Arithmetic mean of values under LOD were given LOD/2 in the calculation of mean concentrations.
[b] Concentration ranges (Min – Max) in positive samples.

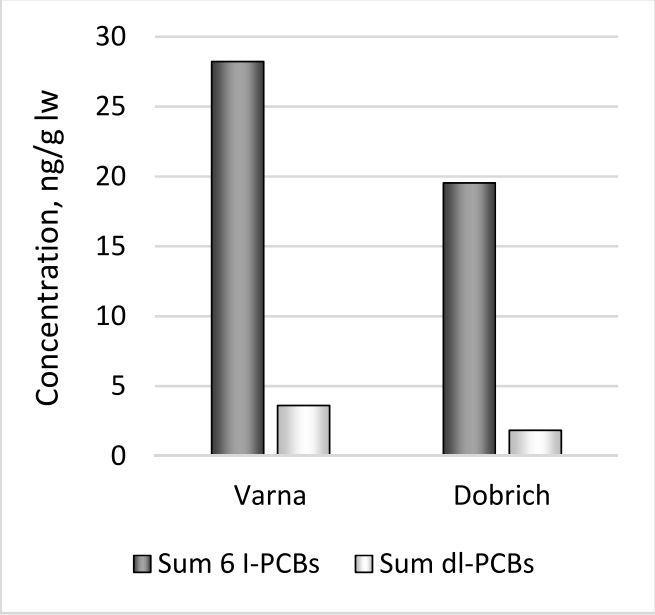

Fig. 1. Sum six Indicator PCBs (I-PCBs) and sum dl-PCBs (ng/g lipid weight) in breast milk from Varna and Dobrich regions.

and 180) multiplied with 1.64 (total PCB) showed a median of 19.2 ng/g lw, which is significantly lower than the data for breast milk samples from two German federal states show – 50.1 ng/g lw (Fromme et al., 2022).

3.3. Impact of maternal age, parity and BMI

There are several individual characteristics that affect PCB levels, such as maternal age, parity, dietary habits, etc. The relation between PCBs and the maternal age of both primiparae and multiparae mothers is given in Fig. 2. Our results showed a rise in PCB concentrations in breast milk with the increase of primiparae mothers' age in both regions studied.

The mothers' age as a determinant of body burden with PCBs were reported in several studies on PCB levels in milk samples from Belgium, Norway, Germany, Tunisia and Poland (Aerts et al., 2019; Polder et al., 2009; Zietz et al., 2008; Ennaceur et al., 2008; Grešner et al., 2021).

The positive correlation between PCB levels in breast milk and the maternal age is clearly expressed in the primiparae group. The mean sum of PCBs in breast milk from primiparae mothers (34.9 ng/g lw) was found higher than that in samples from multiparae mothers (29.1 ng/g lw) for Varna region. The total PCBs in Dobrich milk samples were 24.2 and 15.8 ng/g lw from primiparae and multiparae mothers, respectively. Glynn et al. (2011) showed a positive correlation between PCB levels and mothers age with both younger and older Swedish women and pointed age as the most important determinant of body burdens with PCBs.

Table 3
Comparison of median concentrations (ng/g lipid weight) of PCBs and TEQ (pg/g lipid weight) in human milk from European countries.

Country	Period	N	Sum I-PCBs	WHO TEQ_{2005} dl-PCB	Ref.
Bulgaria	2019–2021	72	18.82	1.09	Present study
Ireland	2016–2018	92[a]	19.25	1.4	Houlihan et al. (2021)
Spain	2016–2019	60	66[b]	0.2	Rovira et al., 2022
Czech	2019–2021	231	80.69	–	Parizek et al. (2023)
Germany	2016–2018	100	51.90	2.45	Fromme et al. (2022)
Slovakia	2010–2012	37	144.10	–	Čechová et al. (2017)
The Netherlands	2011–2014	120	39.08	–	Čechová et al. (2017)
France	2011–2014	96	85.19	4.3	Antignac et al., 2016
Belgium	2009–2010	84	39.7	1.71	Croes et al. (2012)
Norway	2001–2006	388	62.07	–	Čechová et al. (2017)

[a] - Analyzed as 16 pool samples.
[b] - Sum PCB138, 153 and 180.

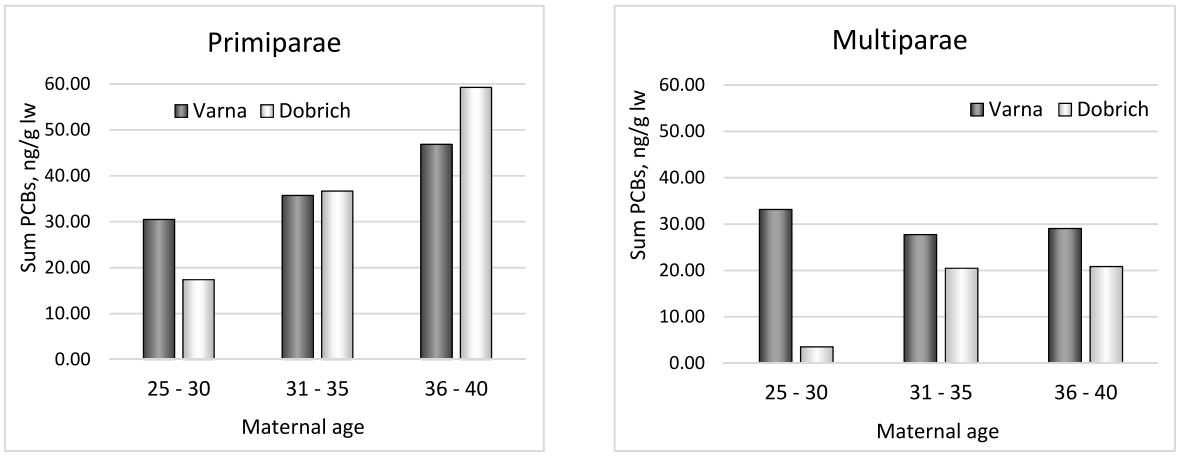

Fig. 2. Total PCB levels in breast milk by parity and age group of participants from Varna and Dobrich regions.

The highest PCB levels were found in milk samples from primiparae mothers in the 36–40 age group (for both regions). The data obtained may be due to the longer exposure to PCBs of the 36–40 year mothers through their diet (as the main route). Lower PCB concentrations (27 ng/g lw) in multiparae mothers' milk in the same age group might be due to previous breastfeeding. Fernández-Cruz et al. (2017) showed breastfeeding as the main route of excretion of lipophilic organic pollutants, so that maternal POPs body burden decreases from 20 to 70% in the course of six months of exclusive breastfeeding.

It is important to note that PCB body load was also associated with the participants' individual BMI. Most mothers (69.4%) had a healthy body mass index (in the range of 18.5–24.9 kg/m^2) before delivery. The results for sum PCBs in milk samples from primiparae mothers increased with the increase of BMI (Fig. 3). The trend was more pronounced in primiparae mothers, while there was no statistically significant correlation in multiparae mothers.

This can be explained by the transfer of human milk lipids, based on a study by Koletzko et al. (2001). The authors found that 70% of the milk lipids in breast milk come from the maternal depots and only 30% from the diet.

3.4. Impact of maternal diet and smoking

The mothers' diet and smoking are among the factors which may affect the residue levels of PCBs in breast milk over the lactation period. All donors in the present study consumed food of animal origin – an important dietary exposure route to persistent pollutants. Seafood consumption has been suggested as a major contributor to human exposure to POPs from a number of authors (Lee et al., 2013; Aerts et al., 2019). In our study group, 93% of the participants consumed fish and seafood much less than the recommendations of the World Health Organization (twice a week) – Table 1. Data from the questionnaires showed that meat was part of the daily food intake of 1/3 of the mothers. About 60% of the mothers consumed meat 2 to 4 times a week. Statistical analysis of the data showed no statistically significant associations between PCB levels in breast milk and the consumption of fish or meat. These results can be explained by the simultaneous influence of different factors. Grešner et al. (2021), found statistically significant relationships between the frequency of fish and dairy consumption and the concentrations of lipophilic pollutants in breast milk.

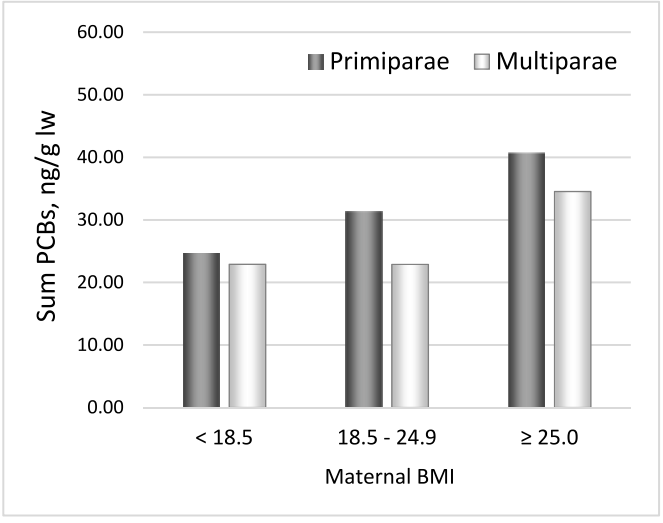

Fig. 3. Total PCB levels in breast milk by BMI group of participants from Varna and Dobrich regions.

The effect of active smoking on persistent pollutant levels in human milk and blood serum has been studied and discussed by different authors (Harris et al., 2001; Glynn et al., 2011; Cerná et al., 2012; Moon et al., 2017). Due to the simultaneous influence of many factors such as age, diet, order of the child, etc. it is difficult to establish a clear relationship between maternal smoking and the content of persistent organic compounds in breast milk. Less than 30% of the participants in the present study defined themselves as smokers before pregnancy – Table 1. Statistical analysis of the data from our study showed that no statistically significant difference was found in the mean PCB levels in the breast milk of smokers and nonsmokers (27.3 and 29.3 ng/g lw, respectively).

3.5. Estimated health risk

Infant exposure to dl-PCBs present in human milk was estimated using toxic equivalents (TEQ), expressed as pg TEQ/g ww (wet weight). TEQ values were calculated by multiplying concentrations of mono-ortho congeners in every sample and toxic equivalency factors (WHO_{2005}-TEF) of dl-PCBs according to Van den Berg et al. (2006). The range of dl-PCB content was in the range 0.0007–0.0179 pg TEQ/g ww (milk samples from Varna) and 0.0004–0.0100 pg TEQ/g ww (milk samples from Dobrich). Commission regulation (EU) No 1259/2011 sets the maximum levels at 0.2 pg TEQ/g ww (Sum of dioxins and dioxin-like PCBs (WHO-PCDD/F-PCB-TEQ)) in infant foods (EC, 2011).

EFSA's CONTAM Panel has set in 2018 a new tolerable weekly intake (TWI) for dioxins and dioxin-like PCBs in food of 2 pg TEQ/kg bw. The main arguments for the reduction of the TWI were the availability of new epidemiological and experimental data on the toxicity of these substances. According recommendation of EFSA the exposure of

breastfed infants should not be compared directly to the TWI (EFSA 2018). The reason is that the new TWI is based on the concentration level in breast milk that would leading to a child serum levels with potentially adverse effects in older children. Instead, it is more appropriate to compare dl-PCBs concentrations to level of 5.9 pg TEQ/g fat, the human milk level likely to result in the NOAEL serum concentration of 7.0 pg WHO_{2005}-TEQ/g fat at 9 years of age (EFSA, 2018; Houlihan et al., 2021).

In Table 2 is present the TEQ values of dl-PCB congeners in the human milk samples from Varna and Dobrich expressed as pg WHO_{2005}-TEQ/g lipid weight in aim to compare the results with European studies. Our results for dl-PCBs in breast milk showed mean levels 1.47 and 1.02 pg WHO_{2005}-TEQ/g lipid weight (for Varna and Dobrich, respectively) and are lower than level of 5.9 pg TEQ/g fat (EFSA 2018). The differences between regions studied are likely due to greater consumption of seafood by the mothers who live in Varna, on the Black Sea coast, compared to mothers from rural region (Dobrich). The percent of participants from Varna, which consume fish and fish products 2 x/month, was more that of mothers from Dobrich. It is well known that seafood is the main contributor to the input of dl-PCBs in humans (Klinčić et al. 2016).

TEQ values for dl-PCB congeners reported in literature are higher (Fromme et al., 2022; Antignac et al., 2016) or comparable (Houlihan et al., 2021; Croes et al., 2012) than those from our study (median values 1.09 pg-TEQ/g lw) – Table 3. The WHO/UNEP global surveys and other international studies of human milk showed a decreasing trend in PCB levels and WHO-TEQs in recent years (van den Berg et al., 2017; Zietz et al., 2008; Čechová et al., 2017; Gyllenhammar et al., 2021). However, even relatively low concentrations of PCBs in human milk, they can cause thyroid hormone disruptions to infants, especially in long-term exposure (Witczak et al., 2022).

Indicator PCBs (PCB 28, 52, 101, 138, 153, 180) are the most frequently analyzed and this group represents about 50% of total non-dioxin like PCB group (ndl-PCBs) measured in food (EFSA, 2018). Commission regulation (EU) No 1259/2011 sets the maximum levels at 1 ng/g ww for sum of I-PCBs in foods for infants. In the current study, the mean values of six I-PCBs in breast milk from Varna and Dobrich were found 0.82 and 0.46 ng/g ww, respectively and did not exceed the maximum levels permitted.

An estimated daily intake (EDI) was calculated to understand the extend of exposure of infants to total PCBs. The calculation was based on assumption that a 5 kg infant ingests 700 g milk per day (Van Oostdam et al., 2005). The EDI (μg/kg body wt./day) was calculated as follows:

$$EDI = \left(c_{milk} \times c_{lipid} \times 700\right) / 5$$

c_{milk} is the mean concentration of total PCBs in the milk samples (μg/kg lw) and c_{lipid} is the lipid content in milk (%).

The guideline standards by Health Canada and WHO proposed tolerable daily intake (TDI) of 1 μg/kg body wt./day (Van Oostdam et al., 2005; Klinčić et al. 2016). Calculated EDI values (0.12 and 0.072 μg/kg body wt./day for Varna and Dobrich, respectively) are one order of magnitude lower than TDI. The results showed that infants are not at risk of any adverse effects caused by PCBs through breast milk. We can conclude that the levels of PCBs in mother's milk are low, and the benefits of breastfeeding far outweigh the possible adverse effects.

4. Conclusions

Positive correlations were found between the mean of the total PCBs levels and two important factors – the age and body mass index of the participants in the primiparae group. The mean values of the analyzed congeners in samples from multiparae were lower than in those from primiparae mothers. The regional differences in PCB concentrations were small, suggesting similar exposures in the studied regions. Women from the more rural area in the northeastern part of Bulgaria (Dobrich) had lower mean levels of indicator PCBs than mothers from Varna. The levels of PCBs in breast milk from the sampled Bulgarian women were found lower than the levels from similar studies in other European countries. The PCB levels were found highest in the breast milk from primiparae mothers in the age group 36–40 years, suggesting bioaccumulation of these pollutants. In conclusion, the presented results show low PCB levels, but it is important to continue the monitoring of organochlorine contamination of both people and other living organisms.

CRediT authorship contribution statement

Stanislava Katelieva Georgieva: Conceptualization, Methodology, Validation, Data curation, Supervision, Project administration, Writing - review & editing. **Temenuga Trifonova**: Methodology, Investigation, Formal analysis, Data curation, Writing - original draft. **Zlatina Peteva**: Methodology, Validation, Investigation, Formal analysis, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114184.

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A case study of neurodevelopmental risks from combined exposures to lead, methyl-mercury, inorganic arsenic, polychlorinated biphenyls, polybrominated diphenyl ethers and fluoride

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ABSTRACT

We performed a mixture risk assessment (MRA) case study of dietary exposure to the food contaminants lead, methylmercury, inorganic arsenic (iAs), fluoride, non-dioxin-like polychlorinated biphenyls (NDL-PCBs) and polybrominated diphenyl ethers (PBDEs), all substances associated with declines in cognitive abilities measured as IQ loss. Most of these chemicals are frequently measured in human biomonitoring studies. A component-based, personalised modified reference point index (mRPI) approach, in which we expressed the exposures and potencies of our chosen substances as lead equivalent values, was applied to perform a MRA for dietary exposures. We conducted the assessment for four different age groups (toddlers, children, adolescents, and women aged 18–45 years) in nine European countries. Populations in all countries considered exceeded combined tolerable levels at median exposure levels. NDL-PCBs in fish, other seafood and dairy, lead in grains and fruits, methylmercury in fish and other seafoods, and fluoride in water contributed most to the combined exposure. We identified uncertainties for the likelihood of co-exposure, assessment group membership, endpoint-specific reference values (ESRVs) based on epidemiological (lead, methylmercury, iAs, fluoride and NDL-PCBs) and animal data (PBDE), and exposure data. Those uncertainties lead to a complex pattern of under- and overestimations, which would require probabilistic modelling based on expert knowledge elicitation for integration of the identified uncertainties into an overall uncertainty estimate. In addition, the identified uncertainties could be used to refine future MRA for cognitive decline.

Abbreviations: ESRVs, endpoint-specific reference values; iAs, inorganic arsenic; IQ, intelligence quotients; LB, lower bound; LOD, level of detection; LOQ, level of quantification; metHg, methyl mercury; MRA, mixture risk assessment; mRPI, modified reference point index; NDL-PCBs, non-dioxin-like polychlorinated biphenyls; PBDEs, polybrominated diphenyl ethers; POD, point of departure; SF, scaling factor; UB, upper bound; UF, uncertainty factor.

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1. Introduction

Populations are exposed to unintentional mixtures of chemicals via their diet, drinking water, inhaled air, dust or contact with consumer products. Until recently, the risks of chemicals to human populations were frequently assessed on a chemical-by-chemical basis and for single exposure routes only. However, increasing awareness of the potential risks from mixtures shifted the focus towards combined exposures to multiple chemicals and routes. Depending on the regulatory framework or region, different terminologies are used, such as cumulative risk assessment (e.g. used by the European Food Safety Authority, EFSA, for combined exposure to pesticides, EFSA 2020a; b, 2022), cumulative impact assessment (e.g. United States Environmental Protection Agency; US EPA, 2022) or mixture risk assessment (MRA). Such an assessment can focus on the combined exposure to chemicals only (e.g. pesticides; EFSA 2020a; b, 2022) or include non-chemical stressors (e.g., social determinants as in US-EPA cumulative impact assessment; US EPA, 2022) In this paper, we will focus on the combined exposure to chemicals only and use the wording MRA.

Considerable efforts have gone into developing concepts, methods, and guidance for MRA ((e.g. Boobis et al., 2008; EFSA, 2007, 2008, 2019, 2021a; Fox et al., 2017; WHO, 2008; Bopp et al., 2018; OECD, 2018). To harmonise MRA within the European Union, EFSA developed two pieces of guidance for human risk assessment of combined exposure to multiple chemicals (EFSA 2019, 2021a). In the 2019 report, EFSA elaborated a tiered approach for several aspects of mixture risk assessment across EFSA's domains (EFSA 2019). The EFSA 2021 report developed criteria for the grouping of chemicals for MRA (EFSA 2021a). Mechanistic information (common mode of action or adverse outcome pathway) through a structured weight of evidence approach is regarded as the gold standard. When such mechanistic data are not readily available, EFSA proposes that grouping may be performed using a common adverse outcome (phenomenon) or a common target organ/-system. EFSA used these grouping principles for dietary exposures to pesticides and proposed common assessment groups derived for chronic effects on the thyroid and for those that have acute effects on the nervous system (EFSA, 2020a; EFSA, 2020b, EFSA et al., 2022).

Biomonitoring studies have shown that humans are exposed to mixtures of contaminants from different chemical classes, such as heavy metals and persistent organic pollutants (Haug et al., 2018; Buekers et al., 2021; Julvez et al., 2021). Despite these findings, MRA is often limited to groups of structurally related contaminants, such as dioxins and dioxin-like polychlorinated biphenyls (PCBs), phthalates or polyfluorinated alkyl substances. We therefore became interested in making a leap to a MRA for chemicals that transcend groups of closely related substances and that would facilitate future scientifically based risk management decisions.

In this paper, we present the results of a MRA case study of developmental neurotoxicants in food in which we applied the EFSA approach to assess possible risks of reduced cognitive function in children. Applying this approach to external dietary exposure allows for identification of risk-driving chemical substance combinations. We focused on chemicals with a high occurrence in human biomonitoring matrices of approximately 1300 English, French, Spanish, Lithuanian, Norwegian and Greek mothers and children of the Early-Life Exposome (HELIX) cohorts, and associations with IQ loss in children after maternal or early childhood exposures, as identified by Grandjean and Landrigan (2006, 2014). Accordingly, we selected the food contaminants lead, methyl mercury, inorganic arsenic, non-dioxin-like polychlorinated biphenyls (NDL-PCBs), and polybrominated diphenyl ethers (PBDEs). Fluoride was added to this list because recent evidence suggests it also may affect cognitive development (Grandjean 2019, 2022). In some European countries, fluoride is added to drinking water (EFSA, 2013). The aim of the paper is 1) to investigate the feasibility of MRA for chemicals from different classes that are associated with IQ loss and 2) to identify challenges and major uncertainties in the input data. The results should not be regarded as formal national risk assessments.

2. Methods

2.1. Cumulative assessment group

Food contaminants were included in the assessment group based on the following criteria:

1. A high occurrence rate in human biomonitoring matrices of approximately 1300 English, French, Spanish, Lithuanian, Norwegian and Greek mothers and children of HELIX cohorts, defined as quantifiable in >50% of blood and urine samples, as shown by Haug et al. (2018). Polychlorinated organic pollutants, brominated flame retardants, per- and polyfluoroalkyl substances, heavy metals, phthalate metabolites, phenols, and organophosphates met this criterion.
2. Evidence of associations with cognitive declines, measured as IQ loss as identified by Grandjean and Landrigan (2006, 2014). Lead, methyl mercury, inorganic arsenic, PCBs, PBDEs and organophosphate pesticides fulfilled this criterion. Although not measured in the study of Haug et al. (2018), fluoride was included because high intake levels are associated with IQ loss (Grandjean 2019, 2022).
3. Sufficient data to derive a point of departure (POD) and an endpoint-specific reference value (ESRV) from epidemiological studies. This criterion was met by lead and methyl mercury as their health-based guidance value is based on IQ loss (EFSA 2010a; US EPA 2001). ESRV for PBDEs are extrapolated from developmental neurotoxicity (locomotion and total activity) in rodents. The available epidemiological data for PCBs, inorganic arsenic and fluoride allowed estimations of POD and ESRV, but the data basis for organophosphates was judged to be insufficient. They were therefore not included in the present assessment. Accordingly, the cumulative assessment group for this study was composed of lead, methyl mercury, inorganic arsenic, PCBs, fluoride and PBDEs.

2.2. Estimation of PODs and ESRVs

For all the substances included in the assessment group, we collated quantitative dose estimates for declines in IQ scores and related ESRV for developmental neurotoxicity. The ESRV is defined as the POD of the substance divided by its uncertainty factor (UF) and is used to calculate external exposure. As much as possible, ESRVs were retrieved from existing evaluations of competent authorities (lead, methyl mercury, PBDE). In some cases, however, it was necessary to conduct separate reviews to derive the respective ESRV *de novo* (fluoride, inorganic arsenic). To make the mixture risk assessment as consistent as possible, we attempted to relate all ESRVs to the same effect magnitude, IQ losses by 1 point. However, some studies derived exposures associated with 5-point IQ losses. In such cases, we extrapolated to a 1-point loss. In addition, for some substances an additional UF was applied to take other uncertainties into account.

Table 1 provides an overview of the data used for the derivation of ESRVs. For each substance, details of the derivation of the ESRV are provided below. It should be noted that these ERSVs, unless they are health-based guidance values (e.g. lead), do not have the normative character of such values and should only be used for the purpose of a MRA. Except for iAs, the ESRVs were derived for expected mothers. The same ESRVs were used for all age groups, regardless they were derived from mothers or children.

2.3. Lead

We followed the considerations of EFSA's CONTAM panel (EFSA 2010a). Based on the study by Lanphear et al. (2005) the Panel estimated that a blood lead level of 12 μg/L in children aged 5–10 years old

Table 1
Chemicals in the assessment group, their endpoint-specific reference value (ESRV) used for the scaling factor (SF) calculation and data used for the derivation of the reference dose.

Chemical	Effect	IQ test	Reference point	Sex	Reference type	Species	Conversion to intake dose	Uncertainty factor	ESRV	SF
Lead[a]	IQ loss in children (0–7 years) of exposed mother	FSIQ	1 point IQ loss related to 12 μg/L Pb in blood	Boys and girls	$BMDL_{01}$	Human	Expectant mothers: foetal/maternal Pb blood ratio ~ 0.9	–	0.54 μg/kg bw/d	1
Inorganic arsenic[b]	IQ loss in exposed children, contemporaneous exposure	Raw verbal IQ	2.6 points IQ loss in girls for every 100 μg/L urine	Girls	LOAEL	Human	Conversion to 1 IQ point by linear extrapolation	–	1.3 μg/kg bw/d	0.42
Methyl mercury[c]	IQ loss in children of exposed mothers	Several cognitive test, including FISQ	5 points IQ loss related to 4–25 ppm in maternal hair	Boys and girls	$BMDL_{05}$	Human	Via estimation of blood levels, then kinetic modelling and extrapolation	10	0.1 μg/kg bw/d	5.4
Fluoride[d]	IQ loss in children of exposed mothers	General cognitive index, FSIQ.	0.1–0.2 mg/L urine	Boys and girls	$BMDL_{01}$	Human	With Rugg-Gunn et al., 2011; daily excretion of F at BMDL = 0.1–0.4 mg/d; equivalent to 2.4–12 μg/kg	–	9 μg/kg bw/d	0.06
NDL-PCBs[e]	IQ loss in children of exposed mothers	FSIQ	5 points IQ loss related to 0.63–0.71 μg/g lipid in mother's milk	Boys and girls	$BMDL_{05}$	Human	Via estimation of body burden, kinetic model Factor 2 applied for conversion from 5 to 1 IQ point loss.	2	15 ng/kg bw/d	36
PBDE[f]	developmental neurotoxicity (locomotor, total activity)	–	PBDE-47: 309 μg/kg bw		$BMDL_{10}$	Mice	Via critical body burden in mice and humans to an external dose taking into account kinetic information, except for PBDE 209 since toxicokinetics are assumed to be similar in mice and man[f]. For PBDE-209 the external dose in mice was extrapolated to humans.	PBDE-47: 2.5	PBDE-47: 68.8 ng/kg bw/d	PBDE-47: 7.9
			PBDE-99:12 μg/kg bw/d					PBDE-99: 2.5	PBDE-99:1.68 ng/kg bw/d	PBDE-99: 318
			PBDE-153: 83 μg/kg bw/d					PBDE-153: 2.5	PBDE-153: 3.84 ng/kg bw/d	PBDE-153: 142
			PBDE-209: 1700 μg/kg bw/d					PBDE-209: 100	PBDE-209: 17 μg/kg bw/d	PBDE-209: 0.032

See section 2.2 for explanation. Abbreviations: IQ intelligence quotient; FSIQ full scale intelligence quotient; BMDL Benchmark dose lower limit; LOAEL lowers observed adverse effect level; Pb-lead; F-fluoride; NDL-PCBs non-dioxin-like PCBs; PBDE polybrominated diphenyl ethers; kg kilogram; d day.

[a] Data were retrieved from EFSA (2010a) and were based on Lanphear et al. (2005).
[b] Data were retrieved form Tsuji et al. (2015) and based on Hamadani et al. (2011).
[c] Data were retrieved from Rice et al. (2003).
[d] Data were retrieved from Grandjean (2019).
[e] Data were retrieved from EFSA (2005) and based on Jacobson et al. (2002).
[f] Data were retrieved from EFSA (2011a) and Martin et al. (2017).

Table 2
Description of the food consumption data of nine different European countries, including method of food consumption survey, year(s) in which the food consumption survey was conducted, the name of the survey, the population addressed, the total number of individuals and consumptions days included in the study, and the subpopulation groups and number of individuals included in the cumulative exposure assessment for chemicals relevant for IQ loss.

Country	Food consumption survey						Subpopulation in study	
	Method	Years	Name	Population[a] (years of age)	N total[b]	Consumption days	Subpopulation[c] (years of age)	N[d]
Austria (AT)	24-h dietary recall	2018	ADOLESCENTS-2018-2	10–17	657	2	10–17	657
		2016		18–64	2250	2	F 18-45	1013
		2018	NATIONAL-2016 PREGNANT-2018-2	Pregnant F 19-47	302	2	19–45	299
Croatia (HR)	24-h and 48-h dietary recall	2011–2012	NIPNOP-HAH-2011-2012	18–64	2002	3	F 18-45	629
Cyprus (CY)	24-h dietary recall	2014–2017	2014-2017-LOT1	0–9	848	3	1–2	279
			2014-2017-LOT2	10–76	1016	3	3–9	300
							10–17	274
							F 15-45	287
Czech Republic (CZ)	24-h dietary recall	2003–2004	SISP04	4–64	2353	2	4–9	389
							10–17	298
							F 15-45	419
Denmark (DK)	Food record	2006–2007	IAT 2006-07	0–3	1743	7	1–2	894
		2005–2008	DANSDA 2005-08	4–75	2700	7	3	23
							4–9	298
							10–17	377
							F: 18-45	570
France (FR)	FPQ[e] and 24 h dietary recall	2014–2015	INCA3	General population[f]	4874	3	Toddlers[f]	149
							Other children	921
							Adolescents	1221
Italy (IT)	Food record	2005–2006	INRAN SCAI 2005-06	0–97	3323	3	1–2	36
							3–9	193
							10–17	247
							F 15-45	703
Netherlands (NL)	Food record, 24 h dietary recall	2012–2016	FCS2016_Core	1–80	4313	2	1–2	440
							3–9	853
							10–17	870
							F 18-45	485
Slovenia (SI)	24 h dietary recall	2018	SI.MENU-2018	0.25–75	1981	2	1–2	344
							10–17	493
							F18-45	113

[a] Indicates the age range of the population included in the food consumption survey.
[b] Indicates the number of subjects included in the food consumption survey.
[c] Indicates the age range of the subpopulation included in the case study: toddlers (1–2 years of age), other children (3–9 years of age), adolescents (10–17 years of age) or women in their childbearing age 18–45 years). Unless otherwise stated, the subpopulation included males and females. F means females.
[d] Indicates the number of subjects included per subpopulation in the case study.
[e] FPQ: Food propensity questionnaire.
[f] Due to privacy reasons the French food consumption data contained age groups instead of individual ages.

is associated with an IQ loss of 1 point. By toxicokinetic modelling, EFSA converted this blood lead level into a daily intake of 0.5 μg/kg. Taking account of a foetal/maternal blood lead ratio of 0.9, this is equivalent to a daily intake of 0.54 μg/kg d by expectant mothers, which was used as ESRV in our study. No uncertainty factors were applied.

2.4. Methyl mercury

For our case study, we used the ESRV derived for methyl mercury as described by Rice et al. (2003). Evidence of declines in cognitive ability after maternal methyl mercury exposure during pregnancy comes from three main epidemiological cohorts, those in the Faroe Islands, New Zealand and the Seychelles. Reviewing data from these three cohorts, Rice et al. (2003) used benchmark dose modelling for in-utero exposure for all cognitive effects, including IQ scores of the Faroes cohort and estimated that maternal hair mercury levels of between 4 and 25 ppm are associated with IQ losses by 5 points in their children. Toxicokinetic modelling assuming a hair to blood ratio of 250 and a one compartment model assuming 1) 95% of oral methyl mercury being absorbed, 2) 5.9% of absorbed methyl mercury present in blood, 3) a blood volume of 5 L, 4) an elimination rate of 0.014 day^{-1}, and 5) a fixed body weight of 67 kg for pregnant women (Rice et al., 2003; US EPA 2001) revealed that these hair levels resulted from maternal daily methyl mercury intakes of between 0.447 and 1.9 μg/kg d. It should be noted that this approach assumes a ratio of 1:1 between maternal and cord blood (Rice et al., 2003). By application of an UF of 10 (to account for differences in maternal toxicokinetics and -dynamics), Rice et al. estimated a daily intake of 0.1 μg/kg d as tolerable. We employed this value in our case study. However, it is unclear whether the UF of 10 also caters for an extrapolation to exposures associated with 1 IQ point loss.

2.5. iAs

We adopted the values used by Tsuji et al. (2015) in their systematic review of arsenic-induced developmental neurotoxicity and risk assessment. Tsuji et al. evaluated several epidemiological studies that described associations between inorganic arsenic exposure and verbal IQ scores and rated the data from the Matlab cohort (Bangladesh) communicated by Hamadani et al. (2011) as most suitable for quantitative risk assessments. Hamadani et al. observed a decrease in cognitive ability by 2.6 IQ points in girls for every 100 μg/L increase in speciated urinary arsenic levels. This was related to contemporaneous arsenic exposures; a window of vulnerability for inorganic arsenic and developmental neurotoxicity is poorly defined. Conversion to an IQ loss by 1 point is associated with an increase by 38.5 μg/L speciated urinary arsenic levels. By application of a one-compartment toxicokinetic model, and assuming a urinary excretion rate of 0.4 L/day, 70–90% of oral dose excreted in urine (estimated from monkeys), and a body

weight of 14.9 kg (mean of Matlab cohort) Tsuji et al. (2015) estimated that such urinary arsenic levels result from daily intakes of between 1.1 and 1.47 μg/kg d. We selected the midpoint of this range (1.3 μg/kg d) as ESRV in our study. No UF was applied because the POD was based on human data.

2.6. Fluoride

Grandjean et al. (2022) recently presented a benchmark modelling for IQ losses associated with fluoride exposures in which they used data from two prospective birth cohort studies, the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) cohort in Mexico and the Maternal-Infant Research on Environmental Chemicals (MIREC) cohort in Canada. Assuming a benchmark response of 1 IQ point loss, they derived benchmark concentrations (BMCs) of maternal urinary fluoride and benchmark concentration levels (BMCLs). The BMC for maternal urinary fluoride associated with a 1-point decrease in IQ scores of preschool-aged boys and girls was 0.31 mg/L (BMCL, 0.19 mg/L). The BMD was 0.33 mg/L (BMCL 0.20 mg/L) when pooling the IQ scores from the older ELEMENT children and the MIREC cohort. From these two prospective studies the joint data showed BMCL results about 0.2 mg/L.

Assuming a 24 h urine volume of 1.5 L, this urinary fluoride levels would lead to a daily maternal fluoride excretion of 0.3 mg/d. Rugg--Gunn et al. (2011) have recorded the relationship between total fluoride intake and daily urinary fluoride excretion. Based on 8 studies among adults with a total of 269 data pairs (Fig 3 in Rugg-Gunn et al., 2011) it can be estimated that a daily excretion of 0.3 mg fluoride is to be expected with daily intakes of 0.6 mg. Assuming a body weight of 65 kg, this converts to an intake of 9 μg/kg d which we adopted as ESRV in our study. No UFs were applied.

2.7. NDL-PCBs

From the study of IQ loss in children of PCB-exposed mothers by Jacobson et al. (2002), a benchmark concentration of 0.63–0.71 μg/g lipid in mother's milk is associated with a benchmark response of 5% in terms of full-scale IQ loss (benchmark dose, lower limit, see Table 3 in Jacobson et al.). This value applies to all PCBs. To estimate daily intakes from PCB lipid levels, we followed the assumptions made in EFSA (2005): Adipose tissue constitutes 20% of an adult's body weight, the overall biological half-life of the most persistent PCB congeners is 10 years (3650 days) and the absorbed fraction is 0.9. Based on these assumptions, the daily PCB maternal intakes that will give rise to such PCB lipid levels at steady state can be estimated as 26–30 ng/kg d. For this, the following formula was used: intake [ug/kg/d] = serum lipid level [ug/kg lipid] * 0.138/T1/2 [d]/f, where T1/2 is half-life of excretion, 0.138 a composite of ln 2 and 0.2, and f the absorbed fraction.

Considering that the benchmark concentrations given by Jacobson et al. do not correspond to IQ losses of 1 point, we lowered these values and chose 15 ng/kg d as the ESRV in our study by applying an UF of 2.

PCBs can be split into 12 dioxin-like congeners (DL-PCBS) and 197 NDL-PCBs. We included only NDL-PCBs in our case study for two reasons: 1) According to EFSA, information on neurodevelopmental effects of DL-PCBs is too limited for risk assessment (EFSA 2018) and 2) human body burden in of PCBs in human biomonitoring is frequently assessed based on the sum of three indicator congeners PCB-138, -153 and 180, multiplied by two for inclusion of three additional PCB congeners −28, −52 and −101 (Kraft et al., 2017), which are all NDL-PCBs (EFSA 2005; JECFA 2016).

2.8. PBDEs

We adopted the congener-specific values for PBDE 47, 99, 153 and 209 which EFSA (2011a) used for margin of exposure considerations related to developmental neurotoxicity, applying an UF of 2.5 to PBDE-47, -99 and −153 to account for inter-species difference in toxicodynamics. For PBDE-209 an UF of 100 was applied (Martin et al., 2017). EFSA regarded the available data for other congeners as too unreliable to establish similar values. Therefore, those congeners were not included in the case study. The ESRVs for these PBDE congeners are: PBDE 47–68.8; PBDE 99–1.68; PBDE 153–3.84; PBDE 209–17,000 ng/kg d.

It is noted that these values are derived from motor activity effects observed in a developmental neurotoxicity study in rodents. There is no information how these would relate to IQ loss in humans. However, for the purpose of the present exercise these values were taken as the doses that would lead to 1 IQ point loss in humans.

2.9. Scaling factors

To be able to sum exposures, scaling factors (SF) were used to describe the toxicity of a substance *s* in terms of the toxicity of an index compound and can be used to combine exposures of substances in an assessment group. The SF of substances included in our case study was obtained by dividing the ESRV of lead (Pb) by that of the substance of interest by using the following equation:

$$SF_s = \frac{ESRV_{Pb}}{ESRV_s} \quad \text{(Equation 1)}$$

It should be noted that the scaling factor by definition differs from a relative potency factor (RPF), which is also used to describe the toxicity of a substance s in terms of the toxicity of an index compound to enable combining exposures of substances in an assessment group. Scaling factors can only be called RPFs if chemicals 1) act via a common mode of action; 2) differ only in potency (i.e., their individual dose–response curves should be parallel on log–dose scale), and 3) do not interact (Bosgra et al., 2009; EFSA 2019; Bil et al., 2021). Since this information is lacking for the substances in our case study, we used scaling factors.

2.10. Food consumption data

Food consumption data were obtained from the EFSA data warehouse[1] upon approval of data owners. Consumption data were obtained from nine European Member States and were derived from national food consumption surveys. Table 2 provides an overview of the characteristics of the food consumption data use in this study. Food consumption data were received using the non-hierarchical coding of the harmonized food coding system FoodEx1 (EFSA 2011b) and re-coded in the hierarchical FoodEx1 codes to allow for extrapolation of concentration data.

In their assessments for regulatory purposes, EFSA subdivided the population into age groups, i.e. infants, toddlers, other children, adolescents, adults, elderly and very elderly (EFSA 2011c). In our study, we mirrored the EFSA age groups for children and adolescents as close as possible (i.e. toddlers aged 1–2 years, other children aged 3–9 years, adolescents aged 10–17 years), but selected women aged 18–45 years as proxy for pregnant women. Infants (below the age of 12 months) were not included because the limited availability of food consumption data for this age group among the countries.

2.11. Chemical concentration data in food

Chemical concentration data from the years 2014–2018 were obtained for NDL-PCBs, PBDEs, lead, inorganic arsenic, methyl mercury, and fluoride from the ESFA data warehouse. Data were obtained from 15 European Member States that agreed to share data: Austria, Cyprus, the Czech Republic, Denmark, Estonia, Finland, France, Croatia, Hungary, Ireland, Italy, Luxembourg, the Netherlands, Sweden and Slovenia. Data were formatted according to EFSA standard sample descriptions (SSD1; EFSA 2010b), with food items coded according to the harmonized

[1] https://www.efsa.europa.eu/en/microstrategy/food-consumption-survey.

Table 3
Personalised modified reference point index) for substances relevant to loss of intelligence scores (lead, methyl mercury, inorganic arsenic, fluoride, non-dioxin-like polychlorinated biphenyls and polybrominated diphenyl ethers) calculated for toddlers (1–2 years), children aged 3–9 years, adolescents (10–17 years) and women in their childbearing age (18–45 years).

	Toddlers		Other children		Adolescents		Women child bearing age	
	LB[b]	UB[c]	LB	UB	LB	UB	LB	UB
P50								
AT[a]	–	–	–	–	1.6 (1.5–1.8)[f]	2.9 (2.7–3.2)	2.0 (1.8–2.1)	3.5 (3.2–3.8)
CY	4.7 (4.3–5.3)	9.2 (8.6–9.8)	3.3 (3.0–3.7)	6.3 (5.9–6.9)	1.8 (1.6–2.0)	3.4 (3.1–3.8)	1.5 (1.3–1.7)	2.6 (2.6–3.2)
CZ	–	–	3.6 (3.0–3.6)	6.0 (5.6–6.4)	2.3 (2.1–2.5)	4.1 (3.8–4.4)	1.6 (1.4–1.7)	2.7 (2.5–3.0)
DK	5.4 (4.8–6.0)	9.7 (9.2–10.5)	3.7[d] (3.2–4.0) 4.3[d] (3.2–6.7)	6.9[e] (6.4–7.5) 7.8[e] (6.8–10)	1.9 (1.7–2.2)	3.6 (3.3–3.9)	2.0 (1.8–2.1)	3.4 (3.2–3.7)
FR	4.5 (3.8–5.4)	9.0 (7.8–10.5)	4.2 (3.7–4.7)	7.3 (6.8–7.8)	2.1 (1.8–2.3)	3.7 (3.4–4.1)	–	–
HR	–	–	–	–	–	–	1.4 (1.2–1.5)	2.7 (2.5–3.0)
IT	7.6 (4.1–11)	12 (8.6–16)	4.7 (4.2–5.6)	8.2 (7.2–9.2)	2.7 (2.3–3.1)	4.7 (4.1–5.2)	2.3 (2.0–2.6)	4.0 (3.6–4.7)
NL	4.1 (3.5–5.1)	8.3 (7.4–9.4)	2.6 (2.4–2.9)	5.4 (5.2–5.9)	1.6 (1.4–1.8)	3.2 (3.0–3.5)	1.5 (1.4–1.7)	2.9 (2.6–3.2)
SI	3.4 (3.1–3.8)	7.1 (6.6–7.6)	–	–	1.5 (1.3–1.6)	2.8 (2.5–3.0)	1.3 (1.1–1.5)	2.5 (2.1–2.8)
P95								
AT	–	–	–	–	4.4 (3.5–5.7)	6.5 (5.8–7.7)	6.0 (4.7–7.6)	7.8 (6.8–9.5)
CY	15 (11–18)	21 (17–25)	11 (9.0–14)	15 (13–20)	6.4 (5.8–8.2)	8.8 (7.9–9.9)	6.5 (5.3–9.4)	8.6 (7.3–11)
CZ	–	–	15 (12–20)	20 (15.4–23.2)	11 (8.1–14)	13.1 (9.9–16.6)	7.9 (6.7–9.9)	9.7 (8.2–12)
DK	21 (18–26)	27 (23–31)	13[d] (9.9–16) 14[d] (9.3–20)	17[e] (14–21) 18[e] (12–25)	5.1 (4.0–6.8)	7.3 (6.3–9.1)	5.0 (4.3–6.1)	7.1 (6.5–8.0)
FR	14 (11–21)	20 (16–29)	13 (11–17)	18 (15–22)	6.8 (5.8–8.4)	9.5 (8.5–11)	–	–
HR	–	–	–	–	–	–	5.0 (4.2–6.3)	6.9 (6.0–8.4)
IT	17 (12–23)	24 (19–31)	14 (11–17)	18 (15–22)	9.7 (8.4–12.9)	12 (10.0–15.3)	10 (8.1–13)	12 (10–15)
NL	11 (8.6–14)	16 (14–19)	8.8 (6.8–10)	12 (11–15)	5.0 (4.0–6.4)	7.2 (6.2–8.8)	5.6 (4.5–7.3)	7.4 (6.3–9.5)
SI	11 (8.5–15)	15 (13–20)	–	–	6.9 (5.5–9.4)	8.5 (7.3–11)	6.7 (4.4–12)	8.0 (6.0–14)

[a] AT-Austria, CY-Cyprus, CZ-Czech Republic, DK-Denmark, FR-France, HR-Croatia, IT-Italy, NL-Netherlands, SI-Slovenia.
[b] LB is lower bound scenario. In this scenario analytical values below the limit of detection or limit of quantification were assumed to equal 0.
[c] UB is upper bound scenario. In this scenario analytical values below the limit of detection or limit of quantification were assumed to equal the value of the particular limit.
[d] DANSDA 2005–08 food consumption survey; children aged 4–9 years old.
[e] IAT 2006–07 food consumption survey; children aged 3-years old.
[f] Values between brackets indicate the upper and lower boundaries of the uncertainty interval quantified for uncertainties in food consumption and food occurrence data due limited sample sizes.

FoodEx1 coding system (EFSA 2011b). Wherever possible, food concentration data were used at FoodEx1 level 4, the most detailed level. For example, if sufficient concentration data, defined as at least 50 measurements, were available at the FoodEx1 level 4 code 'cow milk, <1% fat (skimmed milk)' concentration data at this level were used. If sufficient concentration data were not available, concentration data were grouped at a less detailed level. For example, 'cow milk, <1% fat (skimmed milk)' was then recoded into to cow's milk (level 3), liquid milk (level 2) or milk and dairy products (level 1), wherever relevant.

Data with empty cells in any important field of the SSD file, such as level of detection, level of quantification and analytical value, or invalid concentration units, were omitted. If a FoodEx code was missing, but the product name was available, the corresponding FoodEx code was added manually. Only data obtained from random sampling and convenient data were included. For each substance and composite food combination in the dataset it was decided to use the analytical data as such or to convert the food into its ingredients (see matching food consumption and concentration data). Complexity of the food (e.g. the FoodEx1 code represents a broad range of composite foods rather than a single food, such as meat-based dishes), availability of recipe data, and number of measurements for the composite food and its ingredients were important criteria for this decision. Once decided to convert a composite food into its ingredients, the analytical data of the composite food were removed from the data set. Supplemental material A provides information on the decisions made for the foods, and Supplemental material B shows the FoodEx1 level used for each substance in the concentration dataset, together with the number of measurements, the percentage left-censored data, i.e. measurements below the level of detection (LOD) or level of quantification (LOQ) and mean concentrations per food and substance. Below some particularities for the different substances are provided.

2.12. Lead

Of all obtained samples, two aberrant samples (outliers) were removed from the FoodEx1 category 'wine'; one with 14 mg lead/kg and the other 21 mg lead/kg. Of all substances, lead concentration data were most abundantly available, 39,959 entries were obtained from 13 EU countries and for 358 different FoodEx1 codes, after clean-up of the data.

2.13. Methyl mercury

Data for methyl mercury were obtained for fish and sea food (60 foods) from 12 EU countries. Analytical results for both methyl mercury and total mercury were available. Fewer numbers of analytical values were available for methylmercury (n = 165) than for total mercury (n = 6,542). Therefore, we decided to include methyl mercury concentrations calculated out of total mercury concentrations using conversion factors established by EFSA (EFSA 2012a):

- 1 for fish meat, fish products, fish offal and unspecified fish and seafood;
- 0.8 for crustaceans, molluscs and amphibians, reptiles, snails and insects;
- 0 for all other food categories not containing fish or seafood.

For the samples with measured methyl mercury concentrations, total mercury concentrations were also available, allowing comparisons of measured and calculated methyl mercury concentrations. To do this, the mean of positive samples, i.e. samples with an analytical value of methyl mercury or total mercury above the LOQ value, was calculated. The mean calculated methyl mercury concentration was generally slightly higher than mean measured methyl mercury concentration (see Supplementary Material C). Given the smaller number of measured methyl mercury data and the slightly higher concentrations of calculated methyl mercury, it was decided to include only calculated methyl mercury data. After data cleaning, the dataset for methyl mercury contained 6,473 entries.

2.14. Inorganic arsenic

From the EFSA data warehouse, samples containing 'arsenic' between 2014 and 2018 were retrieved. Since we focused on the exposure to inorganic arsenic (iAs), 'organic arsenic' samples were omitted from the data and samples coded as 'arsenic and derivatives' and 'arsenic' were recoded to 'total arsenic' samples, following the approach taken by EFSA (2021b). Of samples for which both 'total arsenic' as well as 'inorganic arsenic' values were reported, the 'total arsenic' samples were omitted from the database. In addition, after closer examination of the original data, samphire ("*zeekraal*") samples analysed for 'arsenic' from the Netherlands were originally coded as 'leafy vegetables' and were consequently recoded as 'sea weeds'. The fraction of iAs was translated from the remaining 'total arsenic' samples according to the median ratios described in EFSA's Scientific Opinion (2021b). Similar to EFSA, total arsenic was not converted into iAs for fish. Supplemental material F lists the factors used for the conversion of total arsenic into iAs. Like EFSA, we used an additional LOQ-cut off of 100 μg/kg for iAS in cereal-based food for infants and young children.

The original dataset also contained 3104 entries for drinking water (tap and bottled). High concentrations of iAs in tap water (typically up to 7920 μg/litre), especially originating from one country, were present in the data set. In addition, the dataset contained non-detects with high LOQs (up to 900 μg/kg). A maximum level of 10 μg/L has been established for water intended for human consumption, without distinguishing among different arsenic forms (EU, 2020). In addition, a maximum level of 10 μg/L was established for total arsenic in natural mineral water (EC, 2003). In the most recent EFSA opinion on iAs, EFSA used concentration data over the years 2013–2018 and excluded values obtained from analytical methods with LOQs higher than 10 μg/L for that reason (EFSA, 2021b). To perform calculations using representative European iAs in drinking water, we did not use the received data from the Data Warehouse but used the mean values for the lower and upper bound as reported by EFSA in 2021. After data cleaning, 3,021 entries for iAs were obtained from 13 EU countries and for 117 different FoodEx1 codes.

2.15. Fluoride

Only fluoride concentrations in drinking water were available. Data were obtained from the EFSA data warehouse for bottled water, carbonated mineral water, still mineral water, well water and drinking water. Because of food conversions containing water, such as soft drinks and liquid infant formulae which are converted to water and other ingredients (see matching food consumption data and concentration data), all types of water were recoded into drinking water (A.15). Within the EFSA data warehouse information from limited countries was available. Therefore, additional fluoride concentration data obtained from the Dutch monitoring program for drinking water between 2014 and 2018 were included. It should be noted that those data were provided as mean values per pumping station. Mean values were calculated using a middle-bound scenario, in which samples below the limit of detection were substituted with a value equal to half the value of the level of detection. After data cleaning, 2011 entries for fluoride in drinking water were available.

2.16. NDL-PCBs

Concentration data were obtained for 6 NDL-PCBs, which are regarded as indicator congeners for the exposure to NDL-PCBs via food (EFSA 2005; JECFA 2016). Concentration data (n = 3,363 samples) for each of the 6 NDL PSBs were obtained from 9 countries. For many samples, the sampling type was not specified. To enlarge the number of observations, those samples were included. NDL-PCB concentrations in food were expressed on a whole weight- or on a percentage fat weight-basis. If for a sample data were available for both whole weight and percentage fat weight, the data expressed on whole weight were selected. If data were expressed based on percentage fat weight, the percentage fat in the original sample was provided in the SSD format. However, the original percentage fat in the sample was not always provided or higher than expected (up to 100%). To calculate the NDL-PCB concentration in those samples, the percentage fat weight according to the Dutch food composition database (NEVO; accessed November 2021)[2] was used. If NEVO provided two or more values for the percentage fat, the average fat weight was used for the calculations. After an initial run, high exposure estimates were obtained for NDL-PCBs in vegetable oil. This was mainly due to extreme NDL-PCBs concentrations analysed in one country. Average concentrations were approximately 250 times higher than those described for vegetable oil in the EFSA opinion (EFSA, 2012b). We therefore omitted the extreme NDL-PCBs concentrations analysed in one country. The mean concentration of the sum of 6 congeners now fell within the range published by EFSA (EFSA, 2012b).

As the sum of 6 indicator congeners comprises 50% of the total exposure to NDL-PCBs (EFSA 2012b), the 6 NDL-PCBs were summed per sample assuming equipotency (see paragraph scaling factors) and multiplied by 2 as a proxy for the total concentration of NDL-PCBs in food. In total, 20,103 data entries for the sum of NDL-PCBs in 60 food categories were used in the case study.

[2] Nederlands Voedingsstoffenbestand (NEVO) | RIVM. https://nevo-online.rivm.nl/Home/En

2.17. PBDEs

Concentration data were obtained from 4 countries and for 24 foods of animal origin (meat and meat products, fish and other seafood, eggs and milk). Like the NDL PCBs, PBDE concentrations in food were expressed based on whole weights or on percentage fat weight. Again, concentrations expressed on a whole weight basis were preferred over those expressed on percentage fat weight. In addition, the fat weight of the original sample was not always available, and the fat weights provided in the Dutch NEVO database was used to calculate PBDE concentrations expressed on whole weight. After data cleaning, the data set contained 1557 measurements for each of the four PBDEs.

Because some exposome studies or aggregated external exposure studies express the sum of PBDEs, concentration data for the four PBDEs −47, −99, −153 and −209 were summed per sample as lead-equivalents thus considering their SFs compared with lead (see paragraph scaling factors). Summing was performed following the lower bound and upper bound scenario (see Exposure scenarios).

2.18. Matching food consumption data and concentration data

As much as possible, food consumption data was linked to concentration data at the same level of detail. If that was not possible, food consumption was linked to a less detailed level FoodEx1 coding using the hierarchical FoodEx1 system. For example, consumption of turnips was linked to concentration data in root vegetables. As concentrations of substances are often available in raw agricultural products rather than processed products, a food translation table was used to link consumed processed food to substance concentrations in its raw agricultural commodity ingredients. For this we used the Dutch food translation table (Boon et al., 2015), which was based on Dutch recipes and contained conversion factors to convert foods classified according to FoodEx1 into their edible raw agricultural commodity ingredients (e.g. 167 g raw spinach is needed to produce 100 g cooked spinach). As this food translation table was developed for pesticide exposure calculations, it focused on fruit and vegetables. As such, the food translation table did not include animal-derived ingredients (fish, meat and milk) in composite food. Therefore, we updated the food consumption table with animal-derived ingredients as much as possible using Dutch recipes for composite foods.

2.19. Exposure scenarios

For each subpopulation the lower and upper bound scenarios following EFSA practice regarding handling concentrations below LOD LOQ EFSA, 2010c) were used for the exposure assessments. In the lower bound scenario, concentration values below the LOD or LOQ, as indicated accordingly in the SSD files, were assumed to equal 0. In the upper bound scenario, concentrations below the LOD or LOQ were assumed to equal the value of the respective limit.

2.20. Mixture risk assessment

Mixture risk assessment was performed using the MCRA tool version 9.1 (https://mcra.rivm.nl) for each country and subpopulation listed in Table 2, assuming dose additivity. Chronic (long-term) exposure was calculated using the Observed Individual Means (OIM) model. For each substance *s* in the assessment group and for each individual *i* in the food consumption data base, the consumed amount of a certain food *f* averaged over the total number of consumption days q_{if} was multiplied with the average concentration present in that food c_{ifs}. This was done for all consumed foods per individual. The subsequent obtained exposures per food were summed for each chemical *s* per individual over the *F* numbers of food consumed and divided by the bodyweight of the individual bw_i, which yielded the chronic exposure E_{is} to chemical *s* of the individual *i*.

$$E_{is} = \frac{\sum_{f=1}^{F} q_{if} c_{ifs}}{bw_i} \quad \text{(Equation 2)}$$

The chronic exposure of each chemical *s* in the assessment group E_{is} was then multiplied by the SF of the chemical (SF_s) and summed per individual to obtain the cumulative exposure per individual *Cum* E_i. As we used lead as the index chemical for deriving the SF, *Cum* E_j is the cumulative exposure of each individual expressed as lead equivalents:

$$Cum\ E_i = \sum_{s=1}^{S} E_{is} * SF_s \quad \text{(Equation 3)}$$

where *s* relates to the chemical considered. This yielded a distribution of the cumulative exposure, from which the median (P50) and the 95th exposure percentile were obtained.

Fold-exceedance of combined potency weighted tolerable exposures to the chemicals in the assessment group were characterised by dividing each individual's combined exposure in lead equivalents (*Cum* E_j) by the ESRV of the index compound lead ($ESRV_{Pb}$). We called the metric obtained in this way a *personalised modified reference point index* (mRPI).

$$personalised\ mRPI_i = \frac{Cum\ E_i}{ESRV_{Pb}} \quad \text{(Equation 4)}$$

This approach is similar to the mRPI introduced by Vejdovszky et al. (2019), as outlined in supplemental material D, and mathematically equivalent to the Hazard Index (Teuschler and Herzberg 1995). According to the EFSA guidance on harmonized methodologies, the hazard index is used in the context of health-based guidance values for the critical effect (such as the acceptable daily intake or the tolerable daily intake), whereas the reference point index (RPI), also known as the point of departure index, could be used for ESRVs that are not necessarily based on the critical effect (EFSA 2019). The RPI could typically use a single group UF (either a default or chemical-specific assessment factor) to assess the risk (EFSA 2019). Since UFs may vary depending on the derivation of the reference points, Vejdovszky et al. (2019) finetuned the RPI approach by applying chemical-specific uncertainty factors and named this the modified RPI (mRPI) approach. Because the reference points for IQ loss are not always based on the critical effect of a chemical and different UFs were applied, the mRPI approach was best suited to estimate the risk related to IQ loss.

Our approach yielded distributions of the personalised mRPI, of which the median and the 95th percentile of personalised mRPI were obtained. The personalised mRPI distributions obtained in this way were evaluated in terms of exceedances of combined "acceptable" exposures to lead equivalents relative to a value of 1. A personalised mRPI larger than 1 either means that a risk of the combined exposure cannot be excluded or that refinement is needed, depending on the direction of the uncertainties.

In addition to the calculation of percentiles, the contribution of substances to the personalised mRPI of the total population was assessed. For a particular substance *s,* the sum of the exposure *E* to that substance (expressed as lead-equivalents) of all individuals (n) in the food consumption database relative to the sum of the cumulative exposures of all individuals was calculated:

$$\%\ contribution\ s = \frac{\sum_{i=1}^{n} E_s}{\sum_{i=1}^{n} Cum\ E_i} * 100 \quad \text{(equation 5)}$$

Calculating the contributions for combinations of foods and substances is done in a similar way.

2.21. Uncertainty

The bootstrapping approach was used to quantify sampling uncertainty in food consumption and concentration data caused by a limited sampling size (Efron 1979; Efron and Tibshirani 1993). This approach

re-samples (with replacement) the original food consumption and concentration dataset to obtain a bootstrap of n observations. In the present calculation, we performed an uncertainty analysis using 100 re-sampling cycles with 10,000 iterations. This yielded 100 alternative exposure distributions, which might have been obtained during sampling from the population of interest and during sampling of foods. The mean and P95 were estimated for each of those 100 alternative exposure distributions, yielding 100 alternative exposure statistics. The median value (regarded as the best estimate) and its 95% uncertainty interval around the exposure estimates were obtained from those 100 alternative exposure statistics.

3. Results

3.1. Personalised modified reference point index

We calculated distributions of the potency weighted lead-equivalent exposures for substances relevant to IQ loss relative to the acceptable level of lead exposure, which we termed personalised modified reference point index (mRPI). Fig. 1 shows the personalised mRPI-distributions of women of child-bearing age from 8 European countries, calculated for the lower bound scenario, where analytical non-detects were set to zero. For the majority of the populations, personalised mRPIs were larger than 1. To make our findings comparable with risk assessments usually performed at the median (P50) or the 95th percentile (P95) of exposures, we additionally listed the P50 and P95 personalised mRPIs in Table 3. In the lower bound scenario, P50 personalised mRPI exceeded the ESRV of lead by between 1.3-fold for Slovenian women in their childbearing age and 7.6-fold for Italian toddlers. Approximately twofold higher P50 personalised mRPI were observed for the upper bound scenario in which we set analytical non-detects to the limit of detection. At P95, the personalised mRPI ranged from 4.4 for Austrian adolescents to 21 for Danish toddlers in the lower bound scenario. In the upper bound scenarios, 1.5-fold higher personalised mRPI were obtained. There was no exposure scenario, population subgroup or country, where the personalised mRPI stayed at or below the value of 1 for the entire population.

3.2. Main substances contributing to the personalised mRPI

Fig. 2 shows the contribution (expressed as percentages) of the different substances to the combined lead-equivalent exposures relative to the acceptable exposure to lead, personalised mRPI, of the various populations we examined in the selected countries.

Lead was an important contributor to the personalised mRPI in both the LB (non-detects set to zero) and UB scenarios (non-detects set at the level of quantification) in most of the countries and for several age groups. Lead alone made up between 15% of the personalised mRPI in Slovenian toddlers and 40% in Austrian adolescents in the LB scenario. In the UB scenario, this rose to between 35% for Danish toddlers and 52% for Austrian adolescents.

Non-dioxin-like PCBs also had a significant impact on the personalised mRPI, ranging from 17% for Austrian adolescents to 57% for Danish toddlers in the LB scenario and 14% for Austrian adolescents to 44% for Danish toddlers in the UB scenario.

For some countries, methyl mercury had a considerable influence on the personalised mRPI while for others its contribution was relatively small. It varied from 8% for Danish toddlers to 38% for Italian women in their childbearing age in the LB scenario. For the UB scenario it ranged from 6% in Austrian adolescents to 27% for Italian women in their childbearing age.

Fluoride showed differing impacts to the personalised mRPI, ranging from 4% for Slovenian toddlers in the LB scenario to 24% for Austrian women in their childbearing age. For the UB scenario, fluoride contributions varied between 4% (Italian toddlers) to 17% (Austrian women in their childbearing age).

Inorganic arsenic did not contribute strongly to the personalised mRPI, making up only 5–10% in the LB scenario and 8–11% in the UB scenario.

The sum of 4 PBDEs were of minor importance to the personalised mRPI in all countries. Their contribution amounted to only 2% or less in both the LB and UB scenarios.

3.3. Risk-driving food-substance combinations

Next, we analysed which food-substance combinations made up most of the intake of chemicals that considerably contributed to the personalised mRPI in the different countries (Table 4).

In all countries, and under both the lower and upper bound assessment scenarios, fluoride in drinking water contributed significantly to the personalised mRPI, varying from 6 to 24%. Methyl mercury in fish and seafood strongly impacted the personalised mRPI in all countries and age groups. This ranged from 8% (Denmark) to 38% (Italy). Lead derived from grains and grain products made up 5–11% of the personalised mRPI. Other notable sources of lead intake were vegetables and products thereof with a contribution to the personalised mRPI of up to 10% and fruit and fruit products (up to 12%). The most important source of NDL-PCB intake was from fish and seafood (8–38% of the personalised mRPI). Dairy products also significantly contributed to NDL-PCB intake (5–10% of personalised mRPI). In some countries, special foods were an important source of NDL-PCBs. This was due to fish oil supplements.

4. Discussion

Our case study shows that the component-based approach for performing MRA following EFSA guidance for grouping and exposure-based prioritisation of chemicals (EFSA 2021a) provides powerful information to risk managers on mixtures of different classes of dietary contaminants. Those mixtures have a high co-occurrence rate in biomonitoring studies. Apart from information on exceedances of the acceptable combined exposures, it provides information of sources of exposure, which could feed into re-evaluations of legal limits of substances in food. Considering chemicals relevant for IQ loss, the median and P95 personalised mRPIs exceeded the value of 1 in all populations. Lead and NDL-PCBs contributed strongly to the personalised mRPI, followed by methylmercury, fluoride and iAs. PBDEs only marginally influenced the combined risk. We also show that the food-substance combinations that contributed most to the combined risk are dairy, fish and seafood for NDL-PCBs, grains and fruits for lead, methyl mercury in fish and seafood, fluoride in drinking water and iAS in grains. There are some strengths and weaknesses in our case study which we discuss below.

4.1. Strengths and limitations of the study

4.1.1. Strengths

A major strength of our case study is in the use of combinations of food consumption data and data on the occurrence of our selected chemicals in food. This allowed us to establish country-specific distributions of personalised mRPIs. This level of detail was not achieved in MRA studies that relied on summary statistics of exposures at the median or the P95 (see for example Vejdovszky et al., 2019, EFSA 2019, Boberg et al., 2021, Sprong et al., 2020, Evans et al., 2016, Martin et al., 2017). The use of such summary statistics cannot deal with the fact that individuals highly exposed to one chemical will not necessarily experience high exposures to another substance. For example, in our study a vegetarian with high lead exposures due to large consumption of vegetables will not also be highly exposed to methyl mercury and NDL-PCBs in fish. The summing of lead exposure equivalents derived from high exposure percentiles is over-conservative. Distributions of personalised mRPIs for MRA provide rather realistic assessments and can therefore be regarded as a high tier MRA. Similar observations were made recently by

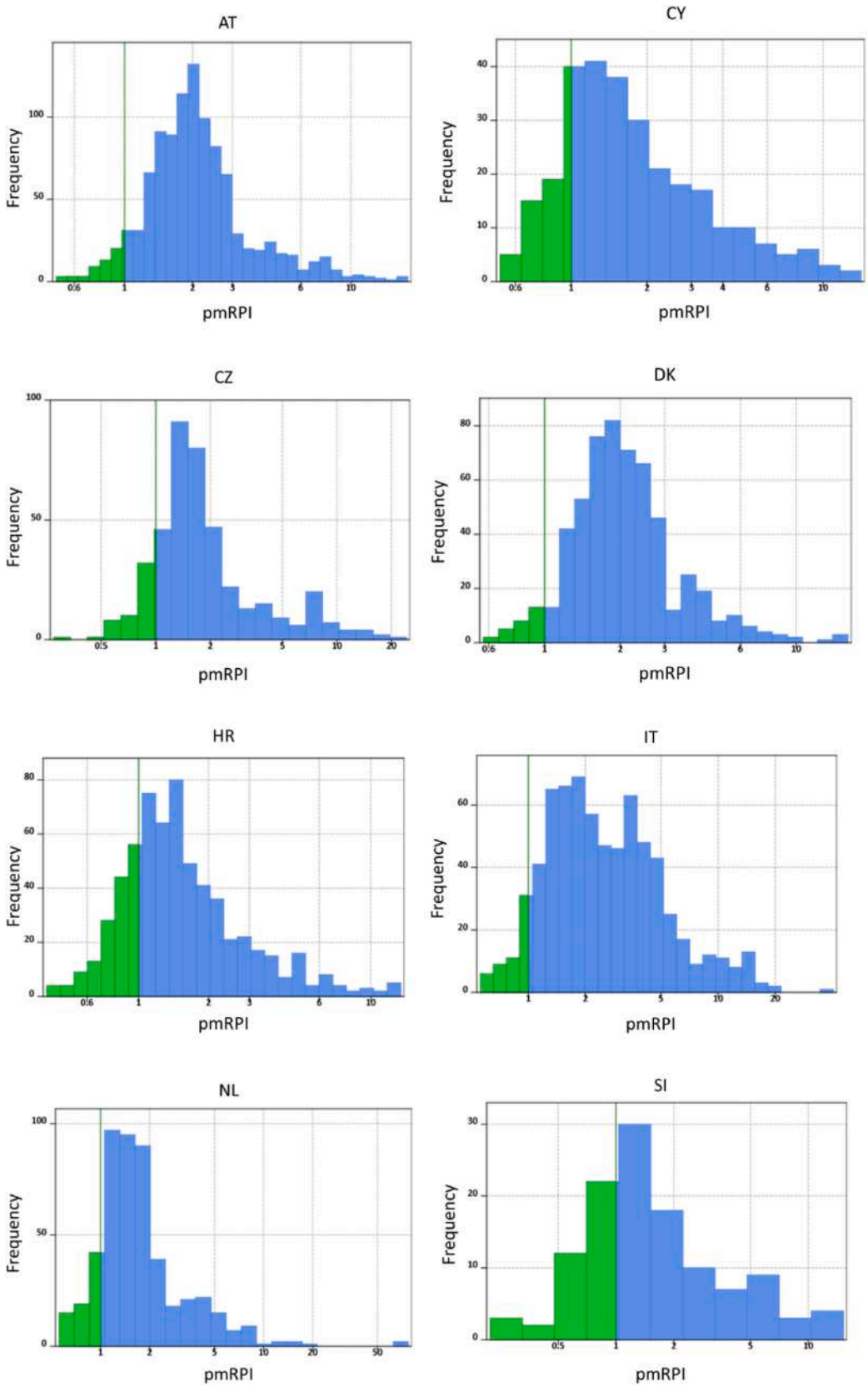
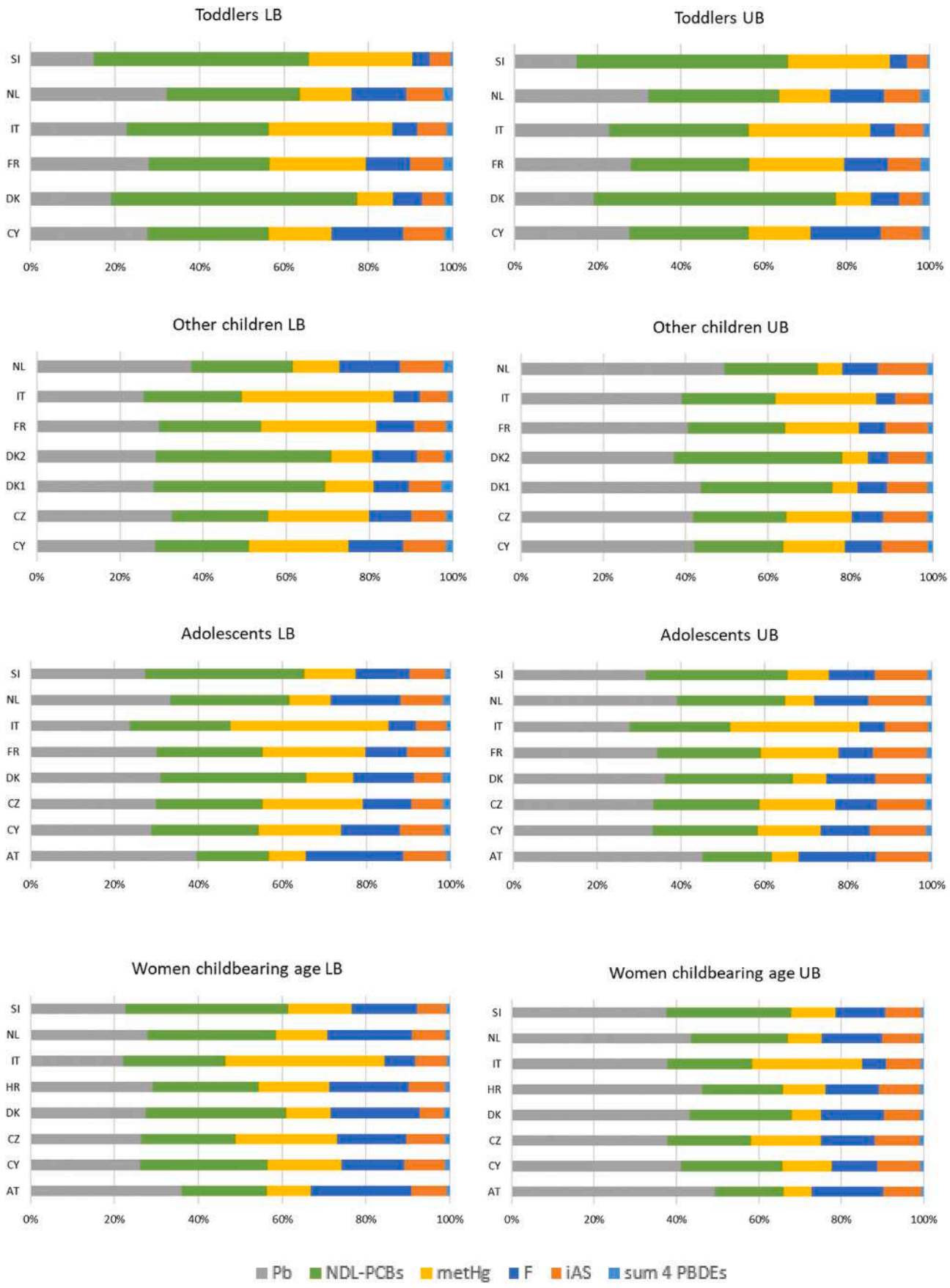


Fig. 1. Distribution of personalised modified reference point indices (pmRPI), which are potency weighted lead-equivalent exposures) for substances relevant to loss of intelligence scores (lead, methyl mercury, inorganic arsenic, fluoride, non-dioxin-like polychlorinated biphenyls and polybrominated diphenyl ethers) relative to the acceptable level of lead exposures (= 1), for women in their childbearing age (18–45 years) in 8 European countries. Results for the lower bound scenario, in which analytical values below the limit of detection or limit of quantification were assumed to equal 0, are shown. AT-Austria, CY-Cyprus, CZ-Czech Republic, DK-Denmark, HR-Croatia, IT-Italy, NL-Netherlands, SI-Slovenia. Values of pm RPI showing acceptable combined exposures (<1) are shaded green, those exceeding the index value of 1 are shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



(caption on next page)

Fig. 2. The percentage contribution of chemicals relevant to IQ loss to the personalised modified reference point index of the total population for toddlers (1–2 years old), other children (3–9 years old), adolescents (10–17 years old) and women in their childbearing ages (18–45 years) of 9 different European countries:Austria-AT, Cyprus-CY, Czech Republic-CZ, Denmark-DK (DK1 and DK 2 denote two different food consumption surveys for the particular subpopulation with DK1 providing the results of 3 years old children and DK2 results of children aged 4–9 years old), France-FR, Croatia-HR, Italy-IT, Netherlands-NL, Slovenia-SI, and for two different scenarios (lower bound-LB and upper bound-UB). For the LB scenario analytical values below the limit of detection or limit of quantification were assumed to equal 0, and for the UB scenario values below the limit of detection or quantification were assumed to equal the value of the particular limit. Pb: lead; NDL-PCBs: Non-dioxin-like PCBs; metHG: methyl mercury; F: fluoride; iAs: inorganic arsenic; PBDE: polybrominated diphenyl ethers.

Van den Brand et al. (2022) in their personalised mRPI distributions for mycotoxins and in the personalised MRA based on the HI approach for deteriorations of semen quality by Kortenkamp et al. (2022).

Another strength of our study is that we used reference values for specific effects, i.e. declines in cognitive ability as measured in terms of IQ loss. This is a rather refined way of performing MRA which avoids the mixing of toxicities as may be the case in low tier assessments based on HBGVs derived for different critical toxicities. Thus, in our case, we could rely on two HBGVs derived for IQ loss (lead, methyl mercury EFSA 2010a; US EPA 2001). In contrast, the HBGV for iAs is based on cancers of the lung, skin and bladder, as well as skin lesions; for NDL-PCBs it is based on liver and thyroid toxicity for NDL-PCBs (EFSA 2009; EFSA 2005). The use of these HBGVs would have biased our assessment. There is currently no HBGV for fluoride. We therefore estimated the corresponding ESRV for IQ loss for iAs, NDL-PCBs and fluoride based on epidemiological data. While there is evidence of associations of PBDE exposures with IQ loss (Eskenazi et al., 2013), there is no information on PBDE congener-specific associations which would have made it difficult to utilize the PBDE congener-specific food occurrence data. We therefore adopted the congener-specific hazard data derived by EFSA (2011a) for developmental neurotoxicity in rodents. Thus, assessments based on reference doses for specific endpoints, as we used in our approach and which shaped the personalised mRPI approach in Vejdovszky et al. (2019), the POD index (EFSA, 2019a), the chemical risk calculator (Boberg et al., 2021) and the normalized total margin of exposure approach (Sprong et al., 2020), provide a more realistic risk assessment.

However, the *de novo* derivation of reference values for specific endpoints can require extensive literature reviews and may be rather resource-intensive, while HBGVs or HBM-GVs are usually more readily available, e.g. in databases such as EFSA's OpenFoodTox database (Kovarich et al. 2016). Approaches based on such values also have merits in that they can provide lower tier MRAs which can be refined if the assessment indicates exceedance of combined acceptable levels (EFSA, 2019a).

4.1.2. Limitations

A major limitation of our study is that non-dietary routes of exposure, such as air, dust and soil, are not considered. Consequently, we very likely underestimated risks from combined exposures. However, for the general population in Europe there is good evidence that non-dietary exposures to lead, iAs, NDL-PCBs and PBDEs are of minor importance compared to dietary exposures. This may not always be the case for children, where uptake via dust and soil can be important routes of exposure to lead, iAs and PBDEs, particularly in highly contaminated areas (EFSA 2010a; EFSA 2011a; EFSA 2012a; EFSA 2005; EFSA 2009).

However, some studies revealed a larger role of non-dietary exposure to PBDE, since ingestion and dermal contact of dust were the major pathways of exposure to PBDE in an American study, accounting for 56–77% of the total exposure in toddlers, children, adolescents and adults, whereas diet only accounted for 20–40% (Johnson-Restepro and Kannan, 2009). In another recent American biomonitoring study, PBDEs exposure was the greatest contributor to IQ loss, followed by lead, organo-phosphates and methyl mercury (Gaylord et al., 2020), while our results show that the contribution of PBDEs to the combined exposure was only limited. This is likely explained by our inability to capture non-dietary exposures to PBDEs in our study; exposure from all routes is accounted for in human biomonitoring studies. Other factors can also explain the observed differences, among them the limited number of analytical data in our study, the differences in PBDE concentrations in dust and food between the US and Europe (Zota et al., 2008; EFSA 2009), the number of PBDEs included, i.e. PBDE- 47, -99, −153, −209 in our study, PBDE-47 in the study of Gaylord et al. (2020) and 20 PBDEs among which the congeners - 47, −99, −153, −209 in the study of Johnson-Restepro and Kannan (2009), and assuming equipotency of all PBDE isomers in other studies.

Some studies also pointed at a larger role for non-dietary sources of NDL-PCBs (Lehmann et al., 2015; Li et al. 2018)), Although banned in the United States and the European Union some decades ago (Lehmann et al., 2015; EFSA 2005), PCBs can be present in the indoor air and dust of many older buildings because of the use of NDL-PCB containing elastic sealants, caulking, paints, and flame retardant coatings (Lehmann et al., 2015). Large contributions of indoor air to the total exposure was shown for all age groups (Lehmann et al., 2015; Li et al. 2018), with contributions observed up to 60.8, 50.5, and 34.6% for children ages 2–3 years and 6–12 years and adults, respectively (Lehmann et al., 2015). Other dietary sources (e.g. tea) and routes of exposure are also relevant for fluoride, such as dental hygiene products, but the information accessible to us was too limited to draw conclusions on their contribution to total fluoride exposures (EFSA 2013). To obtain a more complete picture of the combined exposure to chemicals relevant for IQ loss, other routes of exposure can be included in external exposure assessment. Methodologies to aggregate the exposure from several routes are available (e.g. Husøy et al., 2020: aggregated exposure of di (2-ethylhexyl) phthalate from diet and personal care products) and have been implemented in MCRA (Van der Voet et al., 2020). However, chemical concentration data in consumer products and indoor air was not yet available for the substances included in our case study, and neither were levels in soil and outdoor air (IPCHEM database accessed 22 March 2022).

Another important limitation of our study that leads to an underestimation of the risk is that only certain contaminants were considered. Recently, an endpoint-specific reference value for IQ loss was estimated for cadmium (Chatterjee and Kortenkamp 2022). Cadmium is frequently detected in human biomonitoring samples (Haug et al., 2018; Buekers et al., 2021) at high occurrence rates (e.g. 99.6% and 96.5% quantifiable samples in mothers and child, respectively; Haug et al., 2018). Therefore, cadmium may significantly contribute to the risk of IQ loss.

In addition, human biomonitoring data showed co-exposure to substances from other regulatory domains, such as organophosphate pesticides (Haug et al., 2018), which are also relevant for IQ loss (Grandjean and Landrigan, 2006, 2014). As outlined in the method section, ESRVs of organophosphate pesticides for IQ loss were not readily available. Another issue with adding pesticide exposures to the combined exposure of contaminants is how to integrate different exposure scenarios deemed relevant for the particular regulatory silos. Where the LB and UB scenario is used by EFSA to estimate the risk of contaminants, such as the metals and persistent organic pollutants in our case study, for pesticides other refined scenarios with assumptions for agricultural use based on authorized uses are considered more realistic (EFSA 2020a; and b, EFSA et al., 2022; van Klaveren et al., 2019a and b). Currently, advanced exposure tools calculating exposure distributions are currently unable to deal with different exposure scenarios simultaneously and therefore, combined exposure of substances is often limited to summing percentiles (Sprong et al., 2020). The development of a tool that would be able to aggregate exposures from different regulatory frameworks by allowing simultaneous calculations using different exposure scenarios and

Table 4
Food-substance combinations contributing most to the combined dietary exposure to substances relevant for loss of intelligence presented for toddlers (1–2 years old), other children (3–9 years old), adolescents (10–17 years old) and women in their childbearing ages (18–45 years) of 9 different European countries (Austria-AT, Cyprus-CY, Czech Republic-CZ, Denmark-DK, France-FR, Croatia-HR, Italy-IT, Netherlands-NL, Slovenia-SI) and for two different scenarios (lower bound-LB and upper bound-UB)[a]. Percentages between brackets reflects the fraction of the personalised modified reference point index that can be attributed to the particular food-substance combinations.

Country	Toddlers	Other Children	Adolescents	Women child bearing age
Lower bound				
AT	–	–	F drinking water (23%) Pb grain (products) (11%) MetHg fish & seafood (9%) iAs grain(products) (6%) NDL-PCBs fish & seafood (6%)	F drinking water (24%) MetHg fish & seafood (10%) Pb grain (products) (7%) Pb vegetable (products) (7%) NDL-PCBs fish & seafood (7%)
CY	F drinking water (17%) MetHg fish & seafood (15%) NDL-PCBs fish & seafood (13%) NDL-PCBs dairy (8%) Pb Grain (products) (6%)	MetHg fish & seafood (23%) NDL-PCBs fish & seafood (13%) F drinking water (13%) Pb grain(products) (8%) NDL-PCBs dairy (6%)	MetHg fish & seafood (20%) F drinking water (14%) NDL-PCBs fish & seafood (14%) Pb grains (products) (7%) NDL-PCBs dairy (5%)	MetHg fish & seafood (18%) F drinking water (15%) NDL-PCBs fish & seafood (12%) NDL-PCBs special foods (9%) Pb grain (products) (6%)
CZ	-	MetHg fish & seafood (24%) NDL-PCBs fish & seafood (12%) F drinking water (10%) Pb grain(products) (7%) NDL-PCBs in fats and oils (6%)	MetHg fish & seafood (24%) NDL-PCBs fish & seafood (13%) F drinking water (12%) Pb grain(products) (7%) NDL- PCBs fats and oils (7%)	MetHg fish & seafood (24%) F drinking water (16%) NDL-PCBs fish & seafood (12%) NDL-PCBs fats and oils (5%) Pb grain (products) (5%)
DK	NDL- PCBs fish & seafood (47%) MetHg fish & seafood (8%) NDL-PCBs dairy (7%) F drinking water (7%) Pb grain(products) (6%)	DK1[b] NDL-PCBs fish & seafood (31%) F drinking water (10%) MetHg fish & seafood (9%) Pb grains (products) (8%) NDL-PCBs dairy (7%) DK2[b] NDL-PCbs fish & seafood (41%) MetHg fish & seafood (9%) NDL-PCBs dairy (8%) F drinking water (7%) Pb grains(products) (6%)	NDL-PCBs fish & seafood (22%) F drinking water (15%) MetHg fish & seafood (11%) Pb grain(products) (10%) NDL-PCBs in dairy (7%)	NDL-PCBs fish & seafood (24%) F drinking water (21%) MetHg fish & seafood (11%) Pb grain (products) (6%) Pb vegetable (products) (6%)
FR	MetHg fish & seafood (23%) NDL-PCBs fish & seafood (13%) F drinking water (11%) NDL-PCBs dairy (10%) Pb Grain (products) (7%)	MetHg fish & seafood (27%) NDL-PCBs fish & seafood (15%) Pb grain (products) (10%) F drinking water (9%) NDL-PCBs dairy (6%)	MetHg fish & seafood (24%) NDL-PCBs fish & seafood (14%) Pb grain(products) (11%) F drinking water (10%) iAs grain(products) (5%)	–
HR	-	–	–	F drinking water (19%) MetHg fish & seafood (17%) NDL-PCBs fish & seafood (15%) Pb grain(products) (7%) Pb vegetables (products) (6%)
IT	MetHg fish & seafood (29%) NDL-PCBs fish & seafood (22%) Pb grain (products) products (7%) NDL-PCBs in dairy (6%) F drinking water (6%)	MetHg fish & seafood (35%) NDL-PCBs fish & seafood (15%) Pb grain (products) (7%) F drinking water (6%) NDL-PCBs fats and oils (5%)	MetHg fish & seafood (38%) NDL-PCBs fish & seafood (14%) Pb grain(products) (7%) F drinking water (7%) Pb vegetable (products) (5%)	MetHg fish & seafood (38%) NDL-PCBs fish & seafood (15%) F drinking water (7%) Pb vegetable (products) (6%) Pb grain(products) (5%)
NL	F drinking water (13%) MetHg fish & seafood (12%) NDL-PCBs special foods (9%) NDL- PCBs dairy (9%) Pb grains (products) (8%)	F drinking water (14%) MetHg fish & seafood (10%) Pb grain(products) (9%) NDL-PCBs special foods (8%) NDL- PCBs dairy (8%)	F drinking water (17%) Pb grain (products) (10%) MetHg fish & seafood (10%) NDL-PCBs special foods (9%) iAs grain (products) (6%)	F drinking water (20%) NDL-PCBs special foods (16%) MetHg fish & seafood (12%) Pb vegetable (products) (7%) NDL-PCBs fish & seafood (6%)
SI	NDL- PCBs fish & seafood (13%) F drinking water (13%) MetHg fish & seafood (10%) Pb fruit and fruit products (9%) Pb grain(products) (9%)	–	NDL-PCBs fish & seafood (21%) F drinking water (13%) MetHg in fish & seafood (12%) Pb grain(products) p(8%) NDL-PCBs special foods (7%)	NDL-PCBs fish & seafood (17%) F drinking water (16%) MetHg fish & seafood (15%) NDL-PCBs special foods (15%) Pb grain (products) (5%)

(*continued on next page*)

Table 4 (*continued*)

Country	Toddlers	Other Children	Adolescents	Women child bearing age
Upper bound				
AT	-	-	F drinking water (16%)	F drinking water (17%)
			Pb grain (products) (12%)	Pb vegetable (products) (12%)
			Pb vegetable (products) (9%)	Pb grain (products) (8%)
			Pb fruit (products) (6%)	MetHg fish & seafood (7%)
			MetHg fish & seafood (6%)	Pb drinking water (6%)
CY	F drinking water (11%)	MetHg fish & seafood (15%)	MetHg fish & seafood (13%)	Pb vegetable (products) (12%)
	NDL-PCBs in dairy (9%)	F drinking water (9%)	F drinking water (10%)	MetHg fish & seafood (12%)
	MetHg fish & seafood (9%)	Pb vegetables (products) (9%)	NDL-PCBs fish & seafood (9%)	F drinking water (11%)
	NDL-PCBs fish & seafood (8%)	Pb grain (products) (9%)	Pb vegetable (products) (8%)	NDL- PCBs fish & seafood (8%)
	Pb vegetables (products) ((7%)	NDL-PCBs fish & seafood (8%)	Pb grain (products) (8%)	Pb grain (products) (6%)
CZ	-	MetHg fish & seafood (16%)	MetHg fish & seafood (16%)	MetHg fish & seafood (17%)
		NDL-PCBs fish & seafood (8%)	F drinking water (9%)	F drinking water (13%)
		F drinking water (8%)	NDL-PCBs fish & seafood (9%)	NDL-PCBs fish & seafood (9%)
		Pb grains (products) (8%)	Pb grain (products) (8%)	Pb vegetable (products) (7%)
		Pb vegetable (products) (6%)	Pb vegetable (products) (6%)	Pb grain (products) (6%)
DK	NDL-PCBs fish & seafood (31%)	DK1	NDL-PCBs fish & seafood (13%)	NDL-PCBs fish & seafood (15%)
	NDL-PCBs dairy (9%)	NDL-PCBs fish & seafood (19%)	F drinking water (10%)	F drinking water (15%)
	Pb dairy (7%)	Pb vegetables (products) (10%)	Pb grain (products) (9%)	Pb vegetable (products) (10%)
	Pb grain (products) (6%)	Pb grain (products) (8%)	Pb vegetable (products) (9%)	MetHg fish & seafood (7%)
	MetHg fish & seafood (6%)	NDL-PCBs dairy (8%)	NDL-PCBs dairy (7%)	Pb grain (products) (6%)
		F drinking water (7%)		
		DK2		
		NDL-PCBs fish & seafood (27%)		
		NDL-PCBs dairy (9%)		
		Pb grain (products) (7%)		
		Pb vegetables (products) (7%)		
		Pb dairy (6%)		
FR	MetHg fish & seafood (14%)	MetHg fish & seafood (18%)	MetHg fish & seafood (16%)	-
	NDL-PCBs in dairy (11%)	NDL-PCBs fish & seafood (10%)	Pb grain (products) (12%)	
	Pb foods for infants and small children (9%)	Pb grain (products) (10%)	NDL-PCBs fish & seafood (9%)	
	NDL-PCBs fish & seafood (8%)	Pb vegetable (products) (8%)	Pb vegetable (products) (8%)	
	F drinking water (7%)	NDL-PCBs dairy (7%)	F drinking water (7%)	
HR	-	-	-	F drinking water (13%)
				Pb vegetable (products) ((13%)
				MetHg fish & seafood (10%)
				NDL-PCBs fish & seafood (9%)
				Pb grain (products) (8%)
IT	MetHg fish & seafood (20%)	MetHg fish & seafood (24%)	MetHg fish & seafood (26%)	MetHg fish & seafood (27%)
	NDL- PCBs fish & seafood (15%)	NDL-PCBs fish & seafood (11%)	NDL-PCBs fish & seafood (10%)	Pb vegetable (products) (13%)
	Pb grains (products) (8%)	Pb vegetable (products) (9%)	Pb vegetable (products) (10%)	NDL-PCBs fish & seafood (11%)
	NDL-PCBs dairy (7%)	Pb grain (products) (9%)	Pb grains (products) (9%)	Pb grain (products) (7%)
	Pb vegetables (products) (6%)	Pb fruit (products) (5%)	F drinking water (5%)	F drinking water (6%)
NL	Pb fruit (products) (10%)	Pb grain (products) (9%)	F drinking water (11%)	F drinking water (15%)
	NDL-PCBs dairy (9%)	F drinking water (9%)	Pb grain (products) (10%)	Pb vegetable (products) (11%)
	F drinking water (8%)	Pb vegetable (products) (8%)	Pb vegetable (products) (8%)	NDL-PCBs special foods (10%)
	Pb grain (products) (8%)	Pb fruit (products) (8%)	MetHg fish & seafood (6%)	MetHg in fish & seafood (8%)
	Pb dairy (7%)	NDL-PCBs dairy (8%)	iAs grain (products) (6%)	Pb grain (products) (6%)
SI	Pb fruit (products) (12%)	-	NDL-PCBs fish & seafood (14%)	F drinking water (12%)
	Pb grain (products) (9%)		Pb grain (products) (10%)	NDL-PCBs fish & seafood (12%)
	F drinking water (8%)		F drinking water (10%)	MetHg fish &seafood (11%)
	NDL-PCBs fish & seafood (7%)		MetHg fish & seafood (8%)	Pb vegetable (products) (10%)
	Pb vegetable (products) (7%)		Pb vegetable (products) (7%)	NDL-PCBs special foods (10%)

Pb: lead; NDL-PCBs: Non-dioxin-like PCBs; metHG: methyl mercury; F: fluoride; iAs: inorganic arsenic

[a] For the LB scenario analytical values below the limit of detection or limit of quantification were assumed to equal 0, and for the UB scenario values below the limit of detection or quantification were assumed to equal the value of the particular limit.

[b] Two Danish food consumption sources were available. DK1 reflects the results obtained for the DANSDA 2005–08 food consumption survey (children aged 4–9 years old) and DK2 those of IAT 2006–07 food consumption survey (children aged 3-years old).

results in the generation of exposure distribution would allow for more realistic exposure assessments (Sprong et al., 2020). For the purpose of this case study, only different classes of contaminants were considered.

In our study we summed the indicator NDL-PCBs in food. To obtain the total exposure to NDL-PCBs we multiplied the resulting values by 2 (EFSA, 2005). In doing so, information on the risk driving congener(s) is lost. If such information is needed, additional calculations for NDL-PCBs and PBDEs using the individual congeners could be performed.

In our study, we focussed on MRA of dietary chemicals. However, non-chemical stressors may also affect cognitive development of children. A cumulative impact assessment includes such non-chemical stressors. A recent meta-analysis showed that alongside toxic chemicals, several non-chemical stressors such as maternal health, the mother's ability to access information relevant to a healthy pregnancy, dietary nutrients and quality of social interaction, had a significant impact on the child's cognitive development (Nilsen et al., 2020). It was beyond the scope of our case study to include those non-chemical stressors.

4.1.3. Uncertainties

In common with all risk assessments, the assessment performed in our case study is affected by uncertainties. Their identification is needed to assess whether the assessment represents an over- or an underestimation of risks. This is challenging in the case of a MRA, as the uncertainties behind every single chemical assessment may multiply leading to rather complex patterns of under- or over-estimations of combined risks. For a meaningful interpretation of the risks, an integration of those uncertainties into a final conclusion of the magnitude of over- or underestimation is needed. For pesticide MRAs, EFSA used a probabilistic approach for the integration of uncertainties into an overall conclusion (EFSA 2020a; and b, 2022). They identified 31, 34 and 41 sources of uncertainty (in food consumption, concentration data, hazard data and MRA methodology) for combined exposures to pesticides relevant for chronic effects on the thyroid and for pesticides relevant for acute effects on the nervous system, and craniofacial malformation respectively (EFSA 2020a; and b, 2022). Uncertainties were quantified at the high level of exposures using expert knowledge elicitation following principles of the EFSA guidance (EFSA 2014). This methodology resulted in a factor 3–5 lower combined risks to pesticides relevant for acute effects on the nervous system and a factor 2–4 lower for chronic effects on the thyroid (EFSA, 2020a and b). It was not the purpose of our study to perform such a sophisticated uncertainty analysis as this is resource intensive, and requires several experts with different backgrounds. Instead, we identified the major uncertainties, which future in-depth uncertainty analysis could build upon. Those are described below. Where possible, the direction of the uncertainty (over- or underestimation was given).

4.1.4. Endpoint IQ loss

We selected the loss of 1 IQ point as a measure of cognitive deficits in the developing child, since this degree of cognitive decline at the population level can have an economic impact on societies (Gould, 2009; Grandjean et al., 2012; Bellanger et al., 2013; Trasande and Liu, 2011; Pichery et al., 2011; Gaylord et al., 2020). IQ tests usually consists of several subtests, each measuring a different aspect of cognitive development, such as memory, verbal and spatial reasoning, planning, learning and the comprehension and use of language. Developmental neurotoxicants could affect a certain aspect of cognitive functioning rather than all aspects (EFSA 2010a). The PODs in our study were obtained from heterogenous endpoints, varying from general cognitive indexes (methyl mercury and fluoride), full scale IQ scores (lead, NDL-PCBs) or raw verbal IQ scores (iAs; Table 1). The use of such heterogenous endpoints in a combined assessment could result in uncertainty. This is probably limited for the general cognitive index, which showed concurrent validity with intelligence tests, including the Stanford–Binet IQ (r0.81) and full-scale IQ score (r0.71) from the Wechsler Preschool and Primary Scale of Intelligence (WPPSI) (Grandjean et al., 2022; Kaplan and Sacuzzo, 2010). The uncertainty caused by only including a particular subset (e.g. only verbal IQ or performance IQ) could be larger as exemplified for iAs. Here, the endpoint was based on raw verbal IQ scores. While iAs also affected full scale IQ score, the association between iAs intake and adverse effect in children was by a factor of 3 lower compared with verbal IQ scores as performance and processing speed were not affected (Tsuji et al., 2015). Thus, the use of heterogenous endpoints (full IQ scores or particular IQ scores) may have biased our calculation of personalised mRPI. One could therefore question whether a MRA should be performed based on mixing ESRV for full IQ scores and more specific cognitive functions.

The chemicals in our cumulative assessment group showed sex-specific sensitivities on cognitive development. For example, the ESRV of iAs was based on IQ loss observed in girls, which showed larger IQ losses than boys. In contrast, for fluoride larger effects on IQ loss were observed in boys. Yet, both boys and girls were included for the derivation of the ESRV. As generally the ESRV is based on the most sensitive gender, inclusion of both boys and girls in the derivation of the ESRV would have led to an underestimation of the personalised mRPI.

4.1.5. Model assumptions

Implicit in our adding up of lead-equivalent risk quotients in the personalised mRPI is the assumption of dose addition. The possibility of synergisms or (partial) antagonisms was not considered and this is a potential source of under- or overestimations of risks. However, a recent systematic review of the frequency of synergisms has shown that dose addition provides a good approximation of expected mixture effects (Martin et al., 2022).

4.1.6. Likelihood of co-exposure

In our case study, the likelihood of co-exposure to several pollutants was addressed by reviewing human biomonitoring detection rates (i.e. percentage of measurements above the LOD or LOQ, whatever applicable). For example, in the study described by Haug et al. (2018) occurrence rates varied from 54% for PBDE-153 in children aged 6–12 years to 100% for lead in maternal and children's blood. Occurrence rates of 90% of higher were observed for several PCBs, PBDE-47, mercury and lead, which indicates a high chance of co-occurrence. Besides PBDE-153, a lower occurrence rate was observed for arsenic (59% in maternal blood and 67% in children aged 6–12 years). Hence the chance of co-exposure to PBDE-153 and iAs is smaller, but still present. Assuming 100% co-exposure as we did in our study may have overestimated the personalised mRPI. More sophisticated methods to assess co-exposure patterns based on biomonitoring data are available, such as network analysis (Ottenbros et al., 2021), or external exposures such as the Sparse non-negative matrix under-approximation which has been applied to mothers' milk (EFSA 2021a; Crépet et al., 2022). Application of these methodologies may refine our analysis.

Human biomonitoring studies usually only analyse total mercury and arsenic (e.g. Haug et al., 2018; Julvez et al., 2021). As only methyl mercury and iAs are relevant for IQ loss, establishing co-exposures based on inspecting occurrence rates of total mercury and arsenic may introduce an element of uncertainty regarding co-exposures to methyl mercury and iAs. Measuring different forms of the metals may improve determination of co-exposures. Inclusion based on occurrence rates of total mercury and arsenic may have resulted in overestimations of exposure in the assessment, since occurrence rates of methylmercury and iAs may differ.

We also included fluoride in our case group, because fluoridation of drinking water is common practice in some European regions. Fluoride is not always considered in human biomonitoring studies. As fluoride may be obtained from other (dietary) sources (see 4.2.1 limitations) inclusion of fluoride in biomonitoring programs would be helpful to establish real-life mixtures.

4.1.7. Assessment group membership

With respect to the grouping of substances based on an effect on IQ loss, it should be noted that adverse effects of iAs and fluoride on IQ loss are still under debate. Based on the available evidence, the overall association between low-dose iAs exposure and IQ loss was considered as weak and therefore Tsuji et al. (2015) included only the study of Hamadani et al. (2011), a well-controlled study from the Bangladesh cohort with the most pronounced effect of iAs on IQ loss for the establishment of an ESRV. Some may question whether a substance can be considered as a member of the assessment group for IQ loss, based on overall weak associations with cognitive declines. According to the EFSA guidance on grouping, a higher degree of certainty in grouping efforts can be achieved when knowledge of an adverse outcome pathway (AOP) or the mode of actions is available (EFSA 2021a). Comprehensive AOPs for IQ loss have not yet been constructed and there is limited information on the mode of actions for IQ loss for the substances in our assessment group. Only the metals play a role in the AOP for deficits in learning and cognition (Von Stackelberg et al., 2015), but for the other substances included in our study the available information is limited. A putative AOP for developmental neurotoxicity as part of an integrated approach to testing and assessment was proposed recently by the EFSA PPR panel (EFSA 2021c). The use of AOPs in the classification of substances that have an effect on IQ loss can be evaluated in future studies. As iAs was included in the AOP of von Stackelberg et al. (2015), we included iAs in the assessment group IQ loss.

The detrimental effect of fluoride on cognitive function at low dose exposures in community fluoridation areas has been doubted by Guth et al. (2020, 2021). According to those authors, effects are predominantly observed in highly contaminated areas and from studies with shortcomings in design, such as small sample size and no adjustment for important cofounders, such as maternal IQ and co-exposure to other neurotoxicants. Some well-designed prospective studies from community water fluoridation areas which allowed for controlling well-known confounding factors showed contradictory results.

With respect to PBDE, two recent systematic reviews showed an adverse association between PBDE exposure and cognitive development in children (Lam et al., 2017; Gibson et al., 2018). Lam et al. (2017) concluded that sufficient evidence existed to support and association between developmental PBDE exposure in humans and IQ loss in children, Gibson et al. (2018) were more precautious in their conclusion because several uncontrolled confounders, such as co-exposure to known neurotoxicants, lack of statistical power due to small sample sizes and no statistical correction for multiple comparison, might have affected the outcome and impaired comparison across studies. Therefore, Gibson et al. (2018) advocated standardization of outcome assessment in future work.

4.1.8. ESRVs

Benchmark dose modelling is the preferred approach to establish ESRVs, since it makes a more extended use of dose-response data and it allows for quantification of the uncertainties in the dose-response data, in contrast to more simple approaches such as the NOAEL (EFSA 2017). To take the uncertainty of the benchmark dose into account, the lower bound of the confidence interval BMDL around the bench mark dose is used to derive the POD. In our case study, ESRVs based on BMDLs were obtained for lead, methyl mercury, fluoride, NDL-PCBs (Table 1 main text; all based on epidemiological data) and PBDEs (animal data). For iAs, only a LOAEL was available, which indicates that the ESRV of iAs is less robust.

In our study, we predominantly used ESRVs that were already published. As the aim of our case study was a proof of principle rather than a comprehensive risk assessment, we did not update established ESRVs as this was beyond the scope of our case study. Future research could update and/or refine ESRVs by using data from well-equipped mother/child cohorts addressing cognitive development, such as the HOME cohort; Kalloo et al., 2020, Braun et al., 2017 or the HELIX cohorts (Maitre et al., 2018). In our paper, we describe the uncertainties and indicate, where possible, whether this led to an under- or overestimation of the risk and the subsequent identification of the risk drivers. Table 5 summarizes those uncertainties, together with the direction of the effect on the risk. A detailed explanation on the uncertainties around the ESRVs is provided in Supplemental material E. A general uncertainty was the extrapolation of ESRVs derived for a certain age group to another age group, as was done for several substances (Tables 1 and 5). When extrapolating a reference point in urine or blood into an external dose, differences in kinetics between children and adults should be taken into account. Frequently noted uncertainties leading to overestimations of the personalised mRPI were: uncontrolled confounding (lead and iAs), cumulation of conservative assumptions for kinetic modelling (lead, iAs and PBDEs), and the choice of UFs (methyl mercury and PBDEs). An underestimation of the personalised mRPI was considered due to extrapolation of BMDLs (lead: extrapolation of the BMDL of women in their child-bearing age to toddlers and other children), assumptions for kinetic modelling (for fluoride), uncontrolled confounding for positive effects of fish consumption (methylmercury), inclusion of all dioxin-like PCBs in the ESRV of NDL-PCBS, and ignoring other relevant PBDE congeners.

4.1.9. Exposure data

Uncertainties in exposure data are related to food consumption data, occurrence data and matching food consumption data to concentration data. Sampling uncertainty in food and consumption data due to limited sampling size was quantified by bootstrapping (Efron 1979; Efron and Tibshirani 1993). This yielded the boundaries of the uncertainty interval around the personalised mRPI listed in Table 3, which indicate what the personalised mRPI could have been if other samples from the population and foods were used, assuming that representative sampling was applied. Generally, the upper boundary was about a factor 1.2 higher than the lower boundary at median personalised mRPI estimates, for the P95 the upper boundary was about a factor 1.5 higher. Only for some subpopulations was the ratio between the upper and lower boundary larger. This was predominantly applicable for subpopulations with a smaller size of less than 200 (Table 2). Exposure percentiles obtained for small subpopulations are statistically less robust. EFSA indicated that percentiles calculated over a number of subjects/days lower than 60 for the 95th percentile requires a cautious interpretation of the results since they may not be statistically robust (EFSA 2011b). As none of the lower boundaries of the uncertainty interval around the personalised mRPI is smaller than 1, the impact of sampling uncertainty on MRA is small.

Uncertainty around samples below the LOQ was addressed by the lower and upper bound scenario where those samples were substituted by zero or the value of the LOQ, respectively. Those scenarios were selected as they are frequently performed in risk assessment of contaminants. Other more realistic scenarios are available, such as the median bound (in which samples below the LOQ are assumed to equal half the value of the LOQ) and more sophisticated scenarios considering the distribution of samples below and above the LOQ.

Several other sources of uncertainties could not be quantified in our assessment. Those are listed in Table 6. A detailed description of the uncertainties is provided in Supplemental material F. Uncertainties included the use of the food coding system and assumptions made to handle data gaps. Table 6 also indicates the direction of the uncertainty: over- or underestimation of the personalised mRPI. In many cases, the direction of uncertainty was indeterminate. An exception was the use of conversion factors for methylmercury which resulted in an overestimation of the personalised mRPI and the contribution of methylmercury to the personalised mRPI. In addition, aggregation of foods in higher hierarchical FoodEx groups if less than 50 measurements per food group resulted in an overestimation of the iAs exposure and thus the personalised mRPI. Due to aggregating of foods (e.g. pasta, which could consist of rice-based pasta, such as rice noodles, and wheat-based pasta) and the oversampling of rice-based products compared with products

Table 5
Summary of sources of uncertainty around the endpoint-specific references values for IQ loss for lead, methyl mercury, inorganic arsenic (iAs), fluoride, non-dioxin-like perchlorinated biphenyls (NDL-PCBs) and polybrominated diphenyl ethers (PBDEs) and their effect on the personalised modified reference point index (personalised mRPI).

Substance	Type of uncertainty	Description	Direction effect on personalised mRPI	Reference
Lead	Uncontrolled confounding	Uncontrolled confounding, measurement error and other potential causal factors as common weaknesses were identified in the study of Lanphear, particularly for lead concentrations in blood below 50 or 100 μg/L. Whether this also affected piecewise linear function with breakpoint at 100 μg/L used by EFSA for the derivation of the $BMDL_{01}$ is not known to us.	+/−	Wilson and Wilson (2016), Van Landingham et al. (2021)
	Kinetic modelling	Conservative assumptions used for modelling dietary exposure out of blood concentrations.	+	EFSA 2010a
	Extrapolation $BMDL_{01}$ women childbearing age to other age groups	EFSA derived two $BMDL_{01}$s for IQ loss, one of 0.5 μg/kg bw per day for children aged 0–7 years and another one of 0.54 μg/kg bw/day for women in their child bearing age. Only 0.54 μg/kg bw/day was used in our study.	- (toddlers, other children)	EFSA 2010a
	Choice of UF	EFSA was in their opinion on the risk of lead not very clear which margin of exposure would be adequate, it could be interpreted as both 1 or 10. We used an UF of 1, while 10 could have been more appropriate.	–	EFSA 2010a
Methyl mercury	Point of departure	Considerable study uncertainty in the quantification of IQ loss upon prenatal methyl mercury exposure, with regression coefficients varying from 0 (no effect) to 1.5 (i.e. increase in the maternal hair concentration with 1 μg/g resulted in a loss of 1.5 IQ point). Differences could be explained by distinct exposure patterns, population genetic variability and nutrition (e.g. n-3 fatty acid intake).	+/−	Cohen et al. (2005)
	Uncontrolled confounding	While Rice et al. (2003) investigated the confounding effect of PCBs, they did not consider confounding beneficial effects of n-3 fatty acids in fish. When those were taken into account the ESRV was close to 0.1 μg/kg bw. Appropriate UFs were not provided.	- (if an UF is to be taken into account)	Groth (2017)
	Linear extrapolation $BMDL_{05}$ to $BMDL_{01}$	Not clear whether the UF of 10 covers the uncertainty caused by linear extrapolation of a $BMDL_{05}$ to a $BMDL_{01}$ as we did in our study	+/−	
	Choice of UF	Choice of UF varied from 10 in studies for IQ loss to 6.4 for other DNT effects derived from the same population(s). Difference is based on whether inter-species differences in toxicodynamics would require an additional UF. Rice et al., adapted the UF of US EPA (10), which based their study on 1 population. Rice et al. showed that the ESRV would not change when other (more sensitive) populations were included. EFSA and JECFA concluded that an UF for inter-species differences in toxicodynamics was not needed as a sensitive population was included. It should be noted that JECFA concluded that the UF could be further refined and reduced.	+	Rice et al. (2003) US EPA (2001) JECFA (2004) EFSA (2012)
iAs	Point of departure	Study uncertainty in the quantification of IQ loss due to large variability in studies caused by different study designs. The LOAEL was based on one well-designed study in a possible sensitive population due to malnutrition. Other well-designed studies were performed after the study of Hamadani. BMD modelling from all eligible studies would reduce uncertainty.	+/−	Tsuji et al. (2015)
	Uncontrolled confounding	Hamadani incompletely assessed maternal IQ, which is well-known confounder. Adjustment for study IQ in another study attenuated the association between iAs exposure and IQ loss. Studies performed after the study of Hamadani showed modest declines of IQ scores, with effects being more pronounced in girls than in boys. Residual confounding, such as exposure to other neurotoxicants could not be excluded.	+	Wasserman et al. (2011) Vahter et al. (2020)
	Kinetic model	Choice of parameter values and assumptions: Conservative assumption urinary excretion rates	+	Tsuji et al. (2011)
		Fraction of oral dose excreted in urine based on monkeys	+/−	
Fluoride	Point of departure	Boys more sensitive to the effect than girls. BMCL for pooled data (boys and girls used).	–	Grandjean et al. (2022)
	Kinetic model	The fractional retention of fluoride is only constant (i.e. 36% for adults) at a daily dietary intake of 2 mg/kg or higher (Villa et al., 2010). Below a total daily intake of 0.8 mg/day, fluoride excretion exceeds the intake, resulting in a negative fluoride balance. This means that at a daily urinary excretion of 0.3 mg, the consequent intake would be smaller than 0.3 mg/day instead of 0.6 mg/kg. Taken together, a urinary excretion of 0.3 mg/day leading to 1 IQ point loss is highly uncertain.	–	Rugg-Gunn et al. (2011), Villa et al. (2010)
		Extrapolation of maternal kinetics to children. Kinetics differ for different age groups	–	
		Use of upper boundary of interval around intake levels (highest intake), selection of midpoint would have led to a lower ESRV	–	
NDL-PCBs	Point of departure	ESRV based on total PCBs, which included dioxin-like PCBs, which are not associated with developmental neurotoxicity.	–	Jacobson et al. (2002), JECFA 2016 EFSA 2018

(*continued on next page*)

Table 5 (*continued*)

Substance	Type of uncertainty	Description	Direction effect on personalised mRPI	Reference
	Congener specific toxicity	Toxic potency could also differ between congeners. Preliminary neurotoxicity equivalency factors have been proposed, but not included in our study. The use of well-established neurotoxicity equivalency factors would result in a more precise estimation of the contribution of (individual) NDL-PCB to the combined exposure.	+/−	Simon et al. (2007), Rayne & Forest (2010), Pradeep et al., 2019).
	Kinetic model	Use of half-life of 10 years for all NDL-PCBs. NDL-PCB half-lives differ from 2.6 years for PCB 52 to 14.1 years for PCB 153	+/−	Ritter et al. (2011)
	Choice of UF	UF of 2 was applied for the extrapolation $BMDL_{05}$ to $BMDL_{01}$	+/−	
PBDEs	Point of departure	$BMDL_{10}$ for developmental neurotoxicity in animals rather than humans. It is not clear how the findings in the developmental neurotoxicity study in animals actually relate to IQ loss in children. Ideally data from epidemiological studies should be used. A recent meta-analysis of four prospective cohort studies pointed at a dose-dependent relationship between PBDE exposure and IQ loss. However, it was not possible to derive ESRVs for the different congeners. More human data on individual congeners are needed.	+/−	Lam et al. (2017), EFSA 2011a
		EFSA summarized the uncertainties in the animal studies affecting the ESRVs for developmental neurotoxicity, which included use of technological mixtures instead of pure congeners for toxicity studies, unknown levels of impurities, single dose administration during the pre- and postnatal period, no stratification of litter mates.	+	EFSA 2011a
	Congeners not considered	8 congeners, i.e. −28, −47, −99, −100, −153, −154, −183 and 209 were considered of primary importance by EFSA because of the composition of the technical PBDE mixtures and concentration in the environment and in food. Only PBDE -47, −99, −153, and −209 were included in the case study, because only ESRV were available for those congeners.	–	EFSA 2011a
	Kinetic model	Limited data on half-lives are available for PBDEs in human, and available data pointed at large variability. The largest half-lives of the individual congeners was used.	+	EFSA (2011a)
	Choice of UF	UF of 100 for PBDE-209, based on the study of Martin et al. According to EFSA, the animal $BMDL_{10}$ of 1.7 mg/kw bw expressed as an external dose can be compared with the estimated human dietary exposure, and EFSA related the exposure of PBDE-209 to the minimal margin of exposure of 2.5.	+	Martin et al. (2017) EFSA (2011a)

based on other grains, the personalised mRPI was overestimated. Exclusion of data for which no occurrence data were available (e.g. methylmercury in foods other than fish and seafood, NDL-PCBS in vegetable foods) resulted in an underestimation of the personalised mRPI.

The issue of limited concentration data used for certain substances can be addressed by including the entire EFSA data set which comprises 27 countries, rather than the 4–13 countries included in our study. However, this would not resolve the issue for NDL-PCBs and PBDEs congeners. For NDL-PCBs, concentration data for congeners other than the 6 indicator congeners were very limited. As those 6 indicator NDL-PCBs comprise approximately 50% of the total PCBs (EFSA 2005, 2012b; JECFA 2016), we calculated the exposure by multiplying the sum of the 6 congeners of NDL-PCB with a factor two. Particular if congeners would differ in potency, as described under uncertainties in ESRVs, the sum of 6 indicator congeners multiplied by two could have led to an under- or overestimation of the exposure. Once better information on potencies of the different congeners is available, the NDL-PCB congeners to be analysed in food can be reconsidered. Regarding PBDEs, concentration data (life stock meat, cow milk and eggs) for the four other congeners deemed relevant for dietary exposure by EFSA (2011a) were available in the concentration database.

5. Conclusions

Our case study shows that specific and targeted MRA using a component-based personalised mRPI approach can be performed for mixtures of dietary chemicals. The mixtures were selected in 1) having a high co-occurrence rate in human biomonitoring studies and 2) sharing a common adverse effect. By using this approach to estimate external dietary exposure, we performed MRA for the deleterious effect of combined exposure of lead, methyl mercury, iAs, fluoride, NDL-PCBs and PNDEs on cognitive development, determined by IQ loss.

All included populations exceeded the acceptable level of combined exposure. NDL-PCBs in fish, other seafood and dairy, lead in grains and fruits, methylmercury in fish and other seafoods, and fluoride in water contributed most to exposure and the subsequent risk. PBDEs hardly contributed to the combined exposure.

Uncertainties were identified for the likelihood of co-exposure, assessment group membership, values of ESRVs based on epidemiological (lead, methylmercury, iAS, fluoride and NDL-PCBs) and animal data (PBDE), and exposure data. Those uncertainties lead to a complex pattern of under- and overestimations, which would require probabilistic modelling based on expert knowledge elicitation for integration of the identified uncertainties into an overall uncertainty estimate. In addition, the listed uncertainties could be used to refine future MRA for cognitive decline.

CRediT author statement

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Table 6
Non-quantified uncertainties in exposure data.

Input data	Source	Description	Direction	Comment
Food consumption data	Food coding	Recoding FoodEx2 into FoodEx1	+/−	Not all food consumptions surveys were available in FoodEx1, the food coding used to match food consumption data to occurrence data. FoodEx2 is more refined, but currently the Dutch recipes database is not available in FoodEx2
		Food coding not always discriminate different food products (e.g. crackers which could be rice-based or wheat based).	+/−	FoodEx2 is more refined and could have prevented this issue
	Representativeness	Under sampling of specific consumption patterns	+/−	E.g. vegetarians, vegans
		Infrequently consumed foods	+/−	E.g. fish consumption in the Netherlands
	Number of reporting days	Extrapolation of few days of consumption to long-term exposure	+/−	Higher number of consumption days included (e.g. 7 days for Denmark) resulted in less uncertainty.
	Reporting foods	Underreporting of non-healthy foods and overreporting of health foods, frequency of consumption	+/−	
Occurrence data	Reported concentrations	Errors in reported concentrations or units	+/−	
	Measurement uncertainty	Analytical method not provided, measurement error	+/−	
	Limited data	Use of conversion factors:		Not applicable for other substances
		- iAs, mean of large range ratio iAs to total As (see Annex G)	+/−	
		- Methyl mercury (see Annex C)	+	
		- NDL-PCBs (Sum 6 indicator congeners times 2)	+/−	
		Aggregation of foods if less than 50 measurements were available:		
		- iAs	+	iAs in rice vs lower concentrations in other grains
		- Other substances	+/−	
		Exclusion of foods for which no data was obtained and for which above mentioned assumptions could not be used	-	
	Concentration data expressed per fat weight	Inaccurate description of the percentage fat weight in a sample. Assuming mean fat content of Dutch food composition database (NEVO)	+/−	Accounts only for NDL-PCBs and PBDEs
	Regional variability	Concentration in food and drinking water may vary between regions	+/−	Particularly for lead, iAs and fluoride in drinking water. Sensitivity analysis showed up to 14% lower pmRPIs when Dutch drinking water concentrations were used
	Measurements below LOQ	Assumed to be 0	–	Particularly when non-sensitive analytical methods (high LOQ) are used.
		Assumed to be value of LOQ	+	
	Processed foods	Processing (e.g. washing, cooking) may affect the concentration in food. Concentrations are often provided in raw agricultural commodities (e.g. wheat) or ingredients (wheat flour) but not in processed foods (e.g. wheat bread).	+/−	Processing factors were not used.
Matching food consumption data to occurrence data	Regional variability	Use of (mean of) Dutch food recipes data may not be representative to other countries	+/−	

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Appendix A. Supplementary data

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Air pollution and stroke; effect modification by sociodemographic and environmental factors. A cohort study from Denmark

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ABSTRACT

Objectives: Air pollution increases the risk of stroke, but the literature on identifying susceptible subgroups of populations is scarce and inconsistent. The aim of this study was to investigate if the association between air pollution and risk of stroke differed by sociodemographic factors, financial stress, comorbid conditions, and residential road traffic noise, population density and green space.

Methods: We assessed long-term exposure to air pollution with ultrafine particles, $PM_{2.5}$, elemental carbon and NO_2 for a cohort of 1,971,246 Danes aged 50–85 years. During follow-up from 2005 to 2017, we identified 83,211 incident stroke cases. We used Cox proportional hazards model (relative risk) and Aalen additive hazards models (absolute risk) to estimate associations and confidence intervals (CI) between 5-year running means of air pollution at the residence and risk of stroke in population strata.

Results: All four pollutants were associated with higher risk of stroke. The association between air pollution and stroke was strongest among individuals with comorbidities, with shorter education, lower income and being retired. The results also indicated stronger associations among individuals living in less populated areas, and with low noise levels and more green space around the residence. Estimates of absolute risk seemed better suited to detect such interactions than estimates of relative risk. For example for $PM_{2.5}$ the hazard ratio for stroke was 1.28 (95%CI: 1.22–1.34) and 1.26 (95%CI: 1.16–1.37) among those with mandatory and medium/long education respectively. The corresponding rate difference estimates per 100,000 person years were 568 (95%CI: 543–594) and 423(95%CI: 390–456)

Conclusion: The associations between air pollution and risk of stroke was stronger among individuals of lower socioeconomic status or with pre-existing comorbid conditions. Absolute risk estimates were better suited to identify such effect modification.

1. Introduction

Stroke is a leading global cause of death and morbidity, ranking third among causes of years of life lost (GBD 2017 Causes of Death Collaborators, 2018) and first among causes of disability-adjusted life years (GBD 2016 Stroke Collaborators, 2019). Air pollution affects hypertension, oxidative stress, systemic inflammation, imbalance of the nervous system and atherosclerosis, which are all pathophysiological

Abbreviations: $PM_{2.5}$, Particulate matter <2.5μm diameter; UFP, Ultrafine particles <0.1μm diameter; EC, elemental carbon; DEHM, Danish Eulerian Hemispheric Model; UBM, Urban background model; OSPM, Operational street pollution model; TWA, time weighted average.

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mechanisms associated with stroke (Lederer et al., 2021). Air pollutants with the strongest public health concern include particulate matter with diameter <2.5 μm ($PM_{2.5}$) and nitrogen dioxide (NO_2), and several other pollutants including elemental carbon (EC) and ultrafine particles (UFP, <0.1 μm diameter) indicate risk, but currently with insufficient data (WHO, 2021). A recent meta-analysis found that a 5null$\mu g/m^3$ increase in long-term exposure to air pollution with $PM_{2.5}$ is associated with a 6.3% increased risk of stroke (Alexeeff et al., 2021). Studies on long-term exposure to air pollution with NO_2 are less conclusive but generally indicate a positive association (Olaniyan et al., 2021). Only few studies have investigated the potentially more potent UFP, indicating an association with cardio- and cerebrovascular disease (Downward et al., 2018; Li et al., 2017; Poulsen et al., 2023a,b). Studies on elemental carbon (EC) (or black carbon or $PM_{2.5}$ absorbance) generally indicate no or a weak association with risk of stroke (Beelen et al., 2014; Downward et al., 2018; Ljungman et al., 2019; Poulsen et al., 2023a,b; Stafoggia et al., 2014; Stockfelt et al., 2017; Wolf et al., 2021).

The association between air pollution and risk of stroke may be modified by other factors. In a Danish study, NO_2 was associated with ischemic stroke only at residences with high noise levels (Sorensen et al., 2014). Some studies have linked access to green areas near the residence or living in rural areas with lower incidence and mortality from stroke or cardiovascular disease (Crouse et al., 2019; Hystad et al., 2020; Kim et al., 2019; Klompmaker et al., 2021; Seo et al., 2019; Stafoggia et al., 2014) possibly due to reduced psychological stress, increased physical activity, or by lower levels of air pollution (Nieuwenhuijsen et al., 2017).

Poor socioeconomic conditions are associated with risk of stroke, likely due to an unhealthy lifestyle, including smoking, alcohol consumption, stress, depression, poor diet, obesity, sedentary lifestyle and hypertension, which are all established risk factors for stroke (Boehme et al., 2017). Some studies have indicated stronger associations between air pollution and stroke among people with lower socioeconomic status (Rodins et al., 2020; Stafoggia et al., 2014; Yang et al., 2021), whereas this was not found in other studies (Hystad et al., 2020; Klompmaker et al., 2021).

Elderly people, children, women,and persons with pre-existing comorbidities including stress have been suggested to be more susceptible to the effects of air pollution (Clougherty, 2010; Schwartz et al., 2011). A recent meta-analysis of 11 cohort studies, found evidence of excess risk of ischemic heart disease in women compared to men in relation to long-term $PM_{2.5}$ exposure, but no difference in risk between sexes in relation to stroke (Zhang et al., 2022). Hypertension and diabetes are established risk factors for stroke (Boehme et al., 2017), but studies investigating effect modification by comorbidity have produced conflicting results (Amini et al., 2020; Hart et al., 2015; Hystad et al., 2020; Olaniyan et al., 2021; Shin et al., 2019; Stafoggia et al., 2014). A study found that self-reported stress could modify the short-term association between $PM_{2.5}$ and blood pressure (Hicken et al., 2014), indicating that stress conditions might also modify the association between air pollution and stroke.

At present, there is insufficient evidence to conclusively identify susceptible sub-populations for which the association between long-term exposure to air pollution and stroke is strongest, and where targeted interventions could be merited. When comparing associations between air pollution and risk of stroke in population groups, relative risk (i.e. how many times greater the hazard in one group compared to another, for example hazard ratios (HR)) and absolute risk (additional cases per 100,000 person-years, rate differences) might provide different results. The difference between these two measures arise when the basic disease rates between the groups of interest differ, for example when the incidence of disease is higher in persons of low versus high educational level, which is often the case. In such situations, the hazard ratio for a given exposure between these two groups may be similar, but the absolute rate difference will be higher in the group of low educational level compared to high – provided that the exposure is positively associated with the outcome. From a public health perspective, the knowledge on whether the absolute health effects of exposure to air pollution differ across subpopulations is important. We are only aware of one previous study of air pollution and risk of stroke, which has applied an additive model for estimation of absolute risk (Danesh Yazdi et al., 2021).

The aim of this nationwide study was to investigate if the association between long-term exposure to air pollution and risk of stroke differed by sociodemographic factors, financial stress, comorbid conditions, and road traffic noise, population density and green space at the residence.

2. Methods

The study was set in Denmark, where a unique personal identification number applied since 1968 allows all citizens to be followed in administrative and health registers (Schmidt et al., 2014). From the Danish Civil Registration System (Pedersen, 2011), we retrieved residential address histories until emigration for all persons living in Denmark (excluding Greenland and Faeroe Islands) in 1979 or born in Denmark any time hereafter. Eligible for the present study were 2,048,282 Danes born after 1920 (data on educational level was not available for those born earlier), living in Denmark on 1 January 2005, and who were at least 50 years old any time between this date and 31 December 2017.

2.1. Outcome

From the Danish national patient register (Lynge et al., 2011a,b) and the register of cause of death (Helweg-Larsen, 2011), we identified all stroke cases (ICD8: 431–434, 436, ICD10 I61-I64) recorded as primary cause of death or admission in the period 1977–2017. We excluded prevalent cases at baseline and only counted incident events.

2.2. Air pollution

The identified addresses were geocoded by means of the Building and Housing Registry (Christensen, 2011). Address-specific air pollution concentrations were quantified by adding air pollution contributions modelled at 3 scales using the Danish DEHM/UBM/AirGIS system (Brandt et al., 2003; Jensen et al., 2017; Khan et al., 2019). The system combined 1) the Danish Eulerian Hemispheric Model (DEHM), covering the northern hemisphere, for the long-range transported regional background concentrations, set up with four domains with two-way nesting capabilities for higher resolution over Denmark (Brandt et al., 2012), 2) the Urban Background Model (UBM) (Brandt et al., 2003; Frohn et al., 2022), covering local background at 1 × 1 km resolution for all of Denmark, calculated from Danish emissions of air pollution at the same resolution (Plejdrup et al., 2021) and 3) the Operational Street Pollution Model (OSPM®), modelling air pollution from traffic in the address street if the traffic density was above 500 vehicles per day (Kakosimos et al., 2010; Ketzel et al., 2012). The OSPM calculations took into account traffic density and composition, emission factors, street and building configuration, and meteorology (Jensen et al., 2017; Khan et al., 2019). We modelled $PM_{2.5}$, EC and NO_2 concentrations. Additionally, we modelled particle number concentration, which in the present paper is denoted UFP, as these quantities are highly correlated. This is a new addition to the modelling system, validated and detailed elsewhere (Frohn et al., 2021; Ketzel et al., 2021). The modelling system performs well when compared to measurements. For example for yearly concentrations of UFP at street level, the correlation coefficient was 0.95 (Ketzel et al., 2021). For all pollutants, monthly mean air pollutant levels were aggregated from modelled hourly concentrations. Combining these data with individual address histories, we calculated running 5-year time-weighted average (TWA) exposures for all cohort members.

2.3. Individual socioeconomic covariates

Potential confounders were selected *a priori*. Statistics Denmark provided annually updated data on Civil status (married/cohabiting, other), highest attained educational level (mandatory, secondary/vocational, medium/long), highest occupational level since 1990 (white collar, blue collar, retired), country of origin ("Danish origin": having Danish citizenship or having at least one parent who has; "Other country of origin": all other), personal income and household income. Both income variables were categorized in sex- and calendar-year specific quintiles.

2.4. Financial stress events and comorbidity

From the registers of Statistics Denmark, we identified potentially stressful financial events:

Loss of job, having household or personal income drop more than 50% between two consecutive years or having a household income of less than half the Danish median household income. For each participant, we created a time-dependent variable indicating if they experienced any of these events in the past five years.

We established a running Charlson comorbidity index from National Patient Registry data (Lynge et al., 2011a,b) on diseases in the five previous years. We incorporated a 1-year lag period to ensure that the index did not reflect the stroke outcome under study. The index was categorized as 0, 1 or ≥2.

2.5. Neighborhood characteristics

In the year 2017, Denmark comprised 2160 parishes with a mean area of 16.2 km^2 and a median population of 1032 persons. Using annually updated data from Statistics Denmark, we calculated population density and proportion of parish inhabitants with only mandatory education for each parish. Population density was categorized into <100, 100–2000, and >2000 persons per km^2 and the proportion with only mandatory education was dichotomized by the highest quintile (<11.6% vs. ≥11.6% or more).

2.6. Green space

We used BASEMAP02, a high resolution (10 × 10 m) land-use map of Denmark (Levin et al., 2017) to quantify green space estimated as proportion of greenness within 150 m of the residence including recreational areas, forests and open nature areas, private gardens and agricultural areas. Green space was categorized into tertiles: <55.1%, 55.1–63.5%, ≥63.6%.

2.7. Road traffic noise

Estimation of road traffic noise has been described in detail previously (Thacher et al., 2020). In brief, noise at the most exposed facade of the residence was modelled by the Nordic prediction method (Bendtsen, 1999) with input data on address-specific geocodes, building height, road type, light/heavy vehicle distributions, travel speed, and annual average daily traffic for all Danish road links (Jensen et al., 2019). The model included screening and reflection effects from buildings, terrain and noise barriers. A-weighted sound pressure estimates were aggregated as L_{den} for the years 2000, 2005, 2010, and 2015. Linear interpolation was used to quantify exposures in the intervening years. Noise was categorized into three levels: <55, 55–59, ≥60 dB.

2.8. Statistical methods

We calculated Spearman rank correlations between air pollutants and covariates. The association of air pollutants with risk of stroke was evaluated in two different models: A Cox proportional hazards model, to calculate relative risk estimates (HR) and an Aalen additive hazards model to calculate absolute risk estimates (rate differences per 100,000 person-years). In both models, age was the time-scale, and both models were adjusted for the same covariates: sex, calendar year (categorical, in two-year categories), educational level, occupational status, civil status, country of origin, personal and household income and proportion of parish inhabitants with only mandatory education (above or below highest quintile). All variables were modelled time-dependently, allowing subjects to change levels of exposure and covariates (except sex and country of origin).

Cohort members were followed up from 50 years of age or 1 January 2005, whichever came last, until stroke, age 86 years, more than 14 consecutive days of unknown address, emigration, death, or 31 December 2017, whichever came first.

To facilitate comparison with other studies risk of stroke was investigated per fixed increment ($PM_{2.5}$: 5null$\mu g/m^3$, NO_2: 10null$\mu g/m^3$, EC: 1null$\mu g/m^3$ and UFP: 10,000 particles/cm^3). We stratified analysis by sex, income, educational level, occupation, financial stress event, comorbidity, proportion of parish inhabitants with only mandatory education, population density, road traffic noise and green space within 150 m.

R (version 3.6.3) was used for Aalen analyses; all other analysis was performed in SAS 9.4 (SAS Institute Inc., NC, USA). By Danish law, entirely register based studies do not require ethical approval.

3. Results

From the 2,048,282 eligible Danes we excluded 54,416 persons diagnosed with stroke before baseline and 22,620 with missing covariate information. This left 1,964,702 persons representing 17,790,121 years of follow-up, during which 83,211 cases of incident stroke occurred. The cohort is described in Table 1. Residential exposure to UFP above the median was associated with higher age, being female, non-Danish origin, longer education, higher personal and household income, being retired, high population density, lower proportion of inhabitants with low education in the neighborhood, low level of green space within 150 m of the residence, and higher levels of road traffic noise. The interquartile ranges were 1.85null$\mu g/m^3$ for $PM_{2.5}$, 4248 particles/cm^3 for UFP, 0.28null$\mu g/m^3$ for EC and 7.15null$\mu g/m^3$ for NO_2. The Spearman rank correlations between pollutants ranged from 0.71 ($PM_{2.5}$ vs EC) to 0.86 (UFP vs NO_2) (Table 2).

Overall, we observed a HR for stroke of 1.28 (95% CI: 1.24–1.33) for 5null$\mu g/m^3$ higher $PM_{2.5}$, 1.12 (95% CI: 1.09–1.15) per 10,000 particles/cm^3, 1.06 (95% CI: 1.04–1.09) per 1null$\mu g/m^3$ of EC and 1.06 (95% CI: 1.04–1.07) per 10null$\mu g/m^3$ NO_2 in models adjusted for age, sex, calendar-year, education, occupational status, civil status, personal income, household income country of origin, and area-level deprivation (percentage of parish population with only mandatory education). Similarly, stroke rate (per 100,000 person-years) differences for the same exposure contrasts were 509 (95% CI: 490–529) for $PM_{2.5}$, 282 (95% CI: 266–298) for UFP, 229 (95% CI: 209–250) for EC, and 120 (95% CI: 112–128) for NO_2.

In general, HRs for stroke were similar, and with overlapping CIs, for different levels of the individual sociodemographic characteristics shown in Table 3, whereas rate differences were consistently higher in association with shorter education, being retired and with low income. Table 4 shows a consistent pattern of higher HRs and higher rate differences with higher level of comorbidity. With some exceptions, more green space within 150 m of the residence, low levels of traffic noise, low population density, and low level of education in the neighborhood were associated with higher HRs and higher rate differences.

4. Discussion

This large nationwide cohort study showed that: 1) air pollution with UFP, $PM_{2.5}$, BC and NO_2 was associated with higher risk of stroke, 2)

Table 1
Sociodemographic and exposure characteristics of the study population at baseline; total and by 5-year exposure to ultrafine particles (below and above the median).

Baseline Characteristics	Total (N = 1,964,702)	UFP <11,064 particles/cm^3 (N = 980,586)	UFP ≥11,064 particles/cm^3 (N = 984,116)
Individual-level			
Female (%)	53	51	54
Age, years (median, 5–95 pctl)	58 (50–79)	55 (50–78)	60 (50–80)
Civil status (%)			
Married or cohabiting	73	77	69
Widow(er) or divorced or single	27	23	31
Individual income (%)			
Low (quintile 1)	25	25	25
Medium (quintile 2, 3 and 4)	55	57	53
High (quintile 5)	20	18	23
Household income (%)			
Low	21	20	21
Medium	55	58	52
High	25	22	27
Highest attained education (%)			
Mandatory	36	38	34
Secondary/ vocational	45	46	45
Medium/long	19	16	21
Country of origin (%)			
Danish	98	99	97
Other	2	1	3
Occupational status (%)			
White collar	46	47	46
Blue collar	37	39	35
Retired or unemployed	16	14	19
Financial stress event[a], past 5 years (%)			
Yes	82	81	84
No	18	19	16
Charlson comorbidity index[b] (%)			
0	85	87	83
1	8	7	9
≥2	7	6	8
Address-level			
Road traffic noise, 5-year (%)			
<55 dB	50	57	43
55–60 dB	22	20	23
>60 dB	28	22	34
Green space[c] within 150 m (%)			
<55.1%	38	26	50
55.1–63.5%	27	25	29
≥63.6%	35	48	21
Air pollution, 5-year (median, 5–95 pctl)			
$PM_{2.5}$ (μg/m^3)	11.2 (8.7–12.6)	10.2 (8.2–11.7)	11.7 (10.0–13.2)
UFP (particles/cm^3)	11,106 (7,212–17,239)	9,301 (6,622–10,002)	13,721 (11,304–18,831)
EC (μg/m^3)	0.7 (0.4–1.1)	0.5 (0.4–0.7)	0.8 (0.6–1.4)
NO_2 (μg/m^3)	15.3 (9.3–27.3)	12.5 (8.3–16.4)	19.5 (14.3–31.7)
Area-level			
Parish inhabitants with only mandatory education (%)			
<11.6%	62	52	72
≥11.6%	38	48	28
Population density (%)			
<100 per km^2	26	47	6
100–2000 per km^2	55	49	61
>2000 per km^2	19	4	33

[a] Financial stress events were defined as ≥1 of the following events during the last 5 years: family income below Danish relative poverty limit, personal income drop of ≥50% between two consecutive years, family income drop of ≥50% between two consecutive years, and/or loss of job.

[b] Comorbidity defined as a Charlson comorbidity index of 0, 1 or ≥2 during the last 5 years, with a lag-period of 1 year.

[c] Green space defined as the proportion of gardens, fields, recreational areas, forest, and wet/dry open nature areas within a 150 m of the residence.

Table 2
Spearman correlations between 5-year exposure to $PM_{2.5}$, ultrafine particles, elemental carbon and NO_2 for the study population, 2005–2017.

	$PM_{2.5}$	UFP	EC	NO_2
$PM_{2.5}$	1	0.80	0.71	0.75
UFP	0.80	1	0.84	0.86
EC	0.71	0.84	1	0.82
NO_2	0.75	0.86	0.82	1

both relative and absolute risk of stroke in association with air pollution were higher among individuals with comorbidity, 3) the association between air pollution and absolute risk for stroke was strongest among individuals with shorter education, lower income and being retired; no such patterns was detected for relative risk, and 4) mostly, both relative and absolute risk for stroke in association with air pollution were higher among individuals with more green space, less noise, low population density and low level of education in the neighborhood.

4.1. Individual factors

We found a consistent pattern of stronger associations between air pollution and the risk of stroke among people with higher levels of comorbidity. This was also seen in a large Canadian study where $PM_{2.5}$ was associated with higher relative risk of stroke, particularly among participants with Charlson comorbidity scores ≥5 (Olaniyan et al., 2021). In the US Nurses' Health Study, the association between $PM_{2.5}$ and stroke was strongest among women with diabetes (Hart et al., 2015). However, in one study, the association between$PM_{2.5}$ and stroke was strongest among subjects without pre-existing cardiovascular disease (Hystad et al., 2020) and other studies have not found effect modification by diabetes or cardiovascular diseases (Amini et al., 2020; Shin et al., 2019; Stafoggia et al., 2014). Our consistent pattern of effect modification by comorbidity might relate to the size of our study and that we identified cases both from hospital and mortality registers and that we accounted for multiple medical conditions. Comorbidity might be associated with biological changes potentially leading to higher susceptibility to air pollution effects. In addition, if comorbidity increases the likelihood of having a stroke detected by the health care system, such surveillance effect might have contributed to our result.

We observed stronger associations between air pollution and absolute risk of stroke among individuals with short education, low income and being retired, In line with our study, the absolute risk of stroke from $PM_{2.5}$ and NO_2 was higher among subjects of lower socioeconomic status (SES) in a US study of 63 million Medicare members (Danesh Yazdi et al., 2021). We are not aware of other studies estimating the absolute risk of stroke in relation to long-term air pollution. In terms of relative risk we found no effect modification by SES whereas the Medicare study found that the relative risk of cerebrovascular disease was lower among subjects of low SES (Klompmaker et al., 2021). Two Canadian cohorts have reported stronger associations between $PM_{2.5}$ and NO_2, and relative risk of stroke among less affluent individuals (Olaniyan et al., 2021; Shin et al., 2019), but a multinational cohort did not find income to modify the association with $PM_{2.5}$ (Hystad et al., 2020). With one exception (Andersen et al., 2012) previous studies have, in line with our results, reported no difference in the association between air pollution and relative risk of stroke by educational level (Beelen et al., 2014; Hystad et al., 2020; Ljungman et al., 2019; Stafoggia et al., 2014; Yang et al., 2021).

Table 3
Association between air pollution and risk for stroke by sociodemographic factor.

	cases	$PM_{2.5}$ (5null$\mu g/m^3$)		UFP (10000#/cm^3)		EC (1null$\mu g/m^3$)		NO_2 (10null$\mu g/m^3$)	
		Cox model HR (95% CI)[a]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a and b]	Cox model HR (95% CI)[a]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a and b]	Cox model HR (95% CI)[a]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a and b]	Cox model HR (95% CI)[a]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a and b]
Sex									
Women	38,061	1.29 (1.23–1.35)	490 (467–512)	1.11 (1.07–1.15)	277 (259–296)	1.05 (1.01–1.09)	230 (206–255)	1.05 (1.03–1.07)	116 (107–125)
Men	45,150	1.28 (1.22–1.34)	538 (512–564)	1.13 (1.09–1.17)	287 (267–307)	1.07 (1.04–1.11)	228 (200–256)	1.06 (1.05–1.08)	124 (114–135)
Highest education level									
Short	39,738	1.28 (1.22–1.34)	568 (543–594)	1.13 (1.09–1.17)	366 (341–390)	1.07 (1.03–1.11)	320 (285–354)	1.06 (1.04–1.08)	170 (157–183)
Medium	33,156	1.29 (1.23–1.36)	456 (431–480)	1.12 (1.08–1.16)	233 (214–252)	1.05 (1.01–1.09)	172 (148–196)	1.06 (1.04–1.08)	98 (88–108)
Long	10,317	1.26 (1.16–1.37)	423 (390–456)	1.10 (1.03–1.17)	199 (175–222)	1.07 (1.00–1.15)	164 (130–198)	1.03 (0.99–1.06)	69 (57–81)
Occupation									
White collar	29,446	1.26 (1.20–1.33)	352 (327–377)	1.09 (1.05–1.14)	151 (134–167)	1.05 (1.01–1.10)	113 (92–135)	1.05 (1.03–1.07)	62 (53–71)
Blue collar	29,900	1.31 (1.25–1.38)	355 (328–382)	1.16 (1.12–1.21)	173 (154–192)	1.09 (1.04–1.13)	126 (104–149)	1.08 (1.06–1.10)	77 (67–88)
Retired/ unemployed	23,865	1.27 (1.21–1.34)	681 (651–711)	1.11 (1.07–1.15)	633 (596–671)	1.05 (1.00–1.09)	616 (552–680)	1.04 (1.02–1.06)	304 (283–325)
Personal income, quintiles									
1st (low)	34,401	1.28 (1.22–1.34)	547 (519–574)	1.11 (1.07–1.15)	322 (297–347)	1.06 (1.02–1.10)	257 (224–291)	1.06 (1.04–1.08)	145 (131–159)
2nd–4th	41,938	1.29 (1.23–1.36)	505 (481–529)	1.14 (1.10–1.18)	287 (267–306)	1.07 (1.04–1.11)	243 (218–268)	1.06 (1.04–1.08)	122 (113–132)
5th (high)	6,872	1.21 (1.10–1.34)	367 (334–401)	1.08 (1.00–1.16)	159 (136–182)	1.00 (0.91–1.10)	103 (75–131)	1.02 (0.98–1.07)	53 (41–65)
Household income, quintiles									
1st (low)	31,611	1.27 (1.21–1.33)	606 (578–635)	1.10 (1.06–1.14)	415 (386–444)	1.04 (1.00–1.08)	358 (318–397)	1.04 (1.02–1.06)	179 (163–194)
2nd–4th	42,707	1.30 (1.24–1.37)	461 (438–485)	1.15 (1.11–1.18)	252 (234–270)	1.08 (1.05–1.12)	203 (180–226)	1.07 (1.05–1.09)	110 (101–119)
5th (high)	8,893	1.25 (1.15–1.37)	338 (309–366)	1.11 (1.04–1.19)	145 (125–164)	1.03 (0.95–1.12)	94 (70–117)	1.05 (1.01–1.09)	52 (41–62)

HR = hazard ratio.

[a] Analyses were adjusted for age, sex, calendar-year, education, occupational status, civil status, personal income, household income country of origin, and area-level deprivation (percentage of parish population with only mandatory education).

[b] Risk estimates calculated in Aalen additive hazard model are given as a rate difference per 100,000 person-years (pyrs).

Table 4
Association between air pollution and risk for stroke by financial stress, comorbidity, population density, road traffic noise and green space.

	Cases	$PM_{2.5}$ (5null$\mu g/m^3$)		UFP (10000#/cm^3)		EC (1null$\mu g/m^3$)		NO_2 (10null$\mu g/m^3$)	
		Cox model HR (95% CI)[aa]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a,ba and b]	Cox model HR (95% CI)[aa]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a,ba and b]	Cox model HR (95% CI)[aa]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a,ba and b]	Cox model HR (95% CI)[aa]	Aalen model Rate diff./ 100,000 pyrs (95% CI)[a,ba and b]
Any financial stress event in past 5 years[cc]									
No	71,834	1.29 (1.24–1.34)	520 (499–540)	1.12 (1.09–1.16)	294 (277–311)	1.06 (1.04–1.09)	244 (222–266)	1.05 (1.04–1.07)	125 (117–134)
Yes	11,377	1.27 (1.17–1.36)	434 (398–470)	1.12 (1.06–1.19)	214 (187–241)	1.07 (1.01–1.14)	155 (123–188)	1.08 (1.04–1.11)	91 (77–106)
Charlson comorbidity index[d]									
0	46,470	1.11 (1.06–1.16)	342 (324–361)	1.02 (0.98–1.05)	165 (151–180)	0.97 (0.93–1.01)	115 (99–132)	1.00 (0.98–1.02)	62 (55–69)
1	16,187	1.26 (1.18–1.35)	798 (745–852)	1.08 (1.03–1.13)	505 (455–555)	1.04 (0.99–1.10)	468 (397–540)	1.02 (1.00–1.05)	214 (187–242)
≥2	20,554	1.31 (1.24–1.39)	841 (793–890)	1.14 (1.10–1.20)	594 (546–643)	1.09 (1.04–1.13)	545 (471–620)	1.07 (1.05–1.1)	279 (252–306)
Proportion of parish inhabitants with only mandatory education									
<11.6%	64,979	1.25 (1.20–1.30)	489 (469–509)	1.12 (1.09–1.15)	256 (240–272)	1.06 (1.03–1.08)	198 (178–217)	1.05 (1.04–1.07)	103 (95–111)
≥11.6%	18,232	1.40 (1.32–1.49)	525 (490–560)	1.13 (1.08–1.19)	253 (224–282)	1.12 (1.05–1.20)	290 (249–332)	1.08 (1.05–1.11)	137 (119–154)
Road traffic noise									
<55 dB	40,275	1.36 (1.29–1.43)	541 (516–566)	1.15 (1.11–1.20)	312 (290–334)	1.12 (1.07–1.17)	384 (347–421)	1.13 (1.09–1.16)	219 (203–234)
55–60 dB	18,270	1.29 (1.21–1.38)	559 (525–594)	1.13 (1.08–1.19)	317 (288–345)	1.04 (0.99–1.10)	240 (196–284)	1.07 (1.04–1.11)	172 (154–189)
>60 dB	24,666	1.16 (1.11–1.22)	460 (433–488)	1.05 (1.01–1.09)	239 (217–261)	1.00 (0.96–1.04)	163 (138–188)	1.01 (0.99–1.03)	85 (75–94)
Green space within 150 m[e]									
<55.1%	34,936	1.20 (1.15–1.26)	503 (477–529)	1.06 (1.02–1.09)	269 (247–290)	1.01 (0.97–1.04)	179 (154–203)	1.02 (1.00–1.03)	94 (84–103)
55.1–63.5%	22,155	1.27 (1.20–1.35)	543 (513–572)	1.10 (1.05–1.15)	307 (283–331)	1.07 (1.01–1.14)	307 (269–346)	1.08 (1.05–1.11)	165 (150–180)
≥63.6%	26,120	1.36 (1.28–1.44)	503 (474–532)	1.22 (1.16–1.28)	327 (301–353)	1.13 (1.08–1.19)	315 (270–359)	1.15 (1.11–1.19)	187 (170–204)
Population density									
<100 per km^2	20,446	1.38 (1.29–1.47)	540 (508–572)	1.21 (1.15–1.29)	455 (413–496)	1.05 (1.01–1.10)	189 (143–235)	1.22 (1.16–1.28)	293 (267–319)
100–2000 per km^2	47,360	1.34 (1.28–1.41)	564 (539–590)	1.14 (1.10–1.19)	353 (330–375)	1.10 (1.06–1.14)	331 (299–363)	1.11 (1.08–1.13)	191 (178–204)
>2000 per km^2	15,405	1.13 (1.05–1.20)	485 (451–518)	1.05 (0.99–1.10)	324 (294–354)	0.96 (0.90–1.02)	239 (205–272)	1.00 (0.98–1.02)	99 (86–112)

HR = hazard ratio.
[a] Analyses were adjusted for age, sex, calendar-year, education, occupational status, civil status, personal income, household income, country of origin, and area-level deprivation (percentage of parish population with only mandatory education).
[b] Risk estimates calculated in Aalen additive hazard model are given as a rate difference per 100,000 person-years (pyrs).
[c] Financial stress events were defined as ≥1 of the following events during the last 5 years: family income below Danish relative poverty limit, personal income drop of ≥50% between two consecutive years, family income drop of ≥50% between two consecutive years, and/or loss of job.
[d] Comorbidity defined as a Charlson comorbidity index of 0, 1 or ≥2 during the last 5 years, with a lag-period of 1 year.
[e] Green space defined as the proportion of gardens, fields, recreational areas, forest, and wet/dry open nature areas within a 150 m of the residence.

We found slightly higher relative risk estimates among blue collar workers compared to white collar workers. A German cohort study reported associations between $PM_{2.5}$ and NO_2 and relative risk of stroke to be stronger among those being unemployed or retired (Rodins et al., 2020), which we did not observe. However, we found much higher absolute risk estimates among those being retired; we are not aware of other studies estimating absolute effect of air pollution on risk of stroke by occupational status.

We found a stronger absolute risk among those without financial stress events in past 5 years, which might seem counterintuitive. A possible explanation could be diagnostic bias, if people under stress are less likely to prioritize seeking medical attention for symptoms of milder strokes.

We observed no convincing effect modification by sex, which is in accordance with two recent meta-analyses on $PM_{2.5}$ (Yuan et al., 2019; Zhang et al., 2022). We could not corroborate the results of Danesh Yazdi et al.(Danesh Yazdi et al., 2021) showing stronger associations between $PM_{2.5}$ and stroke in females.

In summary, both the present and previous studies have provided some evidence of stronger associations between air pollution and risk of stroke among the less privileged. Our study showed that an additive model, providing absolute risk estimates, was able to detect interactions, which remained undetected when using the (traditional) multiplicative Cox models and relative risk estimates. We are only aware of one previous study that has applied additive models, and would recommend future studies to do so when investigating risk associations by population groups with different underlying disease/mortality rates. This applies for example to different socioeconomic groups since many aspects of low SES are established or putative risk factors for cardiovascular disease (Powell-Wiley et al., 2022).

4.2. Residential and area factors

Our results showed higher relative and absolute risks of stroke in association with air pollution among individuals living in areas with low educational level. In a US cohort, the association of $PM_{2.5}$ with mortality

from stroke and ischaemic heart disease combined did not differ by area level, income or education; stroke, however, constituted only 20% of cases (Hayes et al., 2020). One explanation for our result could be that the area level indicator acts as proxy for some unaccounted for individual factor, although our model included a range of individual socioeconomic indicators.

The associations of air pollution with stroke, both absolute and relative, were generally stronger in less populated areas and areas with less traffic noise, both indicating rural conditions. In a multinational study, including countries of all income levels, the association between $PM_{2.5}$ and stroke was strongest in rural populations (Hystad et al., 2020), whereas the association between $PM_{2.5}$ and stroke hospitalization was not modified by level of urbanicity in an Italian cohort (Gandini et al., 2018). The Danish nurse cohort study found no clear effect modification by noise level or urbanicity (Amini et al., 2020).Important sources of non-traffic air pollution in Denmark include shipping and non-industrial combustion plants, and we have previously demonstrated that in Denmark, air pollution from non-traffic sources is a stronger risk factor for stroke than air pollution from traffic with $PM_{2.5}$ estimates of 1.004 (95% CI: 0.998–1.011) for traffic sources and 1.091 (95% CI: 1.074–1.108) for non-traffic per IQR (Poulsen et al., 2023a,b). A possible explanation for the weaker association between air pollution and risk of stroke in more urban populations in the present study could thus be that a higher proportion of urban exposure hails from traffic.

Our study showed stronger associations between air pollution and risk of stroke among individuals with much green space around the residence. Previous studies on stroke have found the same (Klompmaker et al., 2021), the opposite (Crouse et al., 2019) or no effect modification (Avellaneda-Gomez et al., 2022). It has been suggested that green space might positively influence cardiovascular health by promoting physical activity and decreasing stress (Nieuwenhuijsen et al., 2017), but it could also be an indicator of differential susceptibility as a consequence of differences in lifestyle or different composition of air pollution (for example a smaller proportion of traffic-related air pollution). Finally, it could also reflect the urban-rural differences discussed above. Further studies will be needed to elucidate the potential effect modifying properties of green space (Poulsen et al., 2023).

4.3. Strengths and limitations

Our study was nationwide and register-based, minimizing the potential for selection bias. Outcome data as well as information on individual and area level SES-covariates were obtained from updated public registers of high quality (Helweg-Larsen, 2011; Lynge et al., 2011a, 2011b; Schmidt et al., 2014, 2015). Furthermore, we had data on financial stress, comorbidity and green space at the residence. Also, we supplemented the standard use of Cox models with additive Aalen models, which accounted for differences in base rates between subpopulations. Such differences are not accounted for by Cox models producing relative risk estimates. Another strength of our study was the detailed address-level assessment of air pollution and noise with high spatiotemporal resolution (Khan et al., 2019).

Some exposure misclassification is, however, inevitable when modelling air pollution, and we did not have information about non-residential exposures. Such misclassification is likely to be independent of case status and may, thus, have attenuated risk estimates. If our 5-year exposure time-window does not optimally capture relevant exposure, this could also attenuate risk estimates. We have, however, previously demonstrated that in this cohort, shorter (1-year) and longer (10-year) exposure windows produce similar risk estimates for the association between air pollution and stroke (Poulsen et al., 2023a,b).

It was a limitation that we did not have data on individual lifestyle factors, such as overweight, diet or smoking, which are considered risk factors of stroke and also potentially linked to choice of residence and thus the individual level of air pollution exposure. However, we have previously shown that if analyses of associations between air pollution and stroke is adjusted for sociodemographic factors from registers, similar to the present study, there was little additional impact of adjusting for individual lifestyle factors (smoking, diet, physical activity and body mass index) (Sorensen et al., 2022).

Even though the validity of our stroke definition has been shown to be high (83.5%) (Luhdorf et al., 2017), our data did not allow a reliable subdivision of stroke by subtype; previous studies suggested that the association with air pollution may differ by stroke subtype (Andersen et al., 2012; Shin et al., 2019).

Our cohort is a predominantly white, middle-aged or older, Western population. Air pollution composition and correlations between environmental and individual risk factors may differ in other settings. These factors should be taken into consideration before generalizing our results to other populations. Another thing to consider in future studies is that all though all four air pollutants are highly correlated and have large contributions from combustion processes and from non-traffic sources (Poulsen et al., 2023a,b) they may represent different aspects of air pollution and we have previously found indications that $PM_{2.5}$ from non-traffic sources, may be closest associated with risk of stroke (Poulsen et al., 2023a,b). It may therefore be worthwhile to apply multipollutant models including multiple sources or types of air pollutants to better identify the true risk factors for stroke. In such analysis one could also consider effect modification by environmental factors such as urbanicity and green space.

5. Conclusion

The associations between air pollution and risk of stroke were stronger among individuals of lower socioeconomic status or with pre-existing comorbid conditions. Absolute risk estimates were better suited to identify such effect modification. Our results substantiate the need for preventive strategies targeted towards reducing air pollution exposure among these specific susceptible groups, with the potential to reduce more cases of stroke.

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Ethics

By Danish law informed consent and ethical approval are not required for entirely register based studies.

Declaration of competing interest

The authors declare they have nothing to disclose.

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Air pollution exposure and social responsiveness in childhood: The cincinnati combined childhood cohorts

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ABSTRACT

Autism Spectrum Disorder (ASD) affects about 1 in 44 children and environmental exposures may contribute to disease onset. Air pollution has been associated with adverse neurobehavioral outcomes, yet little research has examined its association with autistic-like behaviors. Therefore, our objective was to examine the association between exposure to air pollution, including NO_2 and $PM_{2.5}$, during pregnancy and the first year of life to ASD-like behaviors during childhood. Participants (n = 435) enrolled in the Cincinnati Childhood Allergy and Air Pollution Study and the Health Outcomes and Measures of the Environment Study were included in the analysis. Daily exposures to NO_2 and $PM_{2.5}$ at the residential addresses of participants were estimated using validated spatiotemporal models and averaged to obtain prenatal and first year exposure estimates. ASD-like behaviors were assessed via the Social Responsiveness Scale (SRS) questionnaire at age 12. Linear regression models adjusting for confounders were applied to estimate the association between pollutants and SRS scores. After adjusting for covariates, the association between NO_2 and $PM_{2.5}$ and SRS scores remained positive but were no longer statistically significant. Prenatal and first year exposure to NO_2 were associated with total SRS T-scores with an estimated 0.4 point increase (95% CI: −0.7, 1.6) per 5.2 ppb increase in NO_2 exposure and 0.7 point (95% CI: −0.3, 1.6) per 4.2 ppb increase in NO_2 exposure, respectively. For $PM_{2.5}$, a 2.6 $\mu g/m^3$ increase in prenatal exposure was associated with a 0.1 point increase (95% CI: −1.1, 1.4) in SRS Total T-scores and a 1.3 $\mu g/m^3$ increase first year of life was associated with a 1 point increase (95% CI: −0.2, 2.3). In summary, exposure to NO_2 and $PM_{2.5}$ during pregnancy and the first year of life were not significantly associated with higher autistic-like behaviors measured with SRS scores after adjustment of covariates. Additional research is warranted given prior studies suggesting air pollution contributes to ASD.

1. Introduction

Autism spectrum disorder (ASD) is a behavioral condition characterized by deficits in communication and social interaction, and an increase in restricted and repetitive patterns in behaviors, interests, and activities (Diagnostic and Statistical Manual of Mental Disorders, 2013). ASD is associated with lifelong consequences including functional deficits, difficulty maintaining relationships, and challenges with living and working independently (Lord et al., 2018). ASD is among the most common neurodevelopmental disorders of childhood, and its prevalence is increasing worldwide (Zeidan et al., 2022) and within the United States (Christensen et al., 2019). The prevalence of ASD is 4 times higher

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among boys than girls, and about 1 in 44 (2.3%) children were identified with ASD (Werling and Geschwind, 2013) in 2018. According to estimates from the Autism and Developmental Disabilities Monitoring Network, 2.3% represents a 23 percent increase from 2016 (1 in 54), and more than double that of 2000 (1 in 150) (National Center on Birth Defects, 2022). The rise in ASD cases is likely partially attributed to increased monitoring and a broadening of diagnostic criteria (Rice et al., 2012); however, these factors do not fully explain the rising prevalence of ASD (Hertz-Picciotto and Delwiche, 2009).

While genetics and family history play a role in ASD, a growing body of evidence suggests that *in utero* exposure to air pollution, a complex mixture of particles, gases, trace metals and adsorbed organic contaminants, may also be neurotoxic and could contribute to the development of ASD (Ortega et al., 2014; Peters et al., 2006; Muhlfeld et al., 2008; Bekkar et al., 2020; Shang et al., 2021; Costa et al., 2017, 2020). Exposure to air pollution has been linked to adverse physical and developmental effects on the fetus including low birth weight, preterm birth, and high infant mortality (Currie et al., 2009; Stillerman et al., 2008). In children, air pollutants including NO_2 and fine particulate matter ($PM_{2.5}$) have been associated with numerous neurobehavioral outcomes: impaired cognitive abilities (Harris et al., 2015; Suglia et al., 2008), deficits in attention-related behaviors (Chiu et al., 2013; Newman et al., 2013; Siddique et al., 2011), symptoms of anxiety/depression (Ali and Khoja, 2019; Bakolis et al., 2021; Yolton et al., 2019), as well as ASD (Raz et al., 2018; Volk et al., 2013; Roberts et al., 2013; Windham et al., 2013). Toxicological studies have explored plausible biological pathways linked to autism, noting that airborne pollutant particles cause systemic inflammation, alter the neonatal immune system, contribute to neuronal injury, induce oxidative stress, and affect the development of the central nervous system (Peters et al., 2006; Muhlfeld et al., 2008; Li et al., 2003; Pangrazzi et al., 2020). The brain is already vulnerable to oxidative stress due to its high metabolic activity and low levels of antioxidants, and children with ASD may be at greater risk for oxidative stress (Pangrazzi et al., 2020; MohanKumar et al., 2008). Investigating postnatal air pollution exposure is also indicated because brain development continues, doubling in size through a child's first year of life, and environmental toxicant insults can perturb neurodevelopment (Rice and Barone, 2000). Exposure to fine particulate matter in early infancy has been found to influence patterns of structural brain development in childhood, specifically hemispheric differences in gray matter across cortical regions (Cserbik et al., 2020).

Previous studies have found children born to mothers who live close to freeways have twice the risk of ASD (Volk et al., 2011). Furthermore, epidemiological studies have reported associations of ASD diagnosis and prenatal exposure to various air pollutants, including nitrogen dioxide (NO_2) (Volk et al., 2013; Ritz et al., 2018) and fine particulate matter ($PM_{2.5}$) (Volk et al., 2013; Raz et al., 2015; Becerra et al., 2013). The role of postnatal air pollution exposure is uncertain as studies in California (Volk et al., 2013), Pennsylvania (Talbott et al., 2015), Israel (Raz et al., 2018), and Denmark (Ritz et al., 2018) have found that exposure during early childhood is associated with increased odds of ASD, while other studies found the association to be very weak or not significant (Raz et al., 2018; Gong et al., 2014, 2017; Guxens et al., 2016; Pagalan et al., 2019; Kaufman et al., 2019). The objective of this study was to examine the association between NO_2 and $PM_{2.5}$ during early brain development and ASD-related traits and behaviors in two well-characterized longitudinal cohorts with detailed residential histories.

2. Methods

2.1. Study population

We analyzed pooled and harmonized data collected from participants enrolled in two cohorts from the Cincinnati, Ohio region: the Cincinnati Childhood Allergy and Air Pollution Study (CCAAPS) and the Health Outcomes and Measures of the Environment (HOME) Study. The combined study population, referred to as the Cincinnati Combined Childhood Cohort (C4), leverages the two cohorts' compatibility regarding geographic proximity, demographics, available exposure data, neurobehavioral outcomes, and harmonized data for analysis.

A complete description of both cohorts' eligibility, enrollment, and methods is available elsewhere (Yolton et al., 2019; LeMasters et al., 2006; Ryan et al., 2005; Brunst et al., 2015; Braun et al., 2017, 2020). Briefly, CCAAPS is a longitudinal cohort study of children examining the associations between traffic-related air pollutants and respiratory and neurobehavior health (LeMasters et al., 2006; Ryan et al., 2005). Study eligibility required a birth address either <400 or >1500 meters (m) from a major roadway and at least one biological parent with atopy confirmed by skin prick testing (LeMasters et al., 2006). Children were enrolled at approximately age 6 months from 2001 to 2003 and completed study visits at ages 1, 2, 3, 4, 7, and 12. At the 12-year study visit, a comprehensive neurobehavioral assessment battery was completed (Yolton et al., 2019). The HOME Study is a longitudinal pregnancy and birth cohort study that enrolled pregnant women to examine the association between common low-level environmental toxicants and child health and neurobehavioral outcomes (Braun et al., 2017). Study eligibility required living in the study region, <19 weeks pregnant, >18 years old, residing in a home built in or before 1978, not living in a mobile or trailer home, HIV-negative, not taking medications for seizures or thyroid disorders, planning to continue prenatal care and deliver at the collaborating clinics and hospitals, planning to live in the greater Cincinnati area for the next year, fluent in English, and no diagnosis of diabetes, bipolar disorder, schizophrenia or cancer that resulted in radiation treatment or chemotherapy (Braun et al., 2017, 2020). Pregnant women were enrolled from 2003 to 2006 and their children completed study visits at birth, 4 weeks, annually 1–5 years, 8 years, and 12 years. The study visit at age 12 included a comprehensive assessment of mental health and brain development. Both studies were approved by the Institutional Review Boards of the University of Cincinnati and/or Cincinnati Children's Hospital Medical Center. Participants and parents provided informed assent and consent, respectively prior to completing study procedures.

2.2. Air pollutants exposure assessment

We used validated models (Di et al., 2019, 2020) to estimate daily exposure to $PM_{2.5}$ and NO_2 at the homes of C4 participants prenatally through age one year. At each study visit, beginning at enrollment and through the most recently completed 12-year visit, caregivers reported all residential addresses and corresponding dates when the child lived at each location. Daily estimates of air pollutants were aggregated to derive average exposures for each pollutant during two time periods: 1) prenatal exposures during pregnancy (date of conception to date of birth) and 2) exposure during the participant's first year of life (date of birth to first birthday). When more than one address fell into a time interval, we created a weighted average to account for changes in residence.

We estimated NO_2 exposure based on validated spatiotemporal models covering the entire contiguous U.S. with daily predictions on 1-km-level grid cells from 2000 to 2016 (Di et al., 2020). This ensemble model integrated multiple machine learning algorithms, including neural network, random forest, and gradient boosting, with multiple predictor variables, including chemical transport models, to provide estimates with high spatiotemporal resolution (Di et al., 2020). The relationship between daily monitored and predicted NO_2 is almost linear; the ensemble produced a cross-validated R^2 of 0.788 overall, a spatial R^2 of 0.844, and a temporal R^2 of 0.729. Daily estimates of NO_2 concentrations were derived at each participant's home based on the residential history provided. Daily estimates were aggregated and weighted (if residence changed) to calculate the daily average prenatal and first year exposure to NO_2.

We estimated $PM_{2.5}$ exposures using a validated ensemble model that

integrated multiple machine learning algorithms, including neural network, random forest, and gradient boosting (Di et al., 2019). The three machine learning algorithms used more than 100 predictor variables, ranging from satellite data, land-use data, meteorological data, and CTM predictions, with cross-validation controlling for overfitting. A generalized additive model combined $PM_{2.5}$ estimates from the machine learning algorithm to account for geographic differences. The trained model predicts daily $PM_{2.5}$ for the entire contiguous United States from 2000 to 2016 at every 1 km × 1 km grid cell, with excellent performance, a spatial R^2 of 0.89 and temporal R^2 of 0.85. Similar to NO_2 exposure, daily estimates of $PM_{2.5}$ concentrations at the home of each participant were averaged to obtain the average daily exposure prenatally and in the first year of life.

2.3. ASD-related traits and behavior assessment

At the age 12 study visit for both the CCAAPS and HOME cohorts, the Social Responsiveness Scale-2 (SRS-2) was completed by the child's caregiver. The SRS-2 is a 65-item questionnaire that assesses behavioral and social-communicative traits used to identify the presence and severity of social impairment for both the general (non-clinical) population and in clinical settings (Bolte et al., 2008; Constantino and Gruber, 2012; Constantino and Todd, 2005; Frazier et al., 2014). This measure has been used widely in studies with children because of its ease of administration, strong psychometric properties, high internal validity, reliability, and reproducibility (Constantino et al., 2003). It is routinely administered as part of the comprehensive diagnostic assessment for ASD and has been validated against widely implemented clinical diagnostic tools, the Autism Diagnostic Interview-Revised (Duku et al., 2013; Chan et al., 2017) and the Autism Diagnostic Observation Schedule (Bolte et al., 2008, 2011). In addition to a continuous Total score reflecting severity of social deficits associated with the autism spectrum, the SRS-2 generates scores for five treatment subscales: Social Awareness, Social Cognition, Social Communication, Social Motivation, and Restricted Interests and Repetitive Behavior. The SRS-2 also offers two DSM-5 Compatible Subscales, Social Communication and Interaction (SCI) and Restricted Interests and Repetitive Behavior (RRB). Parents of C4 participants completed the SRS-2 during the 12-year visit, and SRS raw scores were created utilizing publisher-supplied software. Sex-specific T scores, with a mean = 50 and standard deviation = 10, were determined based on the publisher's instructions, with higher scores representing more severe impairment and behaviors consistent with ASD.

2.4. Covariates

We collected maternal and child characteristics at enrollment and subsequent follow-up visits using self-report interviews and surveys. Covariates considered for inclusion in adjusted models were identified using a directed acyclic graph and selected from prior literature on their potential roles as confounders of the relationship between air pollution and ASD risk (Fig. S1). These included maternal education (some college education or less/obtained a college degree or graduate/doctoral degree) and a measure of community-level socioeconomic status. We calculated community deprivation, an indicator of community socioeconomic disadvantage, based on residential birth address utilizing a previously developed index that combines several census tract level socioeconomic data to provide a score ranging from 0 to 1, with values indicating greater deprivation (Brokamp et al., 2019).

2.5. Statistical analysis

Descriptive statistics and graphical plots were used to examine the distribution of all variables, examine potential outliers, and describe the cohort, exposures, and outcome measures. Means and standard deviations are reported for continuous variables; number of individuals and frequencies are reported for categorical variables. Missing maternal baseline education levels (n = 21) were replaced with reports obtained in later follow up visits.

We developed separate unadjusted linear regression models to initially evaluate associations between prenatal and first-year of life exposures to air pollutants (NO_2 and $PM_{2.5}$) and the six SRS-2 component scores. Adjusted linear regression models were subsequently developed with covariates. All effect estimates are presented as a interquartile range (IQR) unit increase in exposure concentration. We assessed potential effect modification of NO_2 and $PM_{2.5}$ exposure by child sex by including an interaction term in the adjusted models. We then conducted a stratified analysis by child sex for models that contained an interaction term of significance ($p < 0.1$). We conducted a sensitivity analysis utilizing a logistic regression model with dichotomized outcomes to asses diagnostically-relevant SRS cut points at 60 and 75. Additionally, we explored average exposures during each trimester of pregnancy. All statistical analyses were conducted using R Studio (version 4.1.1; R Development Core Team) and figures were created with GraphPad Prism (version 9.3.1).

3. Results

3.1. Participant characteristics

A total of 435 children (CCAAPS: n = 180, HOME: n = 255) and their caregivers attended the age 12 study visits and completed the SRS questionnaire (Table 1). Children were, on average, 12.4 years at the time of the visit, with slightly fewer males than females (48% vs. 52%). Most participants (64.6%) self-reported their race as White, reflecting the racial distribution of the greater Cincinnati region. On average, mothers were 29.1 years of age at delivery; most of whom had college degree (60.8%), were married or living with a partner (80.9%)) and did not smoke during pregnancy (88.7%).

For the CCAAPS cohort, the SRS questionnaire was added to the study protocol after the start of the age 12 study visits and not all individuals who completed the age-12 visit completed this assessment. No significant differences were identified for participants who completed the age 12 study visit and those who did not (n = 806) with respect to sex, maternal education level, and household income but participants who did not complete the age 12 visit were more likely to be white and be married (Table S1). No significant differences in sex, birthweight, maternal age at delivery, maternal education level, smoking, household income, NO_2 exposure level, and all SRS scores were found between participants of the two cohorts (Table 1). There were significant differences between the cohorts in racial composition, marital status, and community deprivation status with CCAAPS participants more likely to be White, have greater daily exposure to $PM_{2.5,}$ have parents who were married or living together, and live in a census tract with higher deprivation index as compared to HOME Study participants. The mean total SRS score of children in this cohort was 50.8 [standard deviation (SD) = 9.4]. Seventy-four (17.0%) children had total SRS scores ($T \geq 60$) indicating deficiencies in reciprocal social behavior that may interfere with daily social interactions. Ten children (2.3%) had scores considered to be severe ($T \geq 75$) and strongly associated with a clinical diagnosis of ASD (Constantino and Gruber, 2012).

3.2. Exposure to air pollution

Average [SD] exposure to NO_2 among the participants during the prenatal period (32.3 [4.4] ppb) was similar to average exposure during the first year of life (32.0 [4.0] ppb) (Fig. 1A). However, individual participants' exposures to NO_2 differed from prenatal to the first year of life due to changes in both residential locations and temporal variation; the correlation between prenatal and first year of life NO_2 exposure was 0.57. Additionally, exposure to $PM_{2.5}$ among the participants during the prenatal period, 15.7 [1.8] $\mu g/m^3$, was similar to average exposure

Table 1
Child, Maternal, and Household Characteristics of C4, CCAAPS, and HOME cohorts.

Characteristics	C4		CCAAPS		HOME		p-value
	N (%) or Mean (SD)						
Child Characteristics							
N	435		180		255		
Sex							0.06
Male	209	(48.0)	96	(53.3)	113	(44.3)	
Female	226	(52.0)	84	(46.7)	142	(55.7)	
Race							<.01
White	281	(64.6)	131	(72.8)	150	(58.8)	
Black or African American	122	(28.0)	36	(20.0)	86	(33.7)	
Other	32	(7.4)	13	(7.2)	19	(7.5)	
Birthweight (kg)	3.36	(0.6)	3.42	(0.6)	3.31	(0.6)	0.06
Maternal Characteristics (at time of birth)							
Age at Delivery	29.07	(5.9)	29.48	(5.9)	28.78	(5.9)	0.2
Education at Time of Birth							0.7
HS graduate or less	98	(22.6)	41	(22.8)	57	(22.4)	
Some college or technical school	264	(61.1)	45	(25.0)	73	(28.7)	
College graduate or advanced	72	(16.6)	94	(52.2)	124	(48.8.)	
Marital Status							<.01
Married or living with partner	336	(80.1)	155	(88.1)	181	(75.7)	
Not married and living alone	79	(19.0)	21	(11.9)	58	(24.3)	
Smoking	49	(11.3)	21	(11.7)	28	(11.0)	1.0
Household Characteristics (at time of birth)							
Household Income							0.1
<$40,000	157	(37.1)	57	(32.6)	100	(40.3)	
$40,000 - $89,999	185	(43.7)	78	(44.6)	107	(43.1)	
>$90,000	81	(19.1)	40	(22.9)	41	(16.5)	
Deprivation Index	0.36	(0.2)	0.33	(0.1)	0.38	(0.2)	<.01
Air Pollutant Exposure							
NO_2							
Prenatal (ppb)	32.3	4.4	32.3	3.9	32.4	4.7	0.9
First Year of Life (ppb)	32.0	4.0	31.7	4.1	32.3	4.0	0.2
Fine Particulate Matter ($PM_{2.5}$)							
Prenatal ($\mu g/m^3$)	15.7	1.8	16.2	1.4	15.4	2.0	<.01
First Year of Life ($\mu g/m^3$)	15.5	0.9	15.9	0.8	15.3	0.9	<.01
SRS Score							
Total	50.8	(9.4)	51.2	(9.5)	50.5	(9.4)	0.5
Social Awareness	52.8	(9.7)	53.5	(9.7)	52.3	(9.8)	0.2
Social Cognition	50.5	(9.4)	50.5	(9.6)	50.4	(9.4)	0.9
Social Communication	50.6	(9.5)	50.9	(9.5)	50.3	(9.5)	0.5
Social Motivation	50.6	(10.0)	50.9	(10.4)	50.3	(9.8)	0.5
DSM Social Communication and Interaction	51.0	(9.5)	51.4	(9.5)	50.7	(9.5)	0.4
DSM Restricted Interests and Repetitive Behavior	49.8	(9.4)	50.1	(9.7)	49.7	(9.1)	0.6

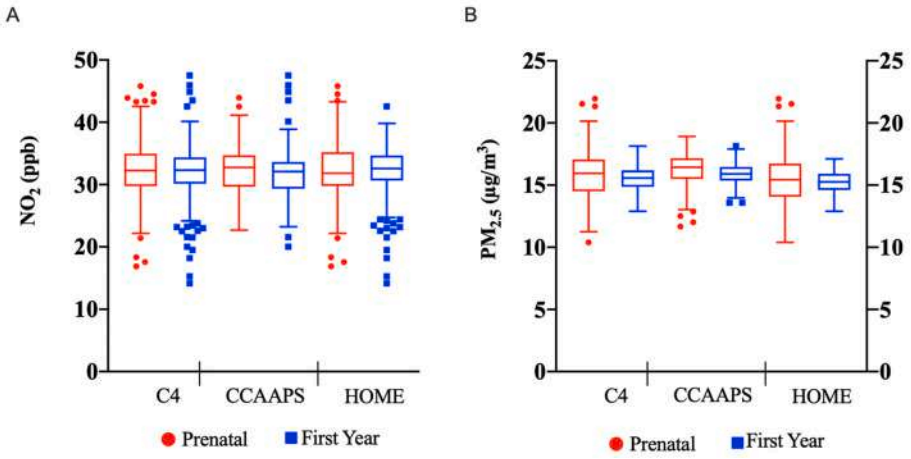

Fig. 1. Prenatal and First Year of Life Air Pollution Exposure
Distribution of prenatal (red, circle) and first year (blue, square) of life NO_2 and $PM_{2.5}$ exposure for C4, CCAAPS, and HOME Study cohorts. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

during the first year of life, 15.5 [0.9] μg/m^3 (Fig. 1B), but the correlation among individual exposure between the two time points was 0.14.

3.3. Air pollution and Social Responsiveness Scale scores

Results are presented in IQR increase of each exposure: prenatal NO_2 (5.2 ppb), first year NO_2 (4.2 ppb), prenatal $PM_{2.5}$ (2.6 μg/m^3), first year $PM_{2.5}$ (1.3 μg/m^3). In unadjusted models, prenatal and first year exposure to NO_2 were significantly associated with total SRS T-scores with an estimated 1.2 point increase (95% CI: 0.17, 2.3) per 5.2 ppb increase in NO_2 exposure (Fig. 2A) and 1.3 point (95% CI: 0.4, 2.2) per 4.2 ppb increase in NO_2 exposure (Fig. 2B), respectively. Prenatal NO_2 exposure was also positively associated with Social Cognition (β = 1.1, 95% CI: 0.009, 2.1), Social Motivation (β = 1.3, 95% CI: 0.2, 2.4), DSM SCI (β = 1.2, 95% CI: 0.1, 2.2), and DSM RRB (β = 1.1, 95% CI: 0.1, 2.2) component scores. First year NO_2 exposure was positively associated Social Awareness (β = 1.1, 95% CI: 0.2, 2.1), Social Cognition (β = 1.5, 95% CI: 0.6, 2.5), Social Motivation (β = 1.3, 95% CI: 0.3, 2.2), DSM SCI (β = 1.3, 95% CI: 0.3, 2.2), and DSM RRB (β = 1.2, 95% CI: 0.3, 2.1). After adjusting for maternal education and census tract deprivation index, positive but nonsignificant associations were observed with prenatal (β = 0.4, 95% CI: −0.7, 1.6) and first year of life (β = 0.7, 95% CI: −0.3, 1.6) NO_2 exposure and SRS Total T-scores. Increased maternal education was significantly associated with decreased SRS Total T-scores in both prenatal and first year of life in the fully adjusted models (Table S2).

For first year $PM_{2.5}$ exposure, in the unadjusted models, a 1.3 μg/m^3 increase in exposure during the first year was significantly associated with a 1.3 point increase (95% CI: 0.02, 2.7) in SRS Total T-scores (Fig. 3B). Additionally, the Social Cognition (β = 1.4, 95% CI: 0.1, 2.8) and DSM SCI (β = 1.4, 95% CI: 0.04, 2.7) component T-scores were significantly associated with the exposure. No significant associations were observed with prenatal $PM_{2.5}$ exposure in the unadjusted model (Fig. 3A). Similar to NO_2 models, increased maternal education was associated with decreased SRS T-scores in the fully adjusted models (Table S2).

When examining potential effect medication by sex, there was a significant interaction between sex and prenatal NO_2 for SRS Total, Social Cognition, Social Communication, and DSM RRB scores (Fig. S2), with female children having increased association (Fig. S2) though the effect of prenatal NO_2 exposure on SRS scores remained not significant. Sensitivity analysis with dichotomized outcomes to asses diagnostically-relevant SRS cut points had no significant findings. In addition, there were no significant associations between trimester specific windows of exposure during pregnancy and SRS scores.

4. Discussion

In this longitudinal study, we found elevated SRS scores associated with exposure to NO_2 and $PM_{2.5}$ during pregnancy and the first year, but these were not statistically significant after adjusting for maternal education and community deprivation. Maternal exposure to NO_2 during pregnancy and child's NO_2 exposure during first year of life seemed to indicate greater ASD risk with elevated SRS total T-scores and component scores for Social Cognition, Social Motivation, DSM RRB, and DSM SCI categories. However, after adjustment for socioeconomic and community confounders, all associations were no longer statistically significant. Similar nonsignificant trends were observed for $PM_{2.5}$ exposure during the first year.

Although prior studies have examined the association between air pollutants and ASD diagnosis, to our knowledge this is the first epidemiologic study to utilize the SRS to study air pollution and ASD-like behaviors in typically developing children. Our findings are consistent with a meta-analysis by Chun et al. which suggested there is weak evidence for a positive association between maternal exposure to NO_2 and ASD in children (Chun et al., 2020). Pagalan et al. conducted a study using a Canadian population-based birth cohort and found that the adjusted odds ratios for NO_2 exposure per interquartile range were not significantly associated with ASD diagnosis, assessed with the Autism Diagnostic Interview–Revised and Autism Diagnostic Observation Schedule (Pagalan et al., 2019). Other studies utilizing ASD diagnosis for case ascertainment have also reached similar conclusions26,38,40. Moreover, studies that studied NO_2 exposure and childhood autistic traits, assessed using the Autism Spectrum Disorder module of the Autism—Tics, Attention Deficit and Hyperactivity Disorders, and Other Comorbidities (A-TAC) inventory, have not found significant associations (Gong et al., 2014; Guxens et al., 2016). However, there is no consensus regarding NO_2 exposure as a risk factor for ASD because some studies reported contradictory findings. Positive association between ASD diagnosis and prenatal and first year NO_2 exposure were observed in the United States (Volk et al., 2013). In Taiwan (Jung et al., 2013), one year preceding diagnosis was found to be a significant exposure window. In a nationwide study of Danish children, Ritz et al. found that pollution exposures in early infancy but not in pregnancy contributed to an increased risk of ASD (Ritz et al., 2018).

Studies analyzing $PM_{2.5}$ exposure and ASD have also reported inconsistent findings. In contrast to our findings, two California based studies reported significant associations between prenatal $PM_{2.5}$ exposure and ASD diagnosis, assessed with the Autism Diagnostic Interview–Revised and Autism Diagnostic Observation Schedule (Volk et al., 2013; Becerra et al., 2013). However, another study of children in California, found no association (Goodrich et al., 2018; Kerin et al., 2018). Consistent with our findings, additional studies have found effect estimates for $PM_{2.5}$ to be elevated but not reaching a significance level of association with ASD diagnosis (Talbott et al., 2015; Pagalan et al., 2019; Kaufman et al., 2019) or ASD-like traits (Guxens et al., 2016). Raz et al. found that while the exposure during the entire pregnancy may not be relevant, exposure during certain time points, specifically the third

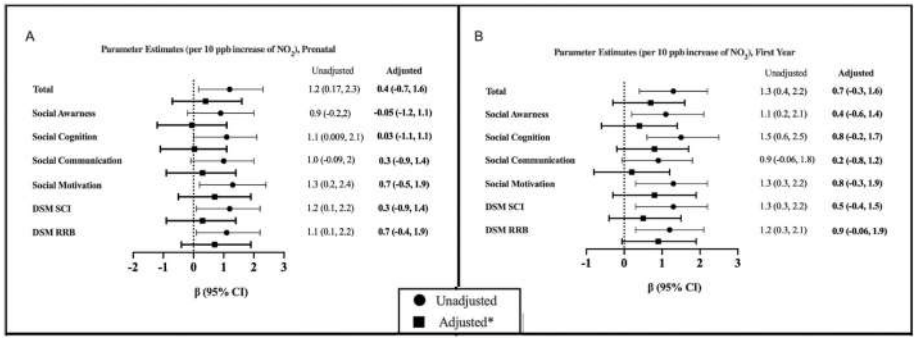

Fig. 2. Linear Regression Parameter Estimates for NO_2 Exposure and SRS Scores
Unadjusted (circle) and adjusted* (square) parameter estimates (per prenatal and first year IQR ppb increase, 5.2 and 4.2, respectivel) for the association between prenatal (A) and first year NO_2 (B) and SRS Total and component scores. *Adjusted for maternal education and community deprivation index.

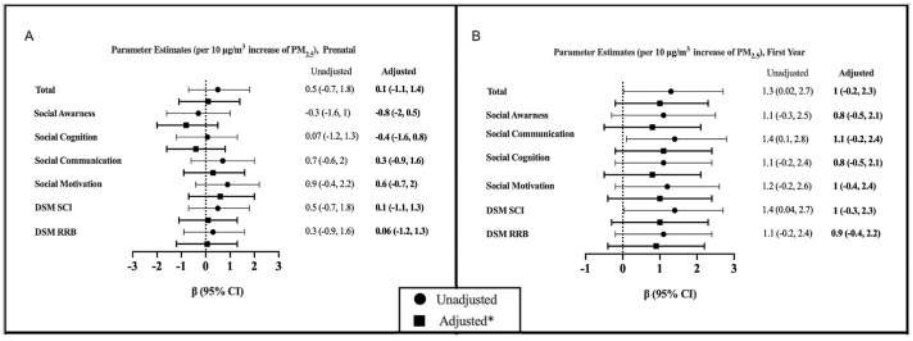

Fig. 3. Linear Regression Parameter Estimates for $PM_{2.5}$ exposure and SRS Scores
Unadjusted (circle) and adjusted* (square) parameter estimates (per prenatal and first year IQR μg/m^3 increase, 2.6 and 1.3 μg/m^3, respectively) for the association between prenatal (A) and first year (B) and $PM_{2.5}$ and SRS Total and component scores. *Adjusted for maternal education and community deprivation index.

trimester, was significant (Raz et al., 2015). Of those studies that specifically looked at first year of life exposure and ASD diagnosis, Volk et al. found this time period to be of significance (Volk et al., 2013) while Talbott et al. and Kaufman et al. did not observe any significant associations (Talbott et al., 2015; Kaufman et al., 2019). Other studies examining postnatal time points, such as Chen et al. examined exposure in the 2nd and 3rd year of life and Ritz et al. at 9 months of age, found statistically significant associations (Ritz et al., 2018; Chen et al., 2018).

Our study did have some limitations that should be considered in the context of our findings and comparisons to previous studies. First, our study population consists of typically developing children and does not represent the phenotypic extreme present in the case–control studies. Thus, only a few participants met the threshold for clinically relevant levels of autistic behaviors. Unmeasured confounders may also be present in our study and affected our observations. Studies have shown that higher gestational concentrations of some phthalate metabolites are associated with higher scores of autistic traits (Oulhote et al., 2020). Similarly, we do not have data regarding maternal folic acid intake which has been shown to modify the effects of air pollution on risk for ASD (Goodrich et al., 2018). In addition, our power to detect statistically significant associations was limited by our sample size. In particular, in models of prenatal and first year of life NO_2 and first year of life $PM_{2.5}$ the parameter estimates were consistently positive and similar between adjusted and unadjusted models.

Our study had multiple strengths including the availability of residential locations for all study participants, allowing us to assign modeled pollutant exposures for developmentally relevant time points while accounting for changes in home address prenatally and during early childhood. In contrast, the majority of previous studies on air pollution and adverse child health outcomes rely on addresses obtained from the birth certificate to classify exposure which may result in misclassification due to maternal residential mobility during pregnancy. A study conducted by Chen. et al. utilizing a New York birth cohort, found that 13% of mothers moved once during pregnancy and 3.5% moved at least twice (Chen et al., 2010). Our ability to characterize exposures throughout changes in residence during pregnancy and the first year of life was advantageous. In addition, we used air pollution estimates from validated models that have been utilized frequently in other studies (Qian et al., 2021; Xi et al., 2022; Qiu et al., 2022; Wyatt et al., 2022; Liao et al., 2021; Rahman et al., 2022; Lu et al., 2021; Shi et al., 2021; Yazdi et al., 2021). Furthermore, our study population is a combination of two well-established longitudinal cohorts, CCAAPS and HOME Study, which both used similar data collection measures allowing them to be merged for increased sample size and power. Also, to our knowledge, this was the first study to utilize the Social Responsiveness Scale as the outcome measure, allowing us to explore the effect of air pollution exposures on an entire range of ASD-associated traits and behaviors that vary in severity. Our study was also the first to analyze this association in this unique geographic region as three major interstates intersect in the Cincinnati, Ohio region to create one of the busiest commercial truck routes in the US (Institute ATR, 2019). The confluence of nearby coal-fired power plants in the Ohio River Valley, industrial emissions, and other sources of $PM_{2.5}$ in the region also result in annual $PM_{2.5}$ concentrations that place the region as the 13th worst metropolitan region in the US for air quality (Association, 2021). Thus, C4 participants reside in a region greatly impacted by NO_2 and $PM_{2.5}$.

5. Conclusions

ASD has lifelong social and behavioral implications with limited treatment options, emphasizing the need to identify modifiable risk factors for these disorders. Air pollution represents an important exposure to consider in the etiology of ASD since brain development and function are susceptible to insult from environmental toxicants during the prenatal and postnatal windows. While our results were not conclusive, we did observe a consistent elevated relationship between air pollution exposure and SRS scores. Collectively, the biological plausibility of air pollution affecting brain development and our results suggest the need for further study, particularly given the limited and inconclusive results from longitudinal cohort studies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114172.

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Ambient ozone exposure and depression among middle-aged and older adults: Nationwide longitudinal evidence in China

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ABSTRACT

Background: Epidemiological studies have linked long-term ozone (O_3) exposure with depression in developed countries. However, available literature is sparse and exists great heterogeneities. We aimed to investigate the association of long-term O_3 exposure with depression among Chinese middle-aged and older adults.
Methods: We designed a repeated measurement study based on longitudinal data from four waves (2011, 2013, 2015, and 2018) of the China Health and Retirement Longitudinal Study (CHARLS). Annual mean O_3 concentrations assessed through machine learning–based spatiotemporal models were assigned to each participant at city level. Depression score was measured using the 10-item Center for Epidemiologic Studies Depression scale (CES-D-10), with scores above the cut-off point of ten defined as depressive symptom. Mixed-effects models were used to evaluate the impact of O_3 on depression score and depressive symptom, and quantify the concentration-response (C-R) relationships. Subgroup analyses were performed to examine the potential effect modifications.
Results: A total of 19,582 participants with 60,125 visits were included in our analysis, with mean depression score of 8.1 (standard deviation: 6.3). Multivariable-adjusted mixed-effects model estimated a 6.34% (95% confidence interval [CI]: 3.34%, 9.43%) increase in depression score and an odds ratio (OR) of 1.29 (95% CI: 1.16, 1.45) for depressive symptom associated with per 10-μg/m^3 rise in annual mean O_3 exposure. Significantly elevated risks were identified only at high concentrations (approximately ≥90 μg/m^3). Participants who suffered from chronic diseases had a significant increased risk of depression (% Change in depression score: 8.42% [95% CI: 4.79%, 12.17%], and OR: 1.42 [95% CI: 1.24, 1.62]), and an evident effect modification was identified for depressive symptom ($P = 0.01$).
Findings: Our study provided novel evidence that long-term O_3 exposure could be a risk factor for depression among Chinese middle-aged and older adults. Our findings may have significant implications for formulating policies in reducing disease burden of depression by controlling air pollution.

1. Introduction

Depression gives rise to a large proportion of the health burden from mental disorder and substantially diminishes quality of life with an increased prevalence across the globe (Moreno-Agostino et al., 2021; Vos et al., 2020). Given the social and health care burden of this disorder, it is imperative to identify modifiable risk factors for prevention of depression. In addition to the identified social and behavioral factors (e.g., low socioeconomic status and smoking) (Li et al., 2021; Ribeiro et al., 2017), ambient air pollution has increasingly been recognized as

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an emerging risk factor for depression (Chen et al., 2018; Pun et al., 2017; Xue et al., 2021). Fine particulate matter ($PM_{2.5}$) has been widely identified as an important environmental determinant of depression onset and aggravation in several recent meta-analysis (Borroni et al., 2022; Braithwaite et al., 2019), while other major air pollutants such as ozone (O_3) has not been adequately investigated.

Associations of O_3 exposure with mental health deserve more epidemiological investigations. Biological mechanism study indicated that exposure to O_3 may be a potential risk factor for depression due to the high neurotoxicity and powerful oxidizing properties (Zhang et al., 2019). Emerging epidemiological studies had reported a positive association between short-term O_3 exposure and depression across the globe (Lu et al., 2020; Nguyen et al., 2021; Tsai et al., 2020), while limited researches investigated the long-term impact of O_3 exposure on depression (Borroni et al., 2022). Available longitudinal O_3-depression evidence was mainly reported in North America (Kioumourtzoglou et al., 2017) and Europe (Bakolis et al., 2021; Pelgrims et al., 2021), wherein great heterogeneities still existed between studies. For instance, a large American cohort study reported increased risk of depression associated with long-term O_3 exposure (Kioumourtzoglou et al., 2017), while non-significant association was observed in a recent regional study in Britain (Bakolis et al., 2021). Besides, the effect of O_3 exposure on depression may vary among subpopulations, which might be due to differential susceptibility of the subgroups to health effects of air pollution (Simoni et al., 2015) and confounding effect of comorbidities (Loop et al., 2013). Related evidence was largely sparse in developing countries such as China, where most locations have been experiencing serious O_3 air pollution (Lu et al., 2018) and rapid increase in depression prevalence during recent decades (Ferrari et al., 2022).

To fill this research gap, we designed a repeated measurement study based on a Chinese nationwide cohort of middle-aged and older men and women during 2011–2018. We primarily aimed to quantify the long-term association between O_3 exposure and depression in Chinese adults, and to depict the concentration-response (C-R) relationship across a wide range of exposure levels.

2. Materials and methods

2.1. Study population

The participants of this study were recruited from the China Health and Retirement Longitudinal Study (CHARLS), an ongoing nationwide longitudinal survey on the health and socioeconomic status of Chinese middle-aged and older adults. A representative sample of ~18,000 Chinese adults from 28 provinces was involved in the baseline survey conducted in 2011–2012, and was generally followed up every 2–3 years through a face-to-face computer-assisted interview (Zhao et al., 2014). Details of the study design and the purpose of the CHARLS are provided elsewhere (Chen et al., 2015; Zhao et al., 2014). The CHARLS study had been approved by the Ethics Committee of Peking University Health Science Center, and all participants gave written informed consent before participation (No. IRB00001052-11015).

Based on publicly available data from CHARLS surveys in 2011, 2013, 2015, and 2018 waves, we involved a sample of 25,458 individual adults with 76,768 observations. To conduct a longitudinal analysis, we focused on participants who had valid CES-D-10 records (n = 23,937). Each visit represented each face-to-face interview with the participant, and could generate a single observation for the participant. We further excluded the adults <40 years old at survey time and participants visited only once. Finally, this study involved 60,125 observations from 19,582 individual adults (Fig. 1) distributed across 125 cities spanning 28 provinces (Fig. 2). Details for description of sample inclusion and exclusion were presented in supplementary materials.

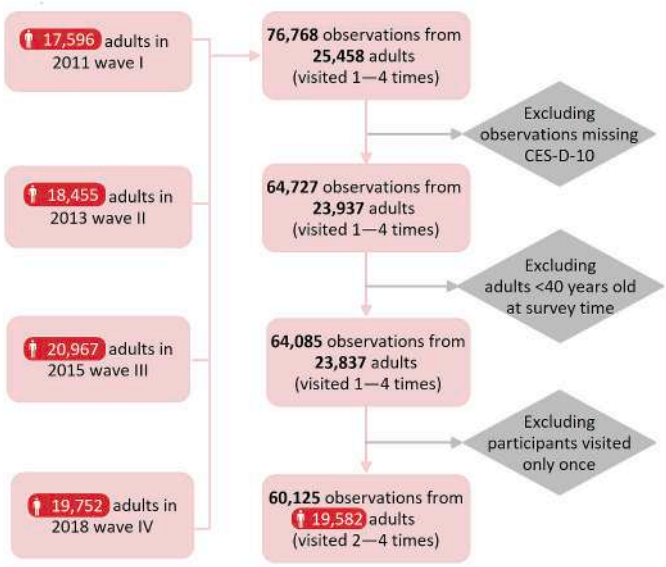

Fig. 1. Flowchart of sample inclusion and exclusion.

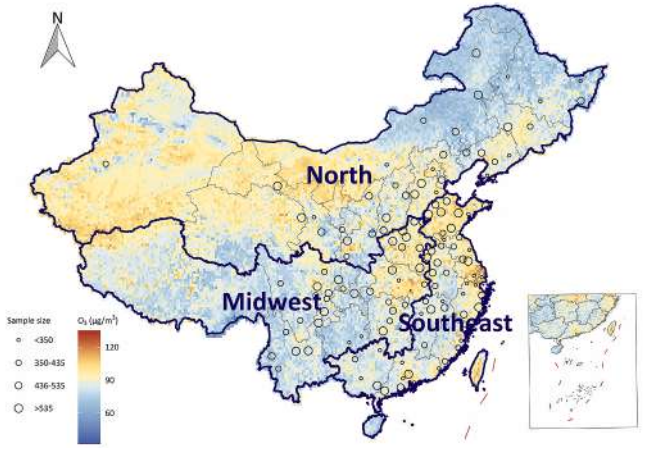

Fig. 2. The map of participants' geographic distribution with long-term averages of O_3 concentrations (2010–2018).

2.2. Environmental exposure

The concrete residential addresses of participants were unable to obtain due to limited access to privacy data in CHARLS, we thus assessed O_3 exposure at the city-level in the longitudinal analysis. By aggregating gridded O_3 estimates at the resolution of 0.1° × 0.1° into city-level averages, we first derived monthly ozone concentrations of 125 prefectural cities from 2010 to 2018, and then calculated average O_3 concentrations over the 12 months preceding the surveyed month for each individual as the exposure. The same method was used to assign annual $PM_{2.5}$ exposure to participants. For privacy consideration, the survey date of CHARLS available for public only included the year and month, which limited our ability to assess O_3 exposure directly based on specific survey date. However, for long-term exposure assessments (e.g., annual mean exposure), calculation using monthly averages instead of daily estimates would not greatly affect the assessments.

Monthly O_3 concentrations across Chinese mainland during 2005–2018 at a resolution of 0.1° were estimated through a data fusion of observations and models. In brief, the daily maximum 8-h O_3 monitoring data (MDA8) of 1713 stations in China from 2013 to 2017 were used to establish a national MDA8 prediction model based on eXtreme

Gradient Boosting (XGBoost). Regional monitors data during 2005–2012 and nationwide measurements in 2018 were applied to test the prediction accuracy with high R^2 values (range: 0.60–0.87) and low root mean square error (RMSE, 12.94–18.41 μg/m^3) in different years (Liu et al., 2020a). The daily ground-level O_3 monitoring data were obtained from the China National Environmental Monitoring Center. More modeling details for O_3 estimates could be found in prior publication (Liu et al., 2020a).

Gridded $PM_{2.5}$ concentrations at a 0.1° × 0.1° resolution from 2010 to 2018 on a monthly scale were derived from Tracking Air Pollution (TAP, http://tapdata.org.cn/en, accessed on April 14th, 2023), which was known as a near real-time air pollutant database in China. Daily $PM_{2.5}$ concentrations were estimated based on a two-stage machine learning model combined with a tree-based gap-filling method and the synthetic minority oversampling technique (Geng et al., 2021). The predicted concentrations of $PM_{2.5}$ showed good consistency comparing against the ground observations, with out-of-bag cross-validation R^2 of 0.80–0.88 and RMSE of 13.9–22.1 μg/m^3 for different years between 2013 and 2020 (Xiao et al., 2021).

2.3. Measurement of depressive symptom

The CHARLS used the 10-item Center for Epidemiologic Studies Depression scale (CES-D-10) to measure the mental health status of the participants, which were collected during a computer-assisted interview by fieldworkers. Cronbach's alpha coefficient was calculated to evaluate the reliability of the CES-D-10 scale used in our analysis. This coefficient ranged between 0.76 and 0.81 in selected waves (2011–2018) of the CHARLS survey (Table S1), which displayed considerably high internal consistencies across the total sample. Prior studies had examined the validity of the CES-D-10 among Chinese adults (Boey, 1999). The CES-D-10 questionnaire included 10 questions and each question measured the frequency of negative mood using a score of 0 (rarely or none of the time), 1 (some or a little of the time), 2 (occasionally or a moderate amount of the time), or 3 (most or all of the time). Two questions were scored in a negative direction. The sum of scores for 10 specific questions were calculated as the depression score to indicate the general status of depressive symptom for each participant. The depression score ranged from 0 to 30, and a higher score represented a higher severity of depression. The depression score greater than or equal to 10 was used as a cut-off for categorizing the status of depressive symptom (Liu et al., 2020b; Yao et al., 2022). Intraclass correlation coefficients (ICC) for depression score during different waves ranged from 0.67 to 0.80 (Table S2), which suggested a relatively high reproducibility of measurements.

2.4. Covariates

We considered a set of covariates in accordance with prior epidemiological studies (Shi et al., 2022; Xue et al., 2021), enabling us to separate the effect of ambient O_3 from potential confounders. The covariates included demographic characteristics (i.e., sex, age, marital status, educational level, employment status, residence, and region), behavioral factors (i.e., cigarette smoking and alcohol drinking), health factors (i.e., chronic diseases, disability in activities of daily living and health status), and environmental factors (i.e., household cooking fuel and outdoor temperature).

To be concrete, marital status was defined as a dichotomous variable, with "yes" representing the participant has been married and lived with a spouse or cohabitation, and "no" otherwise; educational level was divided into below primary school (0 y), primary and middle school (1–9 y), or above middle school (>9 y); employment status was divided into "yes" or "no" according to whether participant was employed; residence was classified as either rural or urban; region was divided into Midwest, Southeast, and North area; cigarette smoking was classified as "yes" or "no"; alcohol drinking were categorized into never, rarely, and frequently; chronic disease was classified as "yes" for participants suffering one or more physician-diagnosed chronic diseases (e.g., stroke, diabetes, coronary heart disease, and hypertension, etc.); health status was divided into "good", "fair" or "poor" according to the self-report judgement; disability in activities of daily living was classified as "yes" or "no"; household cooking fuel was categorized as clean fuel (i.e., electricity, liquefied and natural gas) and solid fuel (i.e., wood, coal, biomass charcoal, and straw). Monthly average temperatures at the city-level were aggregated from daily estimates of the European Center for Medium-Range Weather Forecasts (ECMWF) atmospheric reanalysis data set of the global climate at a resolution of 0.1° × 0.1°.

2.5. Statistical analysis

Continuous variables were described using mean and standard deviation (SD) and category variables were presented by frequency and percentage. Given the identified impact of ambient $PM_{2.5}$ on depression onset and aggravation (Borroni et al., 2022; Braithwaite et al., 2019; Xue et al., 2021), we applied a bi-pollutant model to estimate the associations between O_3 exposure and depression. Considering the longitudinal repeated measurement study design, mixed-effects linear regression model was used to analyze the impact of O_3 on depression score. The model was specified as follows:

$$Log\left(Score_{i,j}+1\right)=\beta_0+\beta x_{i,j}+\gamma z_{i,j}+city_i+\eta(community_i)+\mu(i),$$

where i denotes the subject index; j refers to visit index; β_0 represents the intercept; β is the regression coefficients for air pollutants; $z_{i,j}$ denotes a set of adjusted covariates and γ represents the corresponding regression coefficients; $city_i$ denotes a fixed effect to control the unmeasured city-specific risk factors of depression (Ng, 1997); η and μ denotes two random intercepts to model the correlations between records from the same community or the same subject, respectively. The change in depression score was calculated as $100\% * [exp\ (10 * \beta) - 1]$ to quantify the impact of O_3 on depression score. We adopted a sequential adjustment approach to define the models with different levels of adjustment. The crude model (model 1) was developed to incorporate study cities and the concentrations of air pollutants (O_3 and $PM_{2.5}$) as the fixed-effect term and set individual ID and community as the random-effect terms. Model 2 controlled for demographic characteristics based on the crude model. Model 3 further adjusted behavioral and health factors. Multivariable-adjusted model (model 4) further accounted for environmental factors. Variance inflation factor (VIF) was used to test collinearity in the multivariable-adjusted model, and the VIF-based assessments did not imply appreciable multicollinearity (Table S3). The associations between O_3 and depressive symptom was assessed through mixed-effects logistic regression models with the same adjustments of covariates and parameters as the linear regression models.

To investigate the C-R relationship between O_3 with depression score and depressive symptom, O_3 exposure was fitted as a smoothing term using a restricted cubic spline (RCS) function in the multivariable-adjusted model (Zhang et al., 2022). We adopted three knots to fit the curve according to Akaike information criterion and Bayesian information criterion (Table S4). Likelihood-ratio test was applied to examine the potential nonlinearity of the relationship (Liu et al., 2019). We performed subgroup analyses to examine potential effect modifications, stratified by sex (male or female), age group (<65 or ≥65 years), educational levels (0 y, 1–9 y, or >9 y), employment status (yes or no), cigarette smoking (yes or no), alcohol drinking (never, rarely or frequently), residence (urban or rural), region (Midwest, North, or Southeast) and chronic diseases (yes or no). Meta-regression methods were used to examine whether the differences between subgroups were statistically significant (Liu et al., 2021; Yang et al., 2015).

Sensitivity analyses were performed to assess the robustness of our results. First, we compared the effects of alternative exposure time windows using 2-year average O_3 and $PM_{2.5}$ concentrations. Second, to

Table 1
Constant variables of all studied subjects.

Variable	Subgroup	No. (%)
Total number of Subjects		19582
Educational levels	Below elementary school	7615 (38.9)
	Elementary and middle school	7976 (40.7)
	Above middle school	2377 (12.1)
	Unknown	1614 (8.2)
Sex	Female	10171 (51.9)
	Male	9398 (48.1)
	Unknown	13 (<0.1)
Place of residence	Rural	11698 (59.7)
	Urban	7884 (40.3)
Region	Midwest	6445 (32.9)
	North	5531 (28.2)
	Southeast	7606 (38.8)

Because of using rounding-off method, it may occur that the sum of the percentages does not equal 100%.

comprehensively assess O_3 effects, we re-estimated O_3-depression association using a single-pollutant model without the adjustment of $PM_{2.5}$. Third, considering that air pollutants may have an interactive effect with age on depression (Xue et al., 2021), we adjusted the interaction term of age and O_3 in the multivariable-adjusted model. Fourth, the directed acyclic graph (DAG) was used to identify a minimal sufficient adjustment set of variables (Fig. S1), which was widely used to select covariates for minimizing confounding bias in epidemiological studies (Textor et al., 2016). Fifth, to better capture the long-term effect of O_3 exposure on depression, we excluded participants who only had twice consecutive visits (n = 3238), and re-estimated the associations based on the multivariable-adjusted analysis (model 4). Sixth, we further performed an additional subgroup analysis to explore the potential modification effect of type-specific chronic diseases on O_3-depression associations. Additionally, we used multiple imputation to fill in the missing values and rerun the multivariable-adjusted model (model 4) to verify the stability of our main results.

All analyses were conducted by R software (Version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria), with the "lme 4" package for analyses of mixed-effects models, the "mice" package for multiple imputation, the "rms" package for nonlinear smoothing using restricted cubic spline, and the "effects" package for prediction of C-R relationship. Two-sided test with *P*-value <0.05 was considered statistically significant.

3. Results

Table 1 summarizes sociodemographic and behavioral characteristics of 19,582 middle-aged and older adults included in this study. During a total of 60,125 visits from 2011 through 2018, each participant

Table 2
Longitudinal variables of the studied subjects and environmental exposures at baseline and three consequent waves.

Variable	Total, n (%)	2011 CHARLS	2013 CHARLS	2015 CHARLS	2018 CHARLS
Number of visits	60125	13530	14739	16842	15014
Age (yrs), mean (SD)	59.5 (9.5)	58.3 (9.2)	59.3 (9.4)	59.1 (9.8)	61.1 (9.3)
Depressive symptom	20565 (34.2)	4945 (36.5)	4574 (31.0)	5490 (32.6)	5556 (37.0)
Depression score	8.1 (6.3)	8.3 (6.3)	7.8 (5.8)	7.9 (6.4)	8.5 (6.5)
Married and lived together					
No	10098 (16.8)	2097 (15.5)	2317 (15.7)	2857 (17.0)	2827 (18.8)
Yes	50020 (83.2)	11433 (84.5)	12416 (84.2)	13984 (83.0)	12187 (81.2)
Unknown	7 (0.1)	0	6 (0.1)	1 (0.1)	0
Cigarette smoking					
No	34776 (57.8)	8269 (61.1)	8503 (57.7)	9447 (56.1)	8557 (57.0)
Yes	25321 (42.1)	5260 (38.9)	6235 (42.3)	7380 (43.8)	6446 (42.9)
Unknown	28 (0.1)	1 (0.1)	0	15 (0.1)	11 (0.1)
Alcohol drinking					
Frequent	16253 (27)	3413 (25.2)	4050 (27.5)	4660 (27.7)	4130 (27.5)
Rare	4868 (8.1)	1049 (7.8)	1179 (8.0)	1477 (8.8)	1163 (7.7)
Never	39000 (64.9)	9068 (67.0)	9507 (64.5)	10704 (63.6)	9721 (64.7)
Unknown	4 (0.1)	0	3 (0.1)	1 (0.1)	0
Employment status					
No	18474 (30.7)	3894 (28.8)	4427 (30.0)	5121 (30.4)	5032 (33.5)
Yes	41580 (69.2)	9610 (71.0)	10291 (69.8)	11701 (69.5)	9978 (66.5)
Unknown	71 (0.1)	26 (0.2)	21 (0.1)	20 (0.1)	4 (<0.1)
Health status					
Fair	22027 (36.6)	4426 (32.7)	4786 (32.5)	5312 (31.5)	7503 (50.0)
Good	11629 (19.3)	2145 (15.9)	2506 (17.0)	3167 (18.8)	3811 (25.4)
Poor	26447 (44.0)	6957 (51.4)	7434 (50.4)	8360 (49.6)	3696 (24.6)
Unknown	22 (<0.1)	2 (<0.1)	13 (0.1)	3 (<0.1)	4 (<0.1)
Chronic diseases					
No	17784 (29.6)	4358 (32.2)	4706 (31.9)	4738 (28.1)	3982 (26.5)
Yes	38511 (64.1)	9063 (67.0)	9555 (64.8)	9476 (56.3)	10417 (69.4)
Unknown	3830 (6.4)	109 (0.8)	478 (3.2)	2628 (15.6)	615 (4.1)
ADL disability					
No	44664 (74.3)	11409 (84.3)	11359 (77.1)	11931 (70.8)	9965 (66.4)
Yes	15433 (25.7)	2113 (15.6)	3380 (22.9)	4898 (29.1)	5042 (33.6)
Unknown	28 (0.1)	8 (0.1)	0	13 (0.1)	7 (0.1)
Household cooking fuel					
Clean	34147 (56.8)	6004 (44.4)	7934 (53.8)	10015 (59.5)	10194 (67.9)
Solid	25802 (42.9)	7476 (55.3)	6742 (45.7)	6791 (40.3)	4793 (31.9)
Unknown	176 (0.3)	50 (0.4)	63 (0.4)	36 (0.2)	27 (0.2)
Environmental exposure, mean (SD)					
O_3 concentration($\mu g/m^3$)	88.7 (7.5)	89.9 (7.2)	88.4 (7.4)	88.1 (7.5)	88.4 (7.7)
$PM_{2.5}$ concentration ($\mu g/m^3$)	50.8 (21.9)	58.2 (23.9)	57.3 (24.5)	48.8 (19.2)	40 (14.0)
Temperature (°C)	13.6 (5.0)	13.1 (5.0)	13.6 (5.2)	13.7 (4.9)	13.7 (5.0)

Notes: Because of using rounding-off method, it may occur that the sum of the percentages does not equal 100%. Abbreviations: O_3, ozone; $PM_{2.5}$, fine particulate matter; SD, standard deviation; ADL disability, disability in activities of daily living.

Table 3
Estimated associations of O_3 exposure with depression score and depressive symptom.

	Depression score (% Change, 95% CI)	Depressive symptom (OR, 95% CI)
Model 1	3.72 (0.96, 6.56)	1.18 (1.06, 1.31)
Model 2	3.84 (1.05, 6.69)	1.19 (1.07, 1.32)
Model 3	5.35 (2.50, 8.28)	1.26 (1.13, 1.40)
Model 4	6.34 (3.34, 9.43)	1.29 (1.16, 1.45)

Model 1 was crude model.
Model 2: Adjusted for covariates in model 1 plus demographic characteristics including age, sex, educational level, marital status, employment status, residence and region.
Model 3: Adjusted for covariates in model 2 plus behavioral and health factors including alcohol drinking, cigarette smoking, ADL disability, chronic diseases and health status.
Model 4: Adjusted for covariates in model 3 plus environmental factors including household cooking fuel and outdoor temperature. Model 4 was multivariable-adjusted model.
Abbreviations: O_3, ozone; OR, odds ratio; CI, confidence interval.

was visited 3.1 times on average. Participants were aged 59.5 (SD: 9.5) years (range: 40–108 years), and nearly half of them were males (48.0%). The summary of longitudinal variables for participants in each wave of CHARLS (2011, 2013, 2015, and 2018 waves) were presented in Table 2. Among all visits, the mean depression score was 8.1 (SD: 6.3), and 34.2% were measured as depressive symptom. The characteristics for depressed and non-depressed participants were presented in Table S5. Annual mean concentrations of $PM_{2.5}$ for 125 cities showed a substantial decline from 2011 (58.2 μg/m^3) to 2018 (40.0 μg/m^3), while the average concentrations of O_3 fluctuated around 88.7 μg/m^3 (SD: 7.5 μg/m^3) during same period.

Table 3 estimates associations of ambient O_3 exposure with depression score and depressive symptom. Consistently positive effects of O_3 were observed in different models (model 1–4). According to the multivariable-adjusted model (model 4), a 10-μg/m^3 increase in annual mean O_3 exposures was associated with an excess risk of 6.34% (95% confidence interval [CI]: 3.34%, 9.43%) for elevated depression score. In the secondary analysis based on depressive symptom (depression score ≥10), positive associations were also identified using different models, with comparable odds ratio (OR) estimates ranging from 1.18 (95% CI: 1.06, 1.31) in model 1 to 1.29 (95% CI: 1.16, 1.45) in model 4.

Fig. 3 outlines the shape of C-R curves for long-term associations between annual average O_3 exposure and percentage change in depression score and OR for depressive symptom. We identified a nonlinear relationship between O_3 exposure and depression score (P = 0.02 for nonlinearity), with significantly elevated risks only at high concentrations (approximately ≥90 μg/m^3). In terms of depressive symptom, no evident violation of linear C-R association (P = 0.06) was identified, but we also detected steeper slopes at high concentrations.

Fig. 4 illustrates results from subgroup analysis on O_3-depression relationship stratified by demographic, behavioral and health factors. Males suffered from higher risk of depression (% Change in depression score: 8.33% [95% CI: 3.84%, 13.02%], and OR: 1.44 [95% CI: 1.21, 1.71]) associated with a 10-μg/m^3 rise in O_3 exposure. Participants under 65 years old were at higher risk when exposure to outdoor O_3 pollution, with corresponding excess risk of 6.58% (95% CI: 2.89%, 10.41%), and OR of 1.33 (95% CI: 1.16, 1.52) respectively. O_3-depression associations were more evident among individuals who were smoking or drinking frequently, although effect modifications were non-significant between stratum. Only participants who suffered from chronic disease had a significantly risk of depression (% Change in depression score: 8.42% [95% CI: 4.79%, 12.17%], and OR: 1.42 [95% CI: 1.24, 1.63]), and an evident effect modification were identified for depressive symptom (P = 0.01). The sub-region analyses presented positive associations in the Midwest and Southeast, while non-significant relationship was observed in North. Although participants in Midwest suffered from higher increased risk of depression associated with O_3 exposure than those in North, the difference was only significant for the depression score.

Sensitivity analyses largely supported the findings from our main

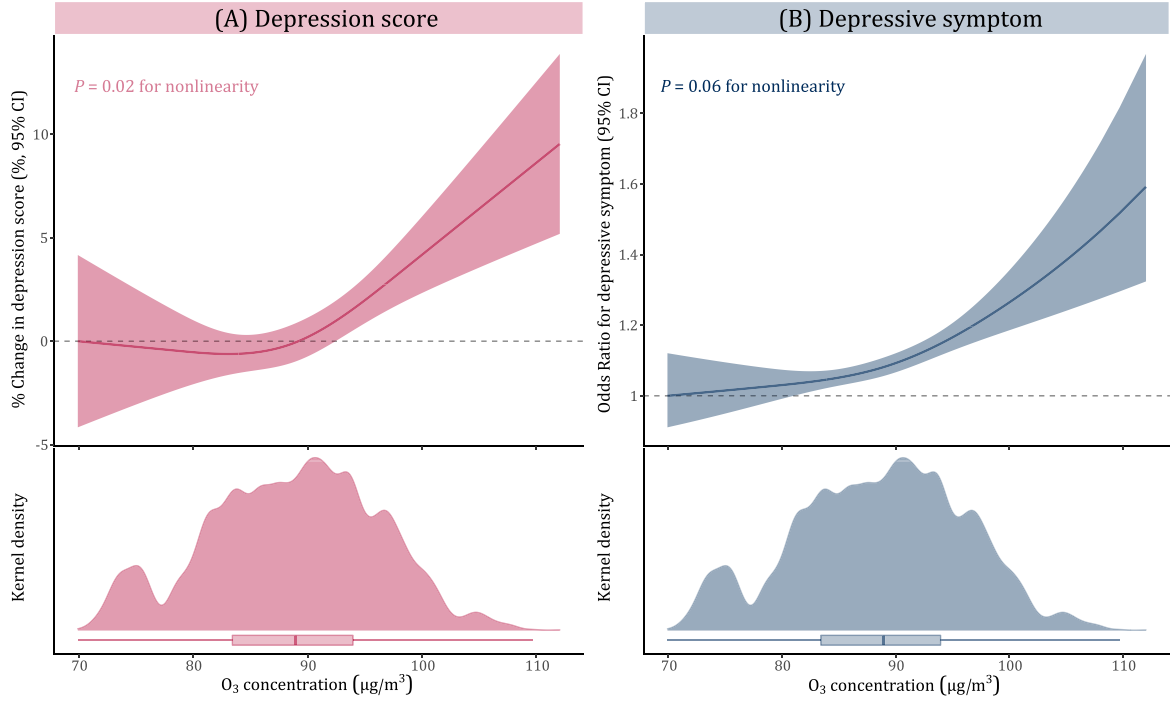

Fig. 3. Shapes of concentration-response relationships of O_3 exposure with depression. Dash area represents the 95% confidence interval (CI). Notes: the kernel density plot and box plot were used to describe the distribution of O_3 exposure for participants during study period, with 83.4 μg/m^3, 88.9 μg/m^3, 94.0 μg/m^3 for 25th, 50th, and 75th percentile O_3 concentrations, respectively.

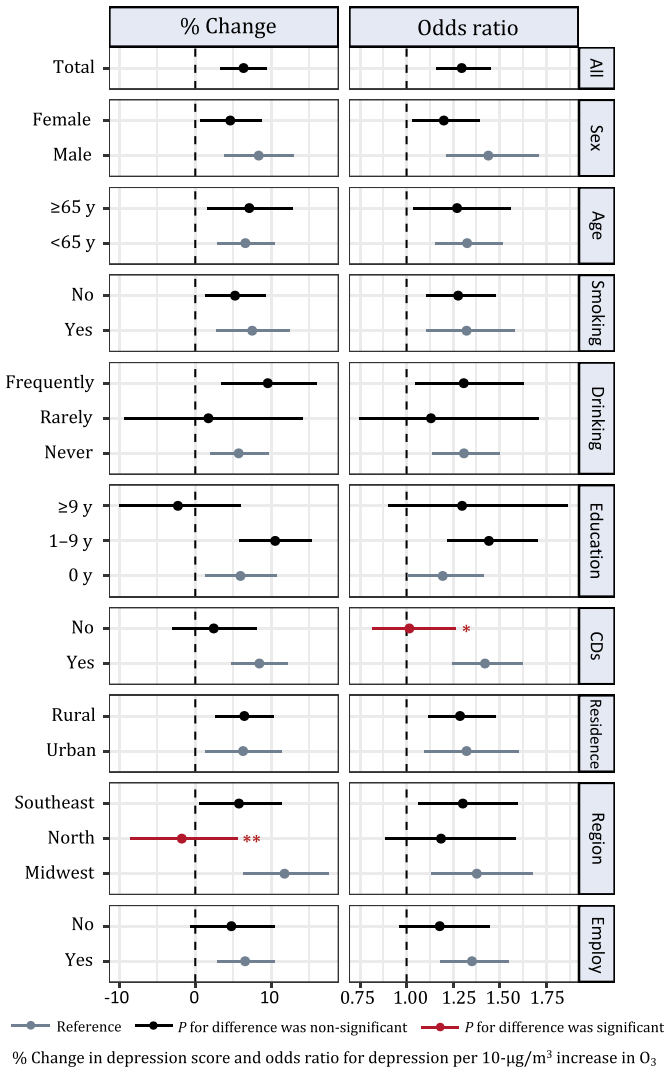

Fig. 4. Subgroup-specific associations of O_3 exposure with depression score and depressive symptom. Notes: † represents the reference group. Error bars, 95% confidence intervals. *P*-values were shown for tests of the null hypothesis that the point-estimate of the association was identical between subgroups; *$P < 0.05$, **$P < 0.01$. The associations were estimated using model 4. Abbreviations: CDs, chronic diseases; O_3, ozone.

analysis (Table S6). The association of O_3 exposure with depression was insensitive to use single-pollutant model (percentage change in depression score: 4.85% [95% CI: 1.91%, 7.86%], and OR: 1.13 [95% CI: 1.02, 1.27]). When using exposure concentrations at the 2-year scale, we observed comparable estimates of increase in depressive score (4.56% [95% CI: 0.41%, 8.87%]) and risk of depressive symptom (1.37 [95% CI: 1.17, 1.60]). The associations were also robust by excluding participants who only had twice consecutive visits, adjusting the minimum set of variables based on directed acyclic graph, and adding the interaction term of age and O_3 in the multivariable-adjusted model. Compared with participants without asthma, significantly higher percentage increase in depression score (29.95, 95% CI: 11.89, 50.94) associated with O_3 exposure was observed among asthma participants, while there was non-significant difference between the stratums when considering the depressive symptom as the outcome (Table S7).

4. Discussion

To the best of our knowledge, this is the first national study to investigate long-term effect of O_3 on depression in China. In this longitudinal study, we found a significantly positive association between long-term O_3 exposure and depression among Chinese middle-aged and older adults. O_3-depression associations were robustness by performing sensitivity analyses and adjusting different categorized covariates, while some evidence for effect modification by chronic diseases was observed. Our findings provided essential longitudinal evidence for O_3-depression associations and may contribute to the primary prevention of depressive disorders.

We observed a positive association between long-term O_3 exposure and depression, which is generally consistent with several prior longitudinal studies (Kioumourtzoglou et al., 2017; Zhao et al., 2020). However, great heterogeneity in epidemiologic evidence existed across investigations. A prospective cohort study suggested an increased risk of depression associated with exposure to O_3 among 41,844 older women from 11 US states (Kioumourtzoglou et al., 2017). Meanwhile, insignificant associations were reported by a prospective longitudinal survey in London enrolling 1698 adults (Bakolis et al., 2021) and a cross-sectional study in Brussels enrolling 1325 inhabitants aged ≥15 years (Pelgrims et al., 2021). These discrepancies may be attributed to differences in study population and design, the magnitude of sample size, as well as demographic characteristics. Risk of depression relating to O_3 exposure is biologically plausible (Martinez-Lazcano et al., 2013; Thomson, 2019) and could be supported by an animal experimental evidence (Li et al., 2019). For instance, inhalation of ambient O_3 could strengthen the release of some hormones (i.e., glucocorticoids and stress hormones) by activating the hypothalamic-pituitary-adrenal (HPA) axis (Henriquez et al., 2019), which was recognized as a pivotal mechanism in the development of mental disorders (Thomson, 2019).

Few prior studies investigated the C-R curve between O_3 exposure and depression. In this study, our analysis highlighted a nonlinear C-R relationship between long-term exposure to O_3 and depression scores. We observed significant increases of depression scores only at high exposure concentrations (approximately ≥90 μg/m^3), suggesting a potential threshold in O_3-related risk of depression. A recent repeated measurement survey (Shi et al., 2022) had reported similar effect of mid-term O_3 exposure on depression scores among 3445 middle-aged and older people in China. This association also could be interpreted by previous studies of neural mechanisms (Martinez-Lazcano et al., 2013; Thomson, 2019). At low levels of O_3 exposure, given the short half-life, it is difficult for O_3 to accumulate in sufficient concentrations to enter the brain to affect the nervous system (Martinez-Lazcano et al., 2013). Despite non-significant evidence ($P = 0.06$) for nonlinear C-R association with depressive symptom, we detected a similar pattern with steeper slopes at high O_3 concentrations. Since depressive symptom was defined by depression scores (depression score ≥10) rather than clinical diagnoses, there may exist possible misclassifications in depression, which would have an inevitable impact on the analysis of O_3-depression relationship (Shi et al., 2022).

Most existing epidemiological studies failed to investigate effect modifications by demographic and behavioral characteristics when assessing relationships of depression with long-term O_3 exposure (Bakolis et al., 2021; Kioumourtzoglou et al., 2017; Pelgrims et al., 2021; Zhao et al., 2020). When performing subgroup analyses stratified by demographic and behavioral characteristics, we did not observe significant differences except for the covariate of region. Comparing with participants living in North, a significantly stronger association of O_3 with depression was observed among individuals in Midwest. This difference may due to the smaller sample size in the north and relatively higher O_3 concentration in Midwest. Similar findings were also reported in a quasi-experimental investigation on the relationship between long-term $PM_{2.5}$ exposure and depression based on CHALRS 2011–2015 (Xue et al., 2021). Meanwhile, there is also some suggestive evidence on population vulnerability in risk of depressive disorders related to mid-term (Shi et al., 2022) or short-term O_3 exposures (Lu et al., 2020). In a multi-community longitudinal investigation spanning 11 Chinese provinces (Shi et al., 2022), older adults aged 65+ years were found to

be at greater risk of depressive disorders associated with mid-term O_3 exposure; In a case-crossover analysis in 13 Chinese cities using records of 111,842 hospital outpatient visits for mental disorders, women exhibited higher vulnerability to short-term O_3-related depression (Lu et al., 2020). Given the wide lack of available population-based longitudinal evidence, more assessments in future studies are warranted to provide a better understanding of susceptible subgroups to depression related to O_3 exposure.

In the stratified analysis, we observed significantly higher risk of depression associated with long-term O_3 exposure in participants with preexisting chronic diseases. This finding was echoed with a time-stratified case-crossover study in Korea, reporting increased air pollution-associated risk of emergency department visits for depressive episode in patients with cardiovascular disease, diabetes mellitus, or asthma (Cho et al., 2014). Although the underlying mechanisms may be difficult to elucidate, a potential hypothesis has been suggested that individuals with some chronic diseases (e.g., diabetes mellitus, asthma) could be more vulnerable to O_3 related depression arising from the influence of O_3 on the psycho-endocrine-immune system through an inflammatory process (Cho et al., 2014; Patterson, 2011). Notably, the modification effects showed inconsistent direction when considering the specific classification of chronic diseases (Lim et al., 2012). Although significantly higher percentage increase in depression score associated with O_3 exposure was observed among asthma participants in our analysis, these results should be interpreted cautiously due to the limited sample size ($n = 553$) of participants prevalent with asthma. Consequently, more large-scale cohort investigations should be conducted to further explore the interactive effects of air pollution and various chronic diseases on depression.

Our study had several limitations. First, we estimated O_3 exposure for individuals at the city level due to limited access to residential address, which may lead to exposure misclassification by ignoring within-city variations in O_3 concentrations (Wu et al., 2019). Although this ignorance would not change direction of the associations, it may introduce bias into point-estimate of the association. Second, since the obvious distinction for O_3 concentrations between ambient environment and household, overestimation of individual O_3 exposure may be caused by unconsidered indoor O_3 exposure (Hu et al., 2020). Third, due to data unavailability to specific survey date for each participant, we assessed annual average O_3 exposure for each participant using monthly O_3 mean concentrations aggregated from daily estimates. This might induce the measurement bias on exposure to some extent. Fourth, unmeasured potential confounders (e.g., drug abuse and history of mental disorders) may still bias O_3-depression relationship, even though we had considered a rich set of covariates in our main analysis. In addition, the findings in our study may not be generalizable to younger population, as we only included middle-aged and older adults who had a higher prevalence of depression (Lu et al., 2021).

5. Conclusions

In summary, this study provided nationwide longitudinal evidence for positive associations between long-term O_3 exposure and depression among Chinese middle-aged and older adults. Our stratified analysis suggested higher susceptibility to O_3-related risk of depression among participants suffering from chronic diseases. These findings highlighted the significance of strengthened clean air action to reduce the global and regional burden of depressive disorders in the context of population ageing, particularly in low- and middle-income countries experiencing high-level air pollution.

Author contributions

Yang Yuan: Writing - original draft, Writing - review & editing, Methodology, Formal analysis; **Kai Wang**: Writing - review & editing, Validation, Visualization; **Zhen Wang**: Writing - review & editing, Supervision; **Hao Zheng**: Resources, Data Curation; **Zongwei Ma**: Resources, Data Curation; **Riyang Liu**: Resources, Data Curation; **Kejia Hu**: Software, Visualization; **Zhiming Yang**: Software, Visualization; **Yunquan Zhang**: Writing - review & editing, Supervision, Funding acquisition. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114185.

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A paradigm shift in cooperation between industry, legislation, and research to protect people and the environment

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1. Crossing the Rubycon

Modern human society is organized in complex ways. Today there are hardly any areas that are not somehow connected to the international trade network. This means that technologies and products manufactured anywhere can reach virtually any other point on the planet to be processed, used, or consumed. For many decades, the focus clearly was on the functionality of a product. Little attention was paid to whether it contained hazardous chemicals, whether they were released, how the product was manufactured, and how the product could be disposed of in an environmentally friendly manner. This attitude only changed when reports about environmental contamination and diseases became common. Within a short period of time, environmental legislation was enforced and the idea of recycling emerged. Suddenly, industry was confronted with various requirements, limit values, and laws, which often led to controversial and sometimes bitter discussions with authorities and environmental organizations.

The distrust was mutual. Authorities accused industry of accepting environmental damage for profit reasons, while industry in turn complained about unnecessarily high hurdles that limited or prohibited the sale of certain products, but hardly helped the environment. Unfortunately, both sides were often right. There were and are individual companies and entire branches of industry that put their economic interests ahead of ecological considerations and consciously try to undermine laws or use legal loopholes to their advantage. *Dieselgate*, with the illegal manipulations of various car manufacturers, made this very clear to us. However, there are also people in public authorities who demand or enforce disproportionately high requirements for political reasons.

Over time, however, the various interest groups have learned that it makes little sense to work against each other. As a rule, only the manufacturer of a product knows its exact composition and properties. Unilateral bans on certain components lead to substitutes, which in turn are also banned and replaced. This concept ends in a tortoise and hare contest that is unsatisfactory for all sides. In addition, the requirements and demands of consumers have grown with increasing environmental awareness in the population. It was therefore advisable for companies to establish voluntary minimum standards for their products, which required extensive investigations even before they were launched on the market. This intensified cooperation with research institutions, as small and medium-sized companies rarely have the appropriate capacities, and large companies often lack the expertise. Authorities fall back on research institutes for the investigation and evaluation of product properties, which should ultimately end in environmental recommendations or regulations. However, it is also often the results of research that trigger further measures. By far the best known example is formaldehyde – interestingly, the first publication on the release of formaldehyde from wood-based materials came from industry (Salthammer et al., 2010). Certainly, no industrial chemical has been more intensively examined and regulated with regard to its release to the indoor

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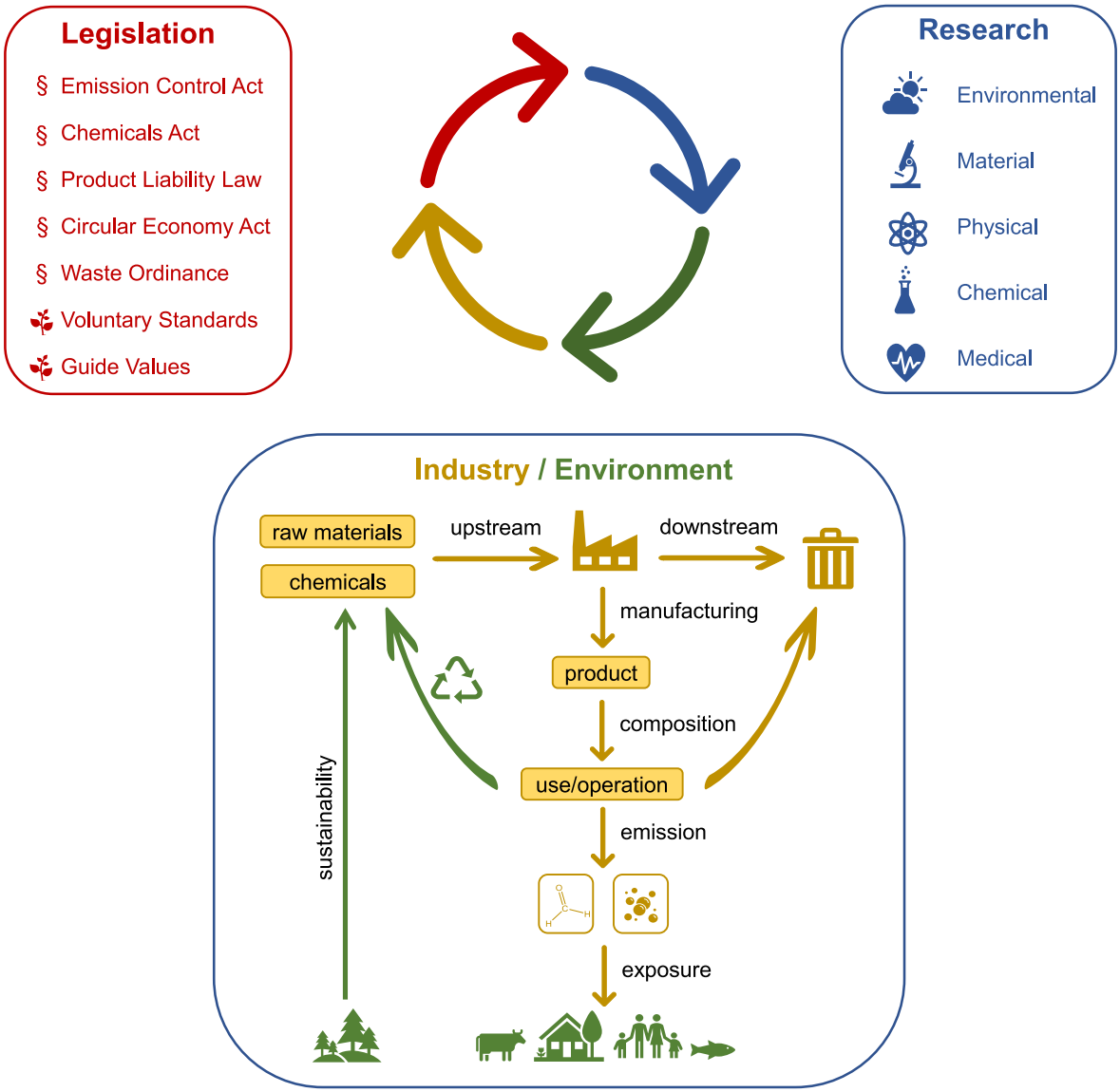

Fig. 1. A product available on the market today has to meet a number of requirements, which usually go beyond legal regulations. The product and production process should be sustainable and have no negative impact on the environment. Recycling has priority over landfill. These complex issues require interdisciplinary research and interplay with stakeholders.

environment. Nevertheless, the call for even lower guide values is heard again and again. Apart from the question of whether this makes sense for health reasons, there is a risk of losing sight of hazards from other substances.

2. The advantages of synergy

The current international regulations require a number of criteria to be observed when manufacturing and distributing products, which affect not only the manufacturer, but also the supply chains (Luthra et al., 2016). This means that the entire framework of the product design must be planned in advance with the help of internal and external expertise. The complicated, nested relationships and requirements are shown in Fig. 1 and demonstrate the necessary interaction between legislation, industry, and research.

Several interest groups have successfully set up joint ventures. For example, the German Federal Ministry for the Environment and the German Chemical Industry Association (VCI) started a cooperation to promote human biomonitoring in 2010. The main goal is to improve knowledge of substances to which the population may be increasingly exposed or which may be of particular relevance to health (Kolossa-Gehring et al., 2017). A very positive outcome of this joint venture was the market launch of the phthalate-free plasticizer DINCH, which was carried out with great transparency and with all the necessary ecological and toxicological data being available. A reliable biomonitoring method was quickly developed so that the internal exposure of the population could be monitored promptly (Kasper-Sonnenberg et al., 2019). This and other initiatives led to a very open dialogue about critical chemicals and possible substitutes (Apel et al., 2017; Salthammer, 2020).

Generally, manufacturing processes and product composition are more transparent today. In the past, the exact chemical recipe of products was often a holy grail whose secret the industry was reluctant to reveal. This has changed for the benefit of a better evaluation of products. For example, the chemical processes taking place in a candle flame could only be understood with information from the manufacturer about the candle ingredients (Salthammer et al., 2021). Such transparency is already being increasingly implemented in the food sector. The “trusted science for safe food” initiative of the European Food Safety Authority (EFSA) requests the full access to study data (EFSA - European Food Safety Authority, 2020) and this approach can in principle be

transferred to other sectors.

The establishment of emission standards for laser printers and copiers can also be seen as a success story. After we characterized the chemical and physical properties of the particles emitted (Morawska et al., 2009), criteria for environmentally friendly devices were quickly developed in research projects that were funded both by the government and by industry (Gu et al., 2020). It is noteworthy that this also changed the previously incorrect public perception that particle emissions from operating printers simply consist of small components of the toner. In fact, the mechanisms of particle formation in the printer are of very complex nature. All in all, this is an excellent example of the working triangle between research, governmental authorities, and industry (Morawska et al., 2019). However, there are also examples in which communication is significantly more difficult. Thus, it has taken an unusually long time to regulate the indoor use of tobacco and nicotine products, despite the social acceptability of protective measures.

Even lessons from wrong decisions or actions are helpful to show that it is beneficial to consider the expertise of others. In 2019, contrary to the advice of many academic and industrial scientists, the European Union classified titanium dioxide in certain forms as carcinogenic. Since then, some products have had to carry a warning label. This classification was declared void by the Court of Justice of the European Union (2022). The judges argued that the European Chemicals Agency (ECHA) had not considered all scientific aspects and therefore reached an implausible conclusion (note that France has appealed the court decision). All in all, this controversial discussion about an important raw material is as unnecessary as it is lengthy and expensive.

Probably the most glaring example of a lack of cooperation in recent times was seen during the SARS-CoV-2 pandemic. It took far too long and needed massive power of persuasion for the scientists to convince the policy makers that the virus is airborne (Morawska and Cao, 2020; Morawska and Milton, 2020). As a result, it became clear how important cooperation between different disciplines and stakeholders is. The protection of the population required an intensive exchange of knowledge. Without the expertise of the industry, the provision of air-cleaning measures (Uhde et al., 2022) would not have been possible. At the same time, however, it had to be ensured that only effective and sensible concepts reached the market and those that are ineffective or potentially dangerous, stopped. The pandemic has also relentlessly shown us the many possibilities for abuse and fraud.

3. Examples of action plans in Europe and Australia

A wide range of activities and concepts exist at different levels to improve communication between interest groups, especially with regard to the climate crisis. In 2019, the European Commission presented the “Green Deal”, which includes a commitment to achieve climate neutrality by 2050. Key points are making transport sustainable, a clean energy system, refurbishing buildings, protecting our planet and our health, and particularly transforming our economy and society. A new way is needed for European industry to take the lead in times of rapid environmental and technological change. In line with the entrepreneurial spirit of this strategy, the EU institutions, Member States, regions, industry and all other relevant stakeholders should work together (European Commission, 2020).

The Australian Life Cycle Assessment Society (ALCAS) has been in existence since 2001. It was established to promote life cycle practices and sustainable development to coordinate the rapidly growing professional community in Australia. ALCAS has members from industry, government, academia and service organizations. Membership of individuals interested in the practice, use, development, education, interpretation and advocacy of life cycle based approaches is also welcomed (ALCAS, 2023).

ALCAS and its New Zealand sister organization LCANZ developed the Environmental Product Declaration (EPD) to communicate transparent and comparable data and other relevant environmental information about the environmental impact of a product throughout its life cycle. EPD has developed into an international system. A wide range of product categories are registered by companies in many countries.

4. Our future is in collaboration

All these arguments and examples show that the days when organizations worked independently on ecological or health related criteria for products should be in the past. The knowledge of the industry is just as necessary as that of the academic scientists and the authorities. Cooperation between industry, legislation, and research is already a reality in many committees dealing with product requirements. International cooperation to overcome national interests is also important. This is the only way to create globally accepted measures and remove unnecessary trade barriers.

The trail is being followed, but the finish line is still far away, although most of the protagonists have seen the benefits of synergy. Nevertheless, the process of rethinking is happening far too slowly and time is running out. This text is therefore an appeal to stakeholders to build mutual trust and accelerate the paradigm shift, so that the concept of sustainability quickly prevails in all types of industrial products. The current experience of the SARS-CoV-2 pandemic has highlighted the importance of trusting cooperation in combating and overcoming problems. Future generations will thank us.

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Application of human biomonitoring data to support policy development, raise awareness and environmental public health protection among countries within the HBM4EU project

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ABSTRACT

Most countries have acknowledged the importance of assessing and quantifying their population's internal exposure from chemicals in air, water, soil, food and other consumer products due to the potential health and economic impact. Human biomonitoring (HBM) is a valuable tool which can be used to quantify such exposures and effects. Results from HBM studies can also contribute to improving public health by providing evidence of individuals' internal chemical exposure as well as data to understand the burden of disease and associated costs thereby stimulating the development and implementation of evidence-based policy.

To have a holistic view on HBM data utilisation, a multi-case research approach was used to explore the use of HBM data to support national chemical regulations, protect public health and raise awareness among countries participating in the HBM4EU project. The Human Biomonitoring for Europe (HBM4EU) Initiative (https://www.hbm4eu.eu/) is a collaborative effort involving 30 countries, the European Environment Agency (EEA) and the European Commission (contracting authority) to harmonise procedures across Europe and advance research into the understanding of the health impacts of environmental chemical exposure. One of the aims of the project was to use HBM data to support evidence based chemical policy and make this information timely and directly available for policy makers and all partners. The main data source for this article was the narratives collected from 27 countries within the HBM4EU project.

The countries (self-selection) were grouped into 3 categories in terms of HBM data usage either for public awareness, policy support or for the establishment HBM programme. Narratives were analysed/summarised using guidelines and templates that focused on ministries involved in or advocating for HBM; steps required to engage policy makers; barriers, drivers and opportunities in developing a HBM programme.

The narratives reported the use of HBM data either for raising awareness or addressing environmental/public health issues and policy development. The ministries of Health and Environment were reported to be the most prominent entities advocating for HBM, the involvement of several authorities/institutions in the national hubs was also cited to create an avenue to interact, discuss and gain the attention of policy makers. Participating in European projects and the general population interest in HBM studies were seen as drivers and opportunities in developing HBM programmes. A key barrier that was cited by countries for establishing and sustaining national HBM programmes was funding which is mainly due to the high costs associated with the collection and chemical analysis of human samples. Although challenges and barriers still exist, most countries within Europe were already conversant with the benefits and opportunities of HBM.

This article offers important insights into factors associated with the utilisation of HBM data for policy support and public awareness.

1. Introduction

Human biomonitoring (HBM) is a valuable tool used to quantify exposures and effects by measuring levels of chemicals and/or their metabolites in biological matrices such as blood, urine, exhaled breath, breast milk, hair, fingernails and teeth (Sexton and Pirkle, 2004; Angerer J et al., 2006; Choi J et al., 2015). HBM studies are always combined with questionnaires to evaluate exposure risk factors and sources such as food, consumer products and the environment. Additionally, markers of effect can be incorporated to facilitate our understanding of the health impacts of these exposures. The applications of the information gained from HBM are broad and include trend analysis (geographical and temporal) in the exposure or health status of a population, identification of emerging threats, undertaking chemical risk assessment, chemical incident and disaster response, addressing policy needs/development and raising awareness.

Results from HBM studies can contribute to improve public health both directly and indirectly by providing evidence of individuals' exposure and data to understand the burden of disease and costs associated with chemicals in the environment (Knudsen and Merlo, 2011a, 2011b), thereby galvanising the development and implementation of evidence-based policy. The value of national HBM programmes has been recognised in many countries across the world for decades (Choi et al., 2015). As a result, some countries have developed regional or national standalone HBM programmes (without the inclusion of health surveys or other studies) while others have sought to integrate/combine HBM with existing Health Examination Surveys (HESs)/diet/nutrition surveys or engage in targeted, specific problem oriented HBM studies. A network of European countries has been working since 2003 to progress the development and implementation of national HBM programmes as part of fulfilling the European Environmental Health Initiatives (Commission, 2020). This has culminated in the current European Human Biomonitoring project which started in 2017 and is a collaborative effort involving 30 countries, the European Environment Agency (EEA) and the European Commission (contracting authority) (HBM4EU, 2022). The European Human Biomonitoring Project (known as HBM4EU) is a multinational effort to harmonise procedures across Europe and advance research into the understanding of the health impacts of environmental chemical exposure. One of the aims of HBM4EU was to assist partner countries to develop and/or strengthen their networks of government departments/agencies/ministries, and stakeholders (from herein these groups are referred to as National Hubs (NHs). Each NH nominated a National Hub Contact Point (NHCP) that acted as the link between the project and the national institutions. The National Hubs constitution was not prescribed by HBM4EU as the national activities were not part of the project activities in HBM4EU. Thus, each country developed a NH to suit their requirements and expertise. For instance, NHs could contain all the partners who were taking part in HBM4EU plus others – such as other academic institutes, national funding bodies, government departments and other stakeholders like the industry and NGOs (Fig. 1).

1.1. HBM, policy and public health

The use of HBM to raise awareness of public health issues, steer policy development and protect population health is evident on a global level. Numerous studies directed to specific chemicals (e.g. lead, polychlorinated biphenyls (PCBs), dioxins) and geographical hotspot investigations (e.g., arsenic in drinking water, cadmium around industrial sources) have applied HBM methods to investigate excess exposures, often leading to targeted policy interventions. Also, in occupational settings, HBM has proven valuable to protect workers' health in many studies. The value of national HBM programmes is demonstrated by the National Health and Nutrition Examination Survey (NHANES) in the USA where a decline of blood lead level was detected (using HBM) following a ban on lead in gasoline (petrol) and its removal from soldered cans for food (Pirkle et al., 1994). The German Environmental

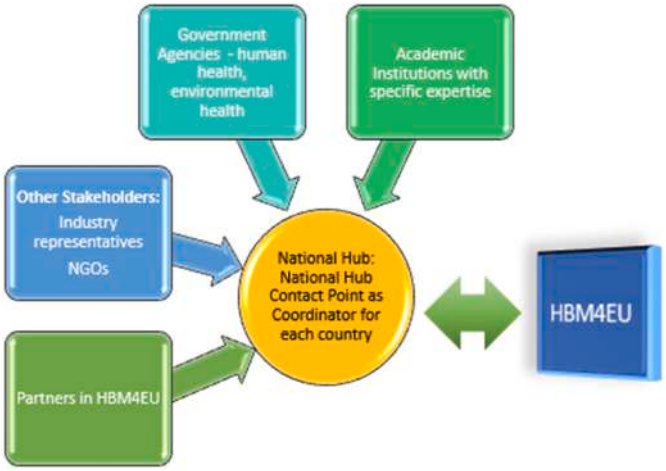

Fig. 1. Relationship between NHs, NHCPs and HBM4EU.

Survey (GerES) and the German Environmental Specimen Bank (ESB) were instrumental in initiating the avoidance of amalgam teeth fillings containing mercury in children (Kolossa-Gehring et al., 2012). Additionally, it contributed to the arguments on restriction of phthalate use in plastics (Göen T et al., 2011). In Belgium, information from the Flemish Environmental and Health Survey (FLEHS) informed the development of policy measures for persistent organic pollutants (POPs); including source-related regulation such as optimising and tightening existing Flemish legislation on open fires (Reynders et al., 2017). Biomonitoring data from the Canadian Health Measures Survey (CHMS) have been used to establish baseline concentrations of chemicals in Canadians; inform public health, regulatory risk assessment and management decisions; and fulfil national and international reporting requirements (Haines DA et al., 2017).

Action 3 of the European Environment and Health Action Plan 2004–2010 required the development of a coherent approach to biomonitoring in Europe (European Commission, 2004). Hence in 2005, the Expert Team to Support Biomonitoring in Europe (ESBIO) project supported the Implementation Group on HBM (IG-HBM) in commencing the preparation of protocols for the selection of study populations and priority chemicals. In 2009, this work was continued by the Consortium to Perform Human Biomonitoring on a European Scale (COPHES, 2009–2012) resulting in the finalisation of the protocols, selection of appropriate biomarkers and recruitment strategy. Demonstration of a study to Coordinate and Perform Human Biomonitoring on a European Scale', LIFE+, 2010–2012 (DEMOCOPHES), then tested the feasibility of a harmonised HBM approach in Europe, by successfully applying the COPHES approach in 17 European countries (Joas et al., 2012). Den Hond et al. (Den Hond E et al., 2015) provide a summary of these data for the exposure to selected chemicals, such as cadmium and phthalate concentrations in urine which are contained in consumer products and food packaging (Koch and Calafat, 2009), and mercury in hair (Grandjean and Landrigan, 2006) in mother-child pairs in those 17 countries (Wittassek and Angerer J, 2011; Budtz-Jørgensen et al., 2004; Akerstrom et al., 2013).

This work continued with HBM4EU, one of the major objectives being to create a harmonised HBM platform leading to consistent comparable and quality assured data throughout Europe. Another objective of HBM4EU is to use HBM data to support evidence based chemical policy and make this information timely and directly available for policy makers and all partners. Furthermore, the programme aims to ensure the sustainability of HBM and support capacity building through the development of a strong NHs network.

This article will assess national narratives provided by the NHCPs in countries within HBM4EU by highlighting good practice and learning gained from partner countries in the application of HBM for chemical risk assessment, policy issues and raising awareness.

2. Methodology

2.1. Research design

A multi-case research approach (a study on multiple cases to understand differences and similarities) was used to explore the use of HBM data to support national chemical regulations, protect public health, raise awareness and/or develop policy among those countries participating in HBM4EU.

Information was collated from 27 countries participating in HBM4EU (three countries did not participate due to issues primarily related to resources and time constraints). At the preliminary stages of HBM4EU the disparity with regards to the use and implementation of HBM nationally was highlighted, documented and provided the platform for this multi-case study. Hence this study has grouped countries according to the establishment of HBM programmes nationally/regionally/locally and/or the status of HBM in the country. The contributions are narratives therefore there is no formal quality control - the information has been referenced as far as possible.

The steps involved in collating and analysing the information are outlined below.

Step 1: Group Categorisation

Participating countries (see Table 1) were asked to self-select their best fit into one of the 3 categories/groups below:

- *Group 1: Countries that have not used HBM data for policy development but through HBM4EU or other initiatives/projects it has contributed to raising awareness of/addressing environmental or specific public health issues.*
- *Group 2: Countries with smaller or recently formed national hubs that have used HBM data for policy development but do not have established national or regional programmes.*
- *Group 3: Countries that have well established HBM programmes and use the data to address policy needs.*

Step 2: Data collection

Following countries' self-selection to the group most suited to their activities/status, the NHCPs (within each group) nominated a group leader to collect and summarise their respective narratives.

Guidelines and templates were developed for each group to ensure standardised/harmonised collection of the data to validate the national situation and the reason for self-selecting the group (see Appendix 1-6).

The template comprised open ended questions such as stating the

Table 1
List of the countries in the 3 groups.

Group 1	Group 2	Group 3
• Denmark	• Croatia	• Austria
• Estonia	• Cyprus	• Belgium
• Finland	• Israel	• Czech Republic
• Hungary	• North Macedonia	• France
• Iceland	• Slovakia	• Germany
• Italy	• Spain	• Sweden
• Lithuania		
• Luxemburg		
• Netherlands		
• Norway		
• Poland		
• Portugal		
• Slovenia		
• Switzerland		
• United Kingdom		

background information on the evolution and status of HBM, describing issues resulting in awareness/policy development, stating ministries, stakeholders or policy makers involved in HBM, describing barriers, challenges, opportunities regarding HBM, describing other players who would be beneficial in promoting HBM and future plans for HBM. Group leaders were also provided with a template/guideline to collate relevant data from the countries in their groups.

Step 3: Data Analysis and Scenario Development:

The first phase included analysis of the use of HBM at a national, regional or local level. In the second phase all examples of HBM use within the respective countries in a group were collated. Further summaries included areas such as ministries involved or advocating for HBM; steps required to engage policy makers; barriers, drivers and opportunities. In the final phase, a cross-case analysis was undertaken. The 3 groups were cross analysed to attempt to identify good practice and transferable lessons learned and make appropriate recommendations.

These narrative from the 3 groups were created to have an in-depth understanding of ministries/departments steering HBM and how to get/sustain the attention of policy makers. A comparison of these cases explores common themes that transcends each individual case and provides the benefit of understanding how countries can progress from using HBM data for public awareness to actual policy development.

3. Results & discussion

This section provides an overview of the information collected from the NHCPs by the group leaders. Where appropriate, the responses have been summarised in tables to show detailed information with additional findings specific to each group presented immediately below (the table). The responses affiliated with all 3 groups have also been presented in sections and text boxes (see case study boxes 1-3) that address factors associated with the utilisation of HBM data, ministries and stakeholders advocating for HBM, steps used in engaging policy makers, barriers, drivers and opportunities involved in the establishment of HBM programmes, potential groups for the promotion of HBM and the future of HBM in European countries.

The countries' self-selection into one of the 3 groups (refer to Table 1) is subjective and therefore it was noted that there are some countries which could have been placed in an alternative group. For example, Austria, which has a well-established NH and is on the verge of establishing a HBM programme, self-selected to Group 3 (well established countries with HBM programmes) rather than Group 2 and Spain, that has established national HBM studies since 2007 but recently included HBM in the Spanish Strategic Action Plan for Health and Environment could have been placed in Group 3 but is seen in Group 2. This limitation was accepted to facilitate categorisation of the groups and with the understanding that the concerned countries will have shared or similar factors.

The reported status of HBM studies/programmes/data within the countries in the 3 groups are shown in Table 1. The focus of this paper is general population-based studies, however, there are many countries which have used HBM in occupational settings hence it will be briefly discussed.

3.1. Group 1: countries that have not used HBM data for policy development but through HBM4EU or other initiatives/projects it has contributed to raising awareness of/addressing environmental or specific public health issues

In this group, relevant points with regards to the utilisation of HBM data for awareness/addressing environmental or public health issues and the establishment of a HBM programme are reported and discussed. The points addressed by countries in Group 1 include background information of HBM, the extent to which HBM data have been used (a maximum of 3 examples per country is presented) and steps used in engaging policy makers.

3.1.1. Background information of HBM and the utilisation of HBM data for awareness/addressing environmental or specific public health issues

The 15 countries in Group 1 (Table 1) reported the use of HBM data either for raising awareness or addressing environmental and public health issues dating back to more than 30 years in some cases. In

Box 1
A case study of dioxins and PCBs in Finland

The benefits of fish consumption which may be counteracted by concomitant exposure to environmental contaminants (in fish) such as dioxins and PCBs prompted the Fishermen Study and the Health 2000 Survey on fish consumption and cardiovascular health in Finland.

The professional Baltic Sea area fishermen and their family members were used to represent a population with high fish consumption (Fishermen Study) and the Health 2000 Survey participants represented the general population of Finland consuming average amounts of fish.

The work investigated:

- The ability of fish consumption biomarkers (fish derived omega-3 PUFAs and environmental contaminants) and other questions to reflect fish consumption.
- The association of fish consumption with the consumption of other foods.
- The associations of fish consumption and fish-derived serum omega-3 PUFAs and environmental contaminants with cardiovascular risk factors.
- Mortality in a population with high fish consumption and presumably high exposure to environmental contaminants.

Results showed that blood concentrations of fish-derived omega-3 PUFAs, dioxins, PCBs, functioned well as biomarkers of fish consumption, fish consumption was positively associated with eating healthy foods both among the professional fishermen and their wives and in the general population. A follow-up study from 1980 to 2005 also showed mortality from many natural causes in epidemiological studies regarding fish consumption and cardiovascular health ischaemic heart disease, was lower among the fishermen and their wives than in the general population of Finland.

In conclusion, the work added to the current advisories that the benefits of salt-water fish consumption and omega-3 PUFA intake outweigh the potential hazardous effects of fish-derived environmental contaminants even at higher levels of exposure (Epidemiological studies on fish consumption, 2000).

addition, 11 countries in this group (Denmark, Hungary, Italy, Luxembourg, The Netherlands, Norway, Poland, Portugal, Slovenia, Switzerland, UK) have participated in at least one of the previous European HBM projects (ESBIO, COPHES and DEMOCOPHES). Ministries/departments/agencies steering HBM were also highlighted of which, the Ministry of Health was the most common (Appendix 7).

Except for Estonia, all countries in Group 1 have participated in one or more of the following fieldwork activities in HBM4EU: collection of new samples from the public, use of bio-banked samples for new analyses and collection of samples from occupational settings (Appendix 1). Many countries reported having a structure that will support chemical management and policy development although they currently have no national or regional HBM programme. The use of HBM and issues which have resulted in the raising of HBM awareness or supported public health interventions in the countries within this group are summarised in Table 2.

3.1.2. Status of the establishment of HBM programme or implementation of an HBM module into Health Examination Surveys

As earlier mentioned, no national HBM programme exists within the countries in Group 1. However, Finland, Luxembourg and the United Kingdom have reported the inclusion of a subsidiary HBM module in Health Examination Surveys (HES) or the development of initiatives to align with HES as part of research activities. Other countries reported the utilisation of HBM studies in other health programmes e.g. the Estonian population health strategy (RAHVASTIKU TERVISE ARENGUKAVA 2020, 2020).

In the UK, a HBM module has been implemented in the Health Survey for England (HSfE) which is undertaken annually since 1991 and monitors trends in the population's health and care (Health Survey for England, 2021). HSfE recruits approximately 10,000 participants (8000 adults over 16years and 2000 children aged 0 to 15) annually and a subset (300 participants, aged 16–49years) of the group will be included in the HBM module.

The Netherlands had a Surveillance Programme titled, "Man, Food and Environment", that terminated in the late 20th century (Fiolet et al., 1998; Zeilmaker et al., 2003). In this programme, pesticides, PCBs and dioxins were measured periodically in mother's milk. HBM is now mainly applied in studies focused on hotspots, occupational settings, targeted research, or determinants of health/chronic or infectious diseases and an element in emergency response to chemical incidents (Behbod et al., 2017; Scheepers et al., 2011).

Norway has established a governmental Human Environmental Biomonitoring programme that utilises the Norwegian Mother, Father and Child Cohort Study (MoBa) as a basis for recruitment. MoBa is an ongoing prospective pregnancy cohort which includes 114 500 children, 95 200 mothers and 75 200 fathers (Magnus et al., 2016).

In Lithuania, the Ministry of Health approved a 2-year (2020–2021) HBM program, during which the concentrations of metals/metalloids, PAHs, dioxins/furans, and PCBs were determined in a random selection of 225 Lithuanian residents and 116 firefighters. A new HBM program for 2023–2027, as a preventive tool, is on the government's agenda.

3.2. Group 2: countries with smaller or recently formed NHs that have used HBM data for policy development

This group of 6 countries (Israel, Spain, Slovakia, Croatia, North Macedonia and Cyprus) have smaller (few participants/stakeholders) or recently formed NHs and have used HBM on an *ad hoc* basis at the local or regional level with some having plans to advance to national HBM efforts (Table 3). Croatia, Spain, Slovakia and Cyprus have participated in at least one of the three previous EU HBM programmes (ESBIO, COPHES, DEMOCOPHES), however, with the exception of Israel, none have yet established formal national/regional initiatives. This may be on the horizon as Cyprus, Spain, Croatia, Slovakia have National Strategies or Plans (including drafts) which include HBM.

The narrative for this group follows a similar pattern as seen in Group 1 except the "*policy uses of HBM data*" section which states how HBM data have been used for both awareness and policy support.

3.2.1. Policy uses of HBM data

Several countries reported using HBM for raising awareness or intervention campaigns (refer to Table 4). For example, Cyprus and Israel reported using cotinine measurement in children to raise awareness about controlling exposure to environmental tobacco smoke in this vulnerable population. In the case of Cyprus, HBM efforts were followed by an intervention campaign and direct communication with policy makers. Whereas in Israel there was media coverage of the HBM data and results were reported to the Parliament and policy makers within the Ministry of Health. In both cases, there is an understanding that even with HBM data and media coverage, there is a need for further policy actions. In Israel, HBM data on nutritional biomarkers (iodine, selenium) collected within the national HBM program was reported to Ministry of Health policy makers and raised awareness regarding iodine deficiency in a country heavily reliant on desalinated drinking water. Also, in Israel, data from a small pilot study on PFAS in blood donors was communicated to stakeholders (energy sector, Ministry of Defense, National Fire Authority) as part of government efforts to reduce use of fluorinated compounds in firefighting foams.

In Croatia, HBM data (mercury in hair) was used in the "Stay Healthy, Stop Mercury" health campaign (Janev Holcer N, 2009). Twelve women participated in the study on mercury in hair; all had detectable levels, some above reference dose level of 1 mg/g which could be linked to fish consumption. As the effects of the campaign were short term, it is recognised that further HBM data and repeated campaigns are needed to impact population behaviors.

Another potential use of HBM data for policy support is developing dietary advice. In Spain, HBM data from a national study of 2000 adults and another on mother-child pairs showed hair mercury concentrations for a fraction of the population above the recommendations proposed by the WHO (Soler-Blasco et al., 2019). This data contributed to the development of dietary advice related to the fish consumption in vulnerable populations with accompanying media campaigns (Aesan.gob.es).

HBM data can be used as part of governmental crisis management. For example, HBM was used in Cyprus to evaluate exposure and potential health risk of citizens exposed to arsenic in drinking water. Toenail arsenic concentrations were measured in hotspot and control populations; although arsenic exposure was higher in the hotspot populations, levels were below health guidance values. These findings were communicated to concerned citizens by high level government officials, and thus were part of holistic assessment, and risk communication, during an environmental crisis.

Spain, Cyprus and Israel reported using HBM data in quantitative risk assessment. In Cyprus and Spain, HBM data on mercury in hair were part of the risk asessment approach incorporating both HBM and dietary data. The Ministry of Health in Israel collected HBM data on urinary concentrations of organophosphate metabolites in children, and found that those consuming more fruit had higher exposure to these pesticides. A portion of the children in the study had estimated pesticide intake above the acceptable daily intake (Berman et al., 2020). The Ministry of Agriculture decision to phase out chlorpyrifos use in edible crops cited this HBM data (see Box 2).

3.3. Group 3: countries that have well established HBM programmes and the use of the data to address policy needs

Countries in Group 3 (Austria, Belgium, Czech Republic, France, Germany, and Sweden) either have established HBM programmes or it is in progress. They have participated in at least one of the previous European HBM projects (ESBIO, COPHES, DEMOCOPHES). All countries have played major roles in HBM4EU and some have participated in one

Table 2
Use of HBM data for awareness and addressing public health issues in countries from Group 1.

Country	Issue/Concern	Population (local/ regional/ national)	Chemical	Ministry or Institute undertaking the study/other	Outcome	Reference
Denmark	Endocrine disruptors and especially child health	National and Local	Endocrine disrupting chemicals (EDCs) such as Bisphenol A, Phthalates, paracetamol	The Danish Environmental Protection Agency (EPA) by funding a center for EDC at the Region H called CEHOS	Provided data for EU regulations and setting standards for concentration of EDCs in toys, food etc	Busch et al. (2021)
	Traffic related exposures to pollutants	National	Polycyclic aromatic hydrocarbons (PAH), particles	EPA and communities	Several monitoring activities and actions taken by the EPA and the local communities	Strak et al. (2021)
	Indoor chemical exposures	National	Polychlorinated biphenyls (PCBs)	Ministries of Health and of Buildings	Guidelines for exposure limits with action levels for evacuation. Local initiatives such as demolition of especially polluted buildings (Brøndby Strand)	Frederiksen et al. (2020)
Estonia	Investigating the impact of oil shale sector on population health	Regional	Heavy metals including mercury; PAH, BTEX	Ministry of Education and Research, Health Board, Ministry of Environment	Results informed the development of the Oil Shale Strategy and highlighted increased Hg levels in fishermen from western Estonia which was not found in oil shale workers	Orru et al. (2020)
Finland	Presence of dioxins and PCBs in fish from the Baltic Sea at levels which exceeded EU limits	National	Dioxins, PCBs	Ministry of Social Affair and Health	Demonstrated that eating the Baltic Sea fish had more positive effects than the negative ones associated with the presence of the chemicals	A. (2012)
	As a result of EFSA 2018 dioxin risk assessment an investigation was undertaken to ascertain levels in vulnerable populations	National (children 7–10 years)	Dioxin, PCBs	Ministry of Social Affairs & Health	Low levels of chemical found encouraged the development of an ongoing government programme to promote eating domestic fish	Rantakokko et al. (2020)
Hungary	Presence of lead in the environment	Local populations	Lead	National Public Health Centre	Linking of lead exposure with neurodevelopment effects (2005). This was followed by a national monitoring campaign for lead in tap water (2018–2020).	Dura Gy et al. (2011)
	Red sludge disaster in 2010	Local populations	Heavy metals	National Public Health Centre	The settled and dried red sludge had no effect on the urinary levels of metals.	Rudnai P et al. (2011)
	Endocrine disruptors	Local populations	Phthalates and certain metals (mercury, cadmium)	National Public Health Centre	The urinary metabolite levels were lower than the corresponding guidance values.	KÖZÉPESY et al. (2017)
Iceland	Arctic monitoring assessment programme investigating the effect of POPs and metals on women of childbearing age and the developing foetus	National (Artic programme)	Persistent organic pollutants (POPs), metals	Ministry of Environment	Supporting ongoing assessment of arctic pollution. Time trends showed reduction in levels since 1995	no (2021)
Italy	Exposure to polyfluoroalkyl substances (PFAS) in an area affected by water contamination	Local	PFAS	Regione del Veneto, Italian National Institute for Health	Characterisation of human exposure, definition of the most affected areas, identification of the population groups at higher risk, definition of a regional health surveillance plan	Ingelido et al. (2018)
	Concerns of citizens about the possible health risks related to the presence of a new Waste-to-Energy Plant	Local	Metals, Hydroxylated polycyclic aromatic hydrocarbons (OHPAHs), Polychlorinated dibenzodioxins (PCDDs), Polychlorinated dibenzofurans (PCDFs), and PCBs	Financially supported by the Metropolitan Area of Turin Piemonte. Realised by Italian National Institute for Health, Department of Epidemiology ASL TO3, Department of Prevention ASL TO1, Department of Prevention ASL TO3, Department of Epidemiology and Environmental Health, Regional Environmental Protection Agency.	Verification of no impact of the WTE plant on human exposure to selected contaminants, support in the definition of the Health Surveillance Program, communication to citizens involved about WTE impact, raising in public awareness	Ingelido et al. (2020)
	Concerns about exposure to contaminants of residents in an area characterised by multiple sources of exposure	Local	Metals, OHPAHs, PCDDs, PCDFs, PCBs, PBDEs, PFAS, pesticides	Ministry of Health, ASL Taranto Dipartimento Prevenzione, Italian National Institute for Health	Characterisation of human exposure, identification of the population sub-groups at higher risk, support to remediation actions taken by local and national authorities	Pitter et al. (2020) Polesello et al. (2013) del Veneto (2016)

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Table 2 (*continued*)

Country	Issue/Concern	Population (local/ regional/ national)	Chemical	Ministry or Institute undertaking the study/other	Outcome	Reference
						Bena et al. (2016) Bena et al. (2021) Bocca et al. (2016) Iamiceli et al. (2020) Iamiceli et al. (2021) Ruggieri et al. (2019) Abate et al. (2016) Comba et al. (2012) Iavarone et al. (2012) De Felip et al. (2015) Ingelido et al. (2017)
Lithuania	Carcinogenicity of cadmium in women	Regional	Cadmium	Lithuanian University of Health Sciences	Assessment of causal association between cadmium exposure and risk of breast cancer (a case-control study)	(Strumylaite et al., 2011, 2014, 2019)
	Environmental exposure of children and adults to lead	Regional	Lead	Lithuanian University of Health Sciences	Lead concentration in children and adults with various exposure levels in Lithuania	Strumylaite et al. (2004)
The Netherlands	Concerns about the emission of Perfluorooctanoic acid (PFOA) from a factory producing Teflon	Local	PFOA	Province of South-Holland; Ministry of Infrastructure & the Environment; Ministry of Health, Welfare and Sports; Ministry of Economic Affairs & Climate Policy; Ministry of Social Affairs; Ministry of Defense	Verification of previous modelling results indicating high levels of exposure to surrounding population	(van Poll et al., 2017; Gebbink WA, 2020)
	Exposure to pesticides of residents in close proximity (<250m) to agricultural fields	Local	Pesticides (asulam, carbendazim (applied as thiophanate-methyl), chlorpropham, prochloraz and tebuconazole)	Dutch Ministry of Infrastructure and the Environment; Ministry of Economic Affairs & Climate Policy	Provided evidence that exposure to pesticides is not limited to the application period but may persist throughout the year	(Figueiredo DM et al., 2020; Oerlemans A et al., 2021)
	Disaster response in victims (1788 adults & 294 children) and relief workers from across the country and neighbouring countries (n = 2114) of the Firework Disaster Enschede (2000)	Local	Metals indicative for firework-related exposures: barium, cadmium, chromium, copper, nickel, lead, antimony, strontium, titanium, zinc.	National response team GGVE, including National Institute for Public Health and the Environment (RIVM) and municipality health service Enschede	Objective was to ascertain results of initial exposure models by HBM in samples collected 2–3 weeks post-disaster. No systematic increases of blood and urine levels, found in the residential group (including children) nor in the relief workers, thus confirming model results. Chance findings, unrelated to the disaster, identified 22 people with relatively high levels on either Cu, Pb, Sr, Ba or Ni that warranted clinical toxicological follow-up.	(Roorda et al., 2004; Enschede, 2001)
Norway	The exposure of anglers to flame retardants	Regional	Flame retardants	NIPH and Norwegian Food Safety Authority	Dietary recommendations were developed following a HBM programme	Thomsen et al. (2008)
	The use of PFAS in skiwax	National	PFAS	NIPH and STAMI (National Institute of Occupational Health)	Elevated levels of PFAS were found in both amateur and professional ski waxers	(Freberg et al., 2010)
Poland	Environmental exposure to metals	National/ Regional	Lead, cadmium, chromium, arsenic, mercury	Ministry of Education and Science; Local Government Organisations	Elevated levels of chemicals in biological material. Identification of the more vulnerable	Kozłowska et al. (2019)

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Table 2 (*continued*)

Country	Issue/Concern	Population (local/ regional/ national)	Chemical	Ministry or Institute undertaking the study/other	Outcome	Reference
					groups among the study population. Search for new biomarkers of exposure, effects and sensitivity	Peplońska et al. (2020) Baszuk et al. (2021) Garí et al. (2022) Kuras et al. (2017) Kuras et al. (2018) Kuras et al. (2019)
Portugal	Population living near a chlor-alkali plant, a solid waste incinerator or a uranium mine	Local	Heavy metals, dioxins and furans	University of Aveiro, Lisbon School of Medicine, Ministry of Health	Results showed that populations presented low levels of the studied chemical agents	(Reis et al., 2007, 2009; Marinho Falcão et al., 2005, 2007)
	Population exposure to plasticisers	Regional	Plasticisers (Di-2-ethylhexyl terephthalate (DEHTP) and DINCH)	Network of Chemistry and Technology (REQUIMTE)/Associated Laboratory for Green Chemistry (LAQV)	Age and processed food consumption were associated with DEHTP exposure. Although average DEHTP exposure was of no concern at the time, increasing exposures are likely. Children are exposed at approximately 5-times higher levels of DINCH than adults. Lower DINCH exposures were observed in children with nutritional guidance.	(Lessmann et al., 2017) (Correia-Sá et al., 2017)
	Population exposure to mycotoxins	Regional	Mycotoxins	National Institute of Health Dr. Ricardo Jorge (INSA)	Exposure assessment of Portuguese population to multiple mycotoxins was confirmed. It was the first time that Alternariol was detected in Europe. Exposure above safe limits for DON.	(Martins et al., 2019, 2020)
Slovenia	Exposure of general population to chemicals	National and regional	Metals/metalloids, PAHs, Glyphosate, Dioxins, PCBs, Polybrominated diphenyl ethers (PBDEs), Phthalates, Bisphenols, Parabens, Triclosan	Ministry of Health	Estimation of exposure levels, was used to identify the main predictors of exposure, and reference values for the selected Slovenian population established	Tratnik et al. (2019)
					The results provided the first insight into the PAH exposure of study group, potential sources of exposure and its spatial variability.	Joksić et al. (2022)
					Estimation of GLY and AMPA exposure in a Slovenian study population showed much lower levels than those in similar studies worldwide.	Stajnko et al. (2020)
					POPs were detected in maternal milk, plasma, and serum; legacy of pollution still visible on a national level. Geographic location of Slovenia might have protective function.	Runkel et al. (2021)
					Exposure of men and lactating women, children and adolescents in Slovenia to a wide range of different EDCs was evaluated connecting it to exposure patterns and exposure sources and susceptibility.	(Runkel et al., 2022; Stajnko et al., 2022; Tkalec et al., 2021)
Switzerland	Political initiation to ascertain the chemical burden in the Swiss population	National	Amongst others quicksilver, glyphosate and a collection of fluorinated substances	Federal Council	Developed the process for establishing a pilot "Swiss Health Study" under the umbrella of HBM4EU	FOPH (2020)

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Table 2 (*continued*)

Country	Issue/Concern	Population (local/ regional/ national)	Chemical	Ministry or Institute undertaking the study/other	Outcome	Reference
UK	Contamination of soil, surface water and wells due to natural bedrock of area and historic tin mining	Local	Arsenic and heavy metals	Health Protection Agency (now UKHSA), British Geological Survey, University of Manchester	Elevated levels of arsenic found in the drinking water samples resulted in the development of an HBM programme to assess population exposure	(Middleton DRS et al., 2017)
	The high infant mortality rate (double that for the England & Wales) within one of the most deprived cities (Bradford) in the UK	Local	Environmental exposures	University of Edinburgh	Identification of the more vulnerable groups among the study population	Wright J et al. (2013)
	Longitudinal study of mothers and babies (referred to as Birth Cohort study) in a county in England of higher SES.	Local	Application for arising environmental exposure research/investigation	University of Bristol, Medical Research Council and Welcome Trust	Continuing evolution of information about how genes, the environment and lifestyle of the participants impact on health. These findings are often applicable to the rest of the UK population	(Bristol)

or more of the following fieldwork activities in HBM4EU: collection of new samples from public, use of biobank samples for new analyses and/or collection of samples from occupational settings.

This section presents background information on the evolution and status of the national or regional HBM programmes, issues which underpinned the sustainability of the national or regional HBM programmes and examples of the use of HBM in policy making.

3.3.1. Background information on national or regional HBM programmes

HBM programmes in Europe have been established since the 1970s. The HBM programmes reported, started in collaboration with or as a component of other surveys e.g. HES before being fully established as a primary initiative even if embedded within another (programme).

The German Environment Agency (Umweltbundesamt -UBA) and the Federal Ministry for the Environment, Nature Conservation, Nuclear Safety and Consumer Protection (BMUV) are responsible for health-related environmental monitoring and human biomonitoring studies at the federal level in Germany. The main components were established in the early 1980s and continue to be used (Schulz et al., 2007). The various components (Kolossa-Gehring et al., 2012) complement each other thereby (a) enabling a comprehensive assessment of human exposure to chemicals, (b) providing indications of sources of exposure, (c) facilitating time trend analyses and (d) helping to monitor the effectiveness of regulatory measures (Kolossa-Gehring et al., 2012). The main components of the system are a) the cross-sectional population-representative German Environmental Surveys (GerES) which combines health and HBM surveillance as well as additional environmental monitoring to enable assessment of human exposure to chemicals, b) the Environmental Specimen Bank (ESB) to facilitate time-trend analyses, c) the co-operation between the BMUV and the German Chemical Industry Association (VCI) to identify specific and sensitive markers and develop new analytical methods for the monitoring of substances to which the general population might be exposed and/or which are of health relevance (Kolossa-Gehring et al., 2017), d) the provision of assessment tools by the German HBM Commission (Apel et al., 2017) and e) the cooperation in European and international HBM networks (Schindler et al., 2014; Nakayama et al., 2019).

The French HBM programme started as a component of the nutritional and health survey. It has therefore been embedded in a wider monitoring of the French population health, nutritional behavior and exposure. More targeted studies were designed in the context of workplace or accidents assessments. In France, the National Nutrition and Health Survey (ENNS) was conducted in 2006–2007 to meet the objectives on biomonitoring, chronic disease surveillance and nutritional surveillance. Thereafter, the Grenelle I Act (No, 2009–967 of August 3, 2009) led to the development of a more sustainable French National Biomonitoring programme, implemented by Santé publique France (SpF - the French National Health Public Agency), in which two distinct studies were designed: 1) Esteban: the Health Study on Environment, Biomonitoring, Physical Activity and Nutrition, a nationwide cross-sectional survey and 2) the ELFE birth cohort (Longitudinal Study from Childhood) (Balicco et al., 2017; Dereumeaux et al., 2016; Fillol et al., 2014; Fréry et al., 2012; Ougier et al., 2021).

In Sweden, the Swedish Environmental Protection Agency (SEPA) is responsible for the monitoring of the environment which has been ongoing since the late 1970s. The monitoring consists of ten programme areas which includes physical, biological and chemical agents. Chemicals are regularly monitored in several biotic and abiotic matrices. In the early 1991 the Swedish parliament decided that there should be a program that includes the connection between the environment and human health (se, 1990). The Institute for Environmental Medicine at the Karolinska Institute developed a structure for the program in 1992 and in 1993 the national programme for health-related environmental monitoring commenced with the inclusion of HBM (Institutet., , 1992). The purpose of the programme is to enable long-term monitoring of environmental factors that can affect population health and track

Table 3
Use of HBM data for policy purposes in countries from Group 2.

Country	Issue/Concern	Population (local/ regional/ national)	Chemical	Ministry or Institute undertaking the study/ other	Outcome	Reference
Spain	Dietary exposure to mercury in children and woman of childbearing age	National	Mercury	Spanish Ministry of Agriculture, Food and Environment	Development of dietary advice related to the fish consumption in vulnerable populations	(De Consumo De Pescado, 2022)
	National Implementation Plan for surveillance on persistent organic compound and other pollutants	National	PCBs, PBDE, OC-pesticides, PAH, PFAS Phtalates&DINCH Metals (mercury, Lead, Cadmium, Cobalt, Selenium, Thallium)	Ministry of Environment Instituto de Salud Carlos III	Monitoring and Reporting Stockholm convention National Implementation plan	(Castaño et al., 2015, 2019; Ramos et al., 2017; Bartolomé et al., 2015, 2017; Cutanda et al., 2015; Lopez-Herranz et al., 2016; Sánchez-Rodríguez et al., 2015)
Croatia	Dietary exposure to mercury in women (25–45 years)	Local	Mercury	Medical School, University of Zagreb Andrija Stampar School of Public Health Department of Environmental and Occupational Health	Development of dietary advice related to the fish consumption in women of childbearing age	Janev Holcer (2010)
	Differences in dietary mercury exposure between women and their newborn babies in coastal and continental region	National	Mercury	Croatian Institute of Public Health	Recognition of the need to implement further studies in areas where environmental pollution and higher exposure have been identified	Capak et al. (2017)
Cyprus	Pesticide exposure in children	Local	Pesticides	Cyprus International Institute for Environmental and Public Health & State General Laboratory/ Ministry of Health	Comment in EFSA public consultation	Makris KC et al. (2019)
	Exposure to arsenic in drinking water	Local	Arsenic	State General Laboratory/Ministry of Health	Risk assessment, communication with citizens	Makris et al. (2012)
	Dietary exposure to Mercury	National	Mercury	State General Laboratory/Ministry of Health	Risk assessment	KATSONOURI et al. (2019)
	Exposure of children to environmental tobacco smoke	Regional	Cotinine	State General Laboratory/Ministry of Health	Policy support	Katsonouri et al. (2009)
Israel	Organophosphate pesticide exposure in children	National (small scale)	Organophosphate pesticides	Ministry of Health	Phase out of chlorpyrifos	Berman T et al. (2020)
	PFAS exposure in general population	National (small scale pilot)	PFAS	Ministry of Health	Raising awareness, data presented in stakeholder meetings	

indicators of exposure by measuring pollutants in human matrices. SEPA and universities, research institutes and other agencies that have a structure and organisation for sample collection, biobanking and are included in a lab network for chemical analysis are included in the programme. Breast milk, blood or serum, urine and hair are monitored on a regular basis.

The Czech Republic national human biomonitoring programme is one of the 7 subsystems of the comprehensive Environmental Health Monitoring System implemented based on the Czech Government Resolution in 1991. The individual subsystems have been in routine operation since 1994. The program of human biomonitoring has been focused on monitoring the most serious substances to which the Czech population is exposed: heavy metals, persistent organic pollutants and emerging organic compounds - phthalates, per- and poly-fluorinated compounds (Batáriová et al., 2006; Černá et al., 2007, 2008, 2017, 2020; Cerna et al., 2015). The toxic substances and their metabolites have been monitored in blood, urine, hair and teeth of adults and children, and in milk of primiparas from selected regions of the Czech Republic. An overview of human biomonitoring activity is presented in Cerna et al. (1997), 2007, 2012 and 2017 (Černá et al., 1997, 2007, 2012, 2017). In addition, the CELSPAC infrastructure of population cohorts, with a biomonitoring element, was established in 2012. It consists of CELSPAC grown-up birth cohort (1991), The Next Generation (TNG) birth cohort (2016) and HAPIEE aging cohort (2002).

Presently, Belgium and Austria do not have national HBM programmes. The absence of a national HBM programme in Belgium is attributed to the division in regional and federal competences; however, the Flemish and Walloon regions have their own programmes and there is collaboration and exchange of knowledge between both regions. Since 2002 the Flemish Government decided to carry out the Flemish Environment and Health Survey (FLEHS), an extended (HBM) programme, which is integrated in the environmental health policy (https://www.milieu-en-gezondheid.be/). The FLEHS studies provided Flemish policy makers with a vast amount of data such as biomarkers of exposure and effect, exposure effect associations, time trends and geographical differences (Schoeters et al., 2012, 2017). The first Walloon biomonitoring program (BMH-WAL) is part of the Walloon environment-health plan adopted in December 2018 by the Walloon government which aims to study and limit environmental risks to human health. BMH-WAL aims to develop Exposure Reference Values of the Walloon population to substances present in the environment, food and products of daily life (P, 2020; Pirard et al., 2020; Jacques et al., 2021).

Table 4
Background information on the regional/national HBM programmes in the countries in Group 3.

Country	Sweden	Belgium	France	Germany	Austria	Czech Republic
Year of establishment of the HBM programme	*1993: Swedish HBM programme*	2002: FLEHS (I-IV) 2019: start BMH-WAL	2006: ENNS 2011: ELFE 2014: Esteban	1985: GerES (I – V) 1981: Environmen-tal Specimen Bank 2010: BMU - VCI Cooperation for further develop-ment of human biomonitoring	*2007: Austrian HBM platform*	1994: Czech HBM programme
Which entities finance/steer the programme?	*financed by the government, coordinated by the SEPA under the ministry of Environment*	FLEHS I-III: financed by dep. science, environment and health, coordinated by the Center on Environment and Health (CEH) BMH-WAL: financed by environment department, coordinated by ISSeP	financed by the ministry of health; coordinated by Santé Publique France.	financed by the ministry for the environment (BMUV), coordinated and scientifically led by UBA	*Studies mostly financed by the minister for the environment (currently named Federal Ministry for Climate Action, Environment, Energy, Mobility, Innovation, and Technology (BMK)); mostly coordinated by EAA*	financed by the ministry for health and ministry for education, youth and sports; coordinated by the National Institute of Public Health and Masaryk University.
HBM included in business/ strategic/ action plan?	(1) follow-up Environmental Quality objective for a non-toxic environment (2) identified chemical risks, reported to SamTox (national authorities working to prevent and manage chemical risks in society)	*instrument for policy making in the Flemish Decree on Preventive Health Care (since 2003).* *Policy use of HBM is mentioned in every yearly policy declaration of the Flemish Minister for environment* BMH-WAL is part of the Walloon environment-health plan (2018)	Strong recommendation for a French national HBM programme in Grenelle I Act (No, 2009–967 of August 3, 2009) HBM is part of the national environment and health plan PNSE 2 (2009–2013), 3 (2015–2019) and 4 (2021–2025)	*HBM is not included in a strategic plan, but data and their evaluation are asked for by ministries and also the parliament at national and Federal State level for policy decision making. Data are also fed into REACH processes, OECD activities and tasks under the Stockholm Convention*	Since 2016, the platform is the official advisory board of the minister for Environment on E&H issues 2017: resolution of the National Council: every 2 years a report *has to be published (BMK, 2019)*	Implemented on the basis of the Resolution of the Government of the Czech Republic No. 369/1991. *HMB-related activities are part of the National Portfolio of Actions implementing the Ostrava Declaration (2017)*
Which entities are involved	Steering group for the HBM programme	Steering committee of the FLEHS studies *BMH-WAL: steering committee*	*The ministry of health and Santé Publique France with the help of a college of experts from different institutions. Constituting the French national hub*	UBA, BMUV (Federal Ministry for the Environ-ment, Nature Conservation, Nuclear Safety and Consumer Protection), HBM Commission and linked third parties involved in HBM4EU	The national hub consists of the members of the Austrian HBM platform	National Institute of Public Health and Masaryk University, national hub

Box 2
A case study of organophosphate (OP) pesticides in Israel

Screening values have not been established for assessing biomarker concentrations of organophosphate (OP) pesticide metabolites. There are also few studies using HBM data on urinary OP concentrations to assess human health risk. In Israel, a study was undertaken to measure OP exposure in a sample of children (103) and explore associations between dietary patterns and OP exposure.

Demographic and dietary information together with urinary samples were collected. Creatinine and dialkyl phosphate (DAP) concentrations were measured in the samples. Urinary DAP concentrations were compared to international populations and its associations with fruit and vegetable consumption were analysed. Using urinary DAP concentrations, estimated daily intakes (EDI) of OP pesticides in each child were calculated and compared to the acceptable daily intake (ADI).

The result showed that children consuming more fruit had higher levels to these pesticides. A portion of the children in the study had estimated pesticide intake above the acceptable daily intake (Berman T et al., 2020)-the levels of dimethyl metabolites were also high compared to other international populations; and that fruit consumption was associated with higher urinary DAP levels.

With the DAP concentration data, it was found that some children in the study may be exposed to OP pesticides above levels that are considered safe. The Ministry of Agriculture's decision to phase out the use of chlorpyrifos in edible crops cited this HBM data.

In Austria, a platform for the establishment of a HBM programme was founded in 2007. The aim was to create a network of the relevant Austrian institutions and to share knowledge to promote health and environmental protection, support national prevention goals and develop national competence in human biomonitoring. So far, several studies on priority substances have been carried out and persistent organic pollutants in mothers' milk are regularly monitored.

A summary and further details relating to regional/national HBM programmes for the countries in Group 3 are provided in Table 4.

3.3.2. Issues which propelled the sustainability of the national or regional HBM programmes

Most countries in Group 3 have reported the utilisation of HBM as a tool to assess levels of pollutants in specific or general populations since the early 1970s. In the early 80s, HBM activities at the federal level have been closely connected to the risk assessment of chemicals and their regulation in Germany and there are many examples of policy translation of such results (Kolossa-Gehring et al., 2012). HBM studies were also initiated by environmental and health crises in most countries. For example, in Belgium, a PCB and dioxin incident (van Larebeke et al., 2001) propelled the establishment and sustainability of the FLEHS studies and in Germany, the lead contamination in the vicinity of a lead smelter (Kolossa-Gehring et al., 2012; Englert et al., 1987) was a prominent driver of the first human biomonitoring studies.

Austria and the Czech Republic reported issues on POPs. In Austria, regular breast milk monitoring for POPs informs an indicator in the frame of the health target "securing sustainable natural resources such as air, water and soil and healthy environments for future generations", which is part of a set of 10 Austrian health targets officially approved by the Federal Health Commission and the Council of Ministers (Bundesministerium für Arbeit, 2019). Additionally, a report on human biomonitoring is submitted to the National Council every 2 years (Bundesministerium für Klimaschutz, 2019). The Czech Republic's long-term goal to have a sustained HBM initiative to evaluate chemical exposure of the Czech population to priority chemicals was set out in a national strategy document "Set up of the national POPs monitoring" (first edition 2009, last updated 2020).

HBM is mentioned in several business, strategic or action plans, which galvanises the sustainability of the existing HBM programmes (see Table 4). In France, the HBM programme is part of the successive national Environmental Health Plan (PNSE) since 2009, following the recommendations of the Grenelle I Act (LOI n° 2009, 2009). Several countries report HBM results in the National Implementation Plan for the Stockholm Convention (e.g. Sweden, Austria, Czech Republic, Germany) or in the Arctic Monitoring Assessment program (Sweden). In most countries implementing HBM programmes, HBM data are used to follow-up environmental quality objective to achieve a "non-toxic environment" (se, 2021) and in Sweden, the need for the programme is further stressed in the governmental strategic document "Towards a non-toxic everyday life - platform for chemical policy" (proposition, 2013).

Societal and political interest in the environment and health have further supported the sustainability of the existing HBM programmes. For example, the results of FLEHS studies provided agenda setting opportunities for politicians and pressure groups and informed the societal debate about environmental health problems in Flanders. Since 1995, the results of the HBM programme and the phased action plans have provided answers to 193 questions addressed to the ministers for environment or health from the Flemish parliament. The HBM program also actively responds to specific (local) concerns and public perceptions. Positive feedback on the FLEHS programme and a call for its continuation from a wide variety of stakeholders, ranging NGOs to industry, has further supported the sustainability of the FLEHS studies. In Germany, the human biomonitoring results, particularly relating to PFAS and glyphosate, have recently made an important contribution to the public and scientific discourse (Duffek et al., 2020; Conrad et al., 2017; Lemke et al., 2021).

3.3.3. Policy translation

Data from the HBM programmes of the countries in Group 3 have been used for a wide variety of local, regional, national or European/international policy actions. In general, HBM outputs have been used to; quantify exposure levels of general or specific populations to harmful substances from the environment, assess health effects of such exposures and long-term trends in exposure and health impacts, identify problems requiring measures to reduce/eliminate exposure, and evaluate the effectiveness of remediation plans. Such results demonstrate the use of objective data for health risks management and the development of health policies and strategies. HBM data are also provided to initiate and support risk management measures under REACH and international conventions (Stockholm Convention on POPs and Minamata Convention on Mercury). Many countries also reported that data on persistent organic pollutants are an important part of reporting in the framework of the Global Monitoring Plan of the Stockholm Convention on POPs and that it enables the effective evaluation of measures on the basis of trends and baselines.

In Germany, the HBM Commission establishes assessment values (reference and HBM values) for selected substances according to defined criteria. These values serve to provide a comprehensive and uniform assessment of HBM results, whether they are derived from the German Environmental Surveys, time-trend analyses, or collected occasionally at the level of the Federal States. The reference values are derived by means of statistical methods and describe the basic exposure of the population. Measured values that are higher than the reference values are an indication of increased exposure compared to the general population but are not apt to interpret health risks (Apel et al., 2017; Angerer et al., 2011). In the HBM4EU context, a scientific exchange took place between HBM4EU staff and members of the HBM Commission on derivation options and methods for reference values, which was also reflected in a corresponding publication (Vogel et al., 2019). The assessment values relevant for a health risk assessment are the HBM-I and II values, which are toxicologically and/or epidemiologically derived. The HBM-I value is defined as the concentration of a substance in human biological material (e.g. urine, blood, hair) at and below which no risk of adverse health effects is to be expected and consequently there is no need for action. The HBM-II value represents an intervention level, where an increased risk for adverse health effects is assumed (Apel et al., 2017; Angerer et al., 2011; der Referenz, 1996; des Umweltbundesamtes, 2007). Building on this concept as well as the concept of Biological Equivalent (BE) values and the work of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES, 2014) on guidance in the occupational field, the strategy to derive HBM-GV under HBM4EU was developed (Apel et al., 2020; Hays et al., 2007; Hays SM et al., 2008; Aylward et al., 2013). The assessment values can be used by policy makers as a basis for possible risk management measures (Choi et al., 2015). UBA regularly reports data and recommendations to the BMUV so that the Ministry of the Environment can take legislative (regulatory) action on the basis of this scientific information if necessary. In addition, other ministries, federal and Federal State authorities are informed by UBA or via the HBM Commission so that they can act accordingly. For example, the provision of HBM-I and II values for PFOA and PFOS was helpful for the Federal States to initiate and control measures in hotspot areas (Hölzer et al., 2021; Schümann et al., 2021). Additionally, they supported the initiative to propose a ban for the whole substance group. Parliamentarians were also informed at their request. Reference values for lead were also useful for local authorities in assessing the body burden of a population in an area with elevated soil lead levels. Additionally, results from the German HBM studies inform voluntary exposure mitigation activities and supply a broad variety of targeted materials for information campaigns to inform citizens and behavioral choices.

In Belgium, a phased action-plan was developed collaboratively by

FLEHS researchers and policy makers to facilitate the science policy process (Keune et al., 2009), and was implemented after each (FLEHS) study. This phased action-plan combines scientific analysis and societal deliberation in a structured and participatory approach. In several successive phases HBM results are prioritised for policy action, explanatory factors are identified and targeted policy interventions are developed.

With this approach, the FLEHS studies have resulted in several action plans, with a diversity of policy actions in addition to existing policies and in cooperation with various national and regional actors (e.g. on POPs, PFAS). Successive action plans were developed for; elevated plasma levels of POPs in rural areas, the increased asthma prevalence in urban areas, exposure to PAHs of the general population and for local increased chemical exposure in the industrial hot spots Menen and Genk-Zuid (Reynders et al., 2017; Colles et al., 2021) and also for lead and arsenic in Hoboken. Each action plan was coordinated by a policy advisor on environment and health leading to targeted policy actions communicated by the ministers for environment and health. The HBM results on endocrine disruptors (pesticides, phthalates, PFAS, BPA) contribute to the yearly revision of the Flemish strategy on endocrine disruption and feed into the national action plan on endocrine disruptors which was initiated in 2021. Recently, HBM results on PFAS (e.g. exceedance of HBM-I values for PFOS and PFOA in almost 80% of adults in FLEHS III, statistical associations with several effect markers and associations between locally grown food (eggs, vegetables) and higher exposure to PFAS) led to the development of a PFAS action plan which is a dynamic plan and involves both generic and hot spot related actions. Wallonia is in the process of developing exposure reference values for the Walloon population and an action plan to achieve targets for reducing exposure to substances of concern will need to be established. For HBM hotspots such as Liège (urban gardeners), the local authorities have been supported to organise an information and awareness campaign for the concerned population (SPW, 2020).

The French population surveys (ENNS, Esteban and ELFE) have highlighted the exposure of the population to a variety of environmental contaminants. These surveys showed a significant level of exposure to endocrine disruptors: Bisphenols (A, S and F), parabens, flame retardants and some metabolites of phthalates and perfluorinated compounds (PFCs-PFOS and PFOA) (Dereumeaux et al., 2017; Fillol et al., 2021). The results from the surveys triggered the strategy on endocrine disruptors in France resulting in stricter regulation of some of these compounds.

Additionally, since 1981 French HBM studies have focused on specific populations or pollutants to gain a better understanding of exposure to environmental chemicals to help regulators to reduce exposure and monitor existing policies of specific concerns. These French HBM studies have been implemented to better understand; the influence of living near an incinerator on serum dioxin and polychlorinated biphenyl (PCB) levels (2005–2007); the influence of consuming fish from contaminated rivers on serum PCBs of fishermen (2009–2011); the evolution of blood lead levels in children from 1 to 6 years old since 1995 (2008–2011) (Fréry et al., 2012) and more recently HBM was combined with community involvement in Southern France to manage soils polluted with lead, arsenic and cadmium in the surroundings of closed metal mines (2015–2017) (Cochet et al., 2020). Another example is a case study in the French West Indies. A HBM study was conducted in 2013–2014 with focus on chlordecone, a legacy pesticide used in the past (until 1993) for the treatment of banana trees in the French West Indies. Results suggest that exposure to chlordecone is persistent and widespread. Chlordecone impregnation appears to have decreased between 2003 and 2013 for most of the population. However, various subgroups of the population remain highly exposed mainly through consumption of contaminated foodstuffs, like fresh fish (all species combined). Supply procedures, mainly those from informal channels, are also associated with exposure to chlordecone (Dereumeaux et al., 2016; Guldner et al., 2010). Since 2002, the Ministry of Health and the Ministry of the Overseas Territories have mobilised significant resources, in the framework of the Chlordecone Action Plans, leading to the raising of awareness and protection of the population (including several HBM studies: KANNARI, HIBISCUS, TIMOUN, KARUPROSTAT), the support of impacted professionals and the improvement of knowledge on this substance (Plan chlordécone 3, 2014).

In Austria, HBM in specific pollution hotspots supported risk assessment and was used to monitor the success of minimising risks (see Box 3). In the Austrian valley Görtschitztal, hexachlorobenzene (HCB) was detected in milk products and meat after waste treatment in a cement plant. An HBM study revealed that affected populations had increased plasma concentrations of HCB. Consequently, several risk managements measures were implemented (e.g. elimination of HCB emission, feed exchange, exchange of animals as well as nutritional recommendations for the affected population) (Steinwider et al., 2019). Additionally, HBM-data are fed into risk management activities of chemicals by the Austrian Competent Authority.

Many examples on the policy use of HBM data exist in Sweden. Noteworthy is the time trend studies to follow up the efficiency of regulatory measures or to detect emerging chemicals of concern and levels of chemicals (PCBs, PFAS, PNDE, DDE, PCDD/F) in mothers' milk and blood. These are used as indicators in the follow-up of the Swedish environmental quality objective "Non-toxic environment" (se, 2019). Data from the Swedish HBM programme has been used, together with other available HBM studies, to provide supporting scientific evidence for human exposure in proposals for different regulatory measures under REACH. For example, several studies of PFAS, time-trends as well as snap-shot studies (Gyllenhammar et al., 2015, 2016; Glynn et al., 2012; Bjermo et al., 2013; Axmon et al., 2014; Gebbink et al., 2015) were used in the REACH Annex XV restriction reports for PFHxS (ECHA, 2020) and C9-C14 PFCAs ((RAC) and E.-C.f.R.A. and C.f.S.-e.A. (SEAC), 2018). In addition, HBM data were used in the risk management option analysis (RMOA) of bisphenol-F and in the REACH Annex XV dossier to identify lead as a Substance of Very High Concern (SVHC).

3.4. Ministries and stakeholders advocating for HBM

3.4.1. Ministries and stakeholders in group 1

The countries in each group listed stakeholders who were included in their national hubs. The Ministries of Health and Environment seem to be the most prominent entities among the 3 groups - all NH had representatives from their ministries/departments of health and environment. It is not possible to evaluate the contribution from each stakeholder or their role in the NHs but it is interesting to see the variety of stakeholders. The level of engagement of health and environment agencies appears to impact on factors that contribute to the establishment of a long-term HBM programme. Ministries and stakeholders are limited in Group 1 in comparison to Groups 2 and 3 which probably indicates that wide networking is the key in raising the profile and status of HBM nationally.

3.4.2. Ministries and stakeholders in group 2

Most countries in Group 1 cited the Ministry/Department of Health as the foremost ministry advocating for HBM. Additionally, some countries cited environment ministries such as the Department of Environment, Food and Rural Affairs-DEFRA (UK), Ministry of Environment and Science (Portugal), Ministry of Infrastructure and the Environment as well as the Ministry of Economic Affairs & Climate Policy (the Netherlands) as their main advocates for HBM. Other ministries supporting HBM include, Ministry/Department of Environment, Social affairs and Food Safety Agencies, Chemical Authorities, University Institutes and Medical Faculties. Other entities who have supported HBM in a smaller scale are, the Artic programme and pesticides research.

The main governmental stakeholders cited by different countries in Group 2 include Ministry of Health, Ministry of Environment, Parliament, Commissioner of the protection of children's rights, Institute of Public Health, Ministry of Agriculture and Rural Affairs and Ministry of

Box 3
A case study of Hexachlorobenzene in Austria

In an Austrian valley called Görtschitztal, Hexachlorobenzene (HCB), a banned persistent organic pollutant, was detected in milk products and meat. The source of the contamination was attributed to waste treatment in a cement plant.

The dietary risk assessment revealed that the minimum risk level to human health for HCB, which is comparable to the usual tolerable daily intake (TDI) value in Europe, of 0.07 μg/kg bw/d exceeded the average and high consumption by all population groups up to 4 and 8-times, respectively. A human biomonitoring survey of the affected population also revealed plasma concentrations of HCB in a broad range (0.1–5.29 μg/l blood plasma). This was above the levels of the reference group of 0.15–0.6 μg/l.

A precautionary warning was issued for the consumption of food with high concentrations of HCB in the affected region. Several risk managements measures e.g. more than 5000 tonnes of contaminated feed was removed from the farms and substituted by HCB-free stock and farmers were advised to interrupt the on-farm cycle of HCB and to rehabilitate the farms and their products to minimize risks. The rather early detection of the HCB release and the risk management measures led to a limited duration of increased uptake of HCB in the population.

Dietary recommendations were established to enable the decrease of the internal HCB burden. Guidance levels for HCB in food for the affected population were calculated. These levels were significantly stricter than the maximum residue levels in EC regulations (Steinwider et al., 2019).

Agriculture, Food and Environment. Additional stakeholders involved in steering and financing of HBM cited by countries include the Ministry of Ecological Transition and the Demographic Challenge, Ministry of Science and Innovation, and Ministry of Consumer Affairs. Additional *potential future* stakeholders include Sanitary and Health Inspectorate, Agriculture Inspectorate, Ministry of Environment and Physical Planning and Ministry of Economy (North Macedonia).

3.4.3. Ministries and stakeholders in group 3

Countries in Group 3 all have a multidisciplinary team who follow-up and give advice on the respective HBM programmes. The formation or expansion of NHs under HMB4EU has enabled more partners to be involved and has strengthened existing collaborations within these countries.

In Sweden, it includes the Swedish Chemicals Agency, the Swedish National Food Agency, the Public Health Agency of Sweden, the County Administrative Board, the National Board of Housing, Building and Planning and the Karolinska Institute. The steering group has an advisory function and meets on a regular basis 4 times per year.

In Belgium, a steering board is also connected to the HBM programme. The steering committee of the FLEHS studies is composed of representatives of the Flemish policy domains on environment, health, agriculture, education, science and innovation. The results are also transferred to the federal administrations on health and environment through the national cell on environment and health (NEHAP). Representatives of the Walloon Ministry of Health are involved in the BMH-WAL steering committee as well as Sciensano, the only federal organisation involved in health studies.

The German HBM Commission which has been in existence for more than 20 years steers the German National Hub. It consists of independent experts from different fields, representatives from the higher scientific federal authorities responsible for chemical risk assessment, management and health surveillance, as well as from universities, hygiene institutes and clinics. In addition to the members, representatives of the BMUV, the Federal Ministry of Health, the Robert Koch Institute, the Federal Institute for Risk Assessment, the Working Group of the Supreme State Health Authorities, and UBA are involved in the work of the HBM Commission as permanent guests, so that a transfer of information between different departments and the national and Federal State level is ensured. The HBM Commission has been providing expert advice to the German Environment Agency on all issues relevant for human biomonitoring studies, be it study design, analytics or the evaluation of collected data. With the inclusion of the research institutions taking part as linked third parties in HBM4EU, the National Hub was extended and further developed, and a further connection between HBM4EU and national activities created.

The French HBM steering board is coordinated by the ministry of health and Santé Publique France. It also includes representatives of the ministries of environment, research and education and scientists from various institutions such as INSERM as well as stakeholders-from industry and non-governmental organisations, constituting the French National Hub. A newly established board has been set up recently for future HBM studies and is expected to meet four times a year to coordinate HBM activities.

In the Czech Republic, the national HBM hub was established in 2013. It is coordinated by the National Centre for Toxic compounds, the joint establishment of the Ministry of Environment and Masaryk University and supervised by the inter-ministerial science-to-policy Board composed of representatives of all relevant ministries. Active involvement of the Ministry of Education, Youth and Sports (MEYS) responsible for the European research programmes enabled mobilisation of resources of academic institutions, and those of large infrastructures for research and innovation as a source of co-financing.

The members of the Austrian NH are experts from ministries and agencies responsible for health, environment, food safety, occupational health and related research institutes and universities. They have committed to a mission statement (Austrian Platform for Human Biomonitoring, 2022) (Umweltbundesamt, 2022). Since 2016, the platform has been the official advisory board of the Federal Ministry for Climate Protection, Environment, Energy, Mobility, Innovation and Technology on issues at the interface between environment and health and usually meets twice a year. The organisation of the platform enabled the participation of an Austrian national hub in the framework of HBM4EU and will find continuation in the upcoming Partnership on the Risk Assessment of Chemicals (PARC).

All countries in all the 3 groups have stated their interest in participating in PARC, the NHs in PARC will have to expand to cover environmental protection as well as institutes with a remit for Human Health risk assessment. The role of the NH will be similar but the remit will expand considerably.

3.5. Steps/processes needed or used to engage policy makers

Almost all the countries in Group 1 highlighted ways used/needed to promote the utilisation of HBM data. Noteworthy, is the association and involvement in HBM4EU which significantly influenced the ongoing process of a national authority's approval for a national HBM programme in Lithuania.

Other steps include the involvement of several authorities/institutes in the NH which can create an avenue to interact, discuss and gain the attention of policy makers through respective channels (Norway). Portugal highlighted the following processes: proceeding further with

the HBM dissemination activities, better communication tools to involve the civil society; extending the national laboratory network and developing research, infrastructures, and capacity building. Portugal and Norway suggested establishing more partnerships/interactions with national bodies. Denmark and Estonia advocated having forum discussions on environmental pollutants of concern. Another mechanism was through the organisation of conferences as in the case of Hungary. Additionally, Switzerland recommended promoting the need for good quality HBM data for political interventions.

For those countries in Groups 1 and 2 who do not have formal national programmes in place, HBM4EU provided a good platform to stimulate discussions and engagement with national authorities to get HBM on the research agenda. The NHs have also sought to establish communications with national authorities as a mechanism for helping to engage with relevant policy makers. Some countries have invited relevant policy leads to participate in conferences where the benefits of engaging and working together can be demonstrated in a more tangible way.

The use of focus groups to galvanize civil society and raise general awareness has also been utilised in some countries. However, in those countries where the profile of HBM is not high, there may be a requirement to have additional disseminating activities. The development of better communication tools could also be helpful in the process - HBM activities communicated to the wider society can be of immense benefit. This process can spur societal and political interest in the environment and health concomitantly supporting the establishment and sustainability of existing HBM programmes as affirmed by most countries in all 3 groups.

Countries in Group 2 have made progress in engaging some policy makers as many describe national plans, programmes or strategies which have included HBM in some way.

Most countries in Group 3 have a strong network involving policy makers and the lessons learned and good practices could be shared through workshops/meetings or in the form of a guidance document or similar.

3.6. Barriers, drivers and opportunities

3.6.1. Group 1

All the countries in Group 1 reported the lack of funding as the major barrier for HBM activities. Other barriers include the difficulty of adding modules to already established cohort studies. Many countries alluded to the fact that setting-up a HBM program is expensive and challenging. The shortage of HBM experts has resulted in underrepresentation of HBM in the national authorities as in the case of Lithuania. In Iceland the recruitment process for HBM studies is becoming difficult because young people are unwilling to participate/complete long questionnaires, most policy makers are not aware of human biomonitoring activities, analytical capacity is dispersed to universities, research centres and government laboratories and there are difficulties in inter-institutional and inter-sectorial collaboration. Participation in HBM4EU was reported to be a major driver for raising political awareness and an understanding of the importance of HBM as a tool in public health protection and chemical management. For example, HBM4EU created an opportunity for the establishment of a UK HBM Steering Group (including health, environment and food ministries/agencies); in Denmark and the Netherlands networks have been strengthened nationally/internationally; and in Luxembourg and the Netherlands it has been the driver of HBM initiatives and future participation in such projects have been welcomed and appreciated by many governmental institutions.

3.6.2. Group 2

For Group 2, the main obstacles for establishing National HBM Programs include lack of funding and resources (Spain, Croatia, Cyprus, North Macedonia, Israel). In Croatia, HBM is included in the Ministry of Health and Ministry of Economy and Sustainable Development Strategy but there is no allocated funding. The National Biomonitoring Programme in Israel was co–funded by a non-governmental agency, with government matching funding, but government funding is not ensured beyond 2021. North Macedonia has mostly received funding for HBM from international organisations such as the UN and work on a public health crisis (COVID-19 pandemic) has more recently diverted technical and human resources from HBM. Lack of legislative framework and permanent allocated resources were described as barriers by Cyprus.

Spain reported the dearth of funding being compounded by lack of clear definition of HBM competences at ministerial level (Environment – Health). Cyprus raised the issue of competing obligations in the fields of chemicals management and environmental health.

North Macedonia cited an additional barrier such as insufficient technical equipment, a slow as well as expensive process of accreditation of research methods, and challenges in recruitment and special training of research staff. Establishment of accredited HBM laboratories was stated as a major goal. In Croatia, cross government collaboration and challenges linking environmental data with exposure measurements are highlighted barriers. Israel described challenges engaging Ministries (Environment, Agriculture) and raising awareness about potential uses of HBM data within the government.

Despite the barriers to advancing national HBM programs, all countries described opportunities. For example, in Croatia participants and the general population showed an interest in participating in HBM studies, and were interested in results. In North Macedonia, HBM4EU, with focus on material for general citizens ("citizens corner"), raised interest in the public and among researchers, demonstrated the need for a national programme, and provided published materials (protocols) to be used in the future. Joining the Horizon Europe Partnership for the Assessments of Risks from Chemicals (PARC; https://www.anses.fr/en/content/european-partnership-assessment-risks-chemicals-parc) provides (a) motivation for developing a national programme in North Macedonia and (b) an opportunity for engaging additional goverment ministries (focus on Ministry of Environment) in HBM acitivities, and increasing political interest (in HBM) in Israel.

The drivers for developing a national HBM programme varies across the countries in this group. In Croatia, the national programme is being developed within the Ministry of Economy and Sustainable Development Climate Change Adaptation Action Plan. This plan (still in draft at the time of writing) will include a component on strengthening the capacity of key existing public health laboratories towards human biomonitoring, with the aim of integrating epidemiological data with the results of chemical analyses in environmental and human samples (hair, serum, urine, etc.) and strengthening the capacity to assess human exposure, including to environmental factors related to climate change. Slovakia's participation in the WHO Europe Ministerial Conference on Environment and Health was a driver for developing a national HBM programme whereas in North Macedonia, concern about exposures at industrial hotspots (chemical industry, mines, lead smelter) is the key motivator.

In Spain, participation in EU projects such as COPHES, DEMO-COPHES and HBM4EU has been a major driver in advancing HBM and has supported strong collaborations with all actors involved including competent authorities and administrations, universities, research institutions and other stakeholders. Participation in HBM4EU led to the creation of a HBM programme in the Strategic Action Plan for Health and Environment, as well as the drafting of a Royal Decree for the establishment of an Inter-Ministerial Commission as basis for the National HBM Hub. Participation in PARC will contribute to the development of the HBM programme in Spain as the Ministry of Health, primary driver of the activity together with the Instituto de Salud Carlos III, is a member of the Governing Board.

The detection of high concentrations of some chemical substances in the population of Spain has also propelled the development of a national HBM programme; exemplified by the high levels of mercury found in

humans associated with food consumption and their potential health risks to vulnerable populations, in particular children. In North Macedonia, the motivation was the need to gather data on exposure of the population to prioritised chemcials including pesticides (Lindane and other HCH), PAH, PCBs, heavy metals (Cd, Hg, As) and mycotoxins. In Israel, non-governmental funding was a major driver for establishing a national programme, combined with interest within the Ministry of Health to collect data on prioritised exposures (environmental tobacco smoke, organophosphate pesticides) and nutritional biomarkers (iodine and selenium).

3.6.3. Group 3

Although the HBM programmes for countries in Group 3 have proven to be useful for chemicals policy (see Table 5), the sustainability of most is hindered by several barriers and challenges. The lack of governmental support and long-term funding for the regional or national HBM initiative is mentioned by most countries. Although in France there has been a general agreement that HBM campaigns should be undertaken regularly, the start has been delayed due to the dependence on secured funding. In the Czech Republic, the HBM programme lacks sufficient funding as it is not the highest priority at the Ministry of Health. Due to increased government saving, funding of the FLEHS studies has decreased by almost 50% for the 4th FLEHS study in comparison to the first 3 rounds. Given the presence of other high impact policy issues (e.g. climate, COVID), it is becoming increasingly more difficult to allocate the necessary budget to continue the Flemish HBM programme.

Several countries stated that fragmentation of (i) competences, (ii) monitoring programmes and (iii) related databases among several ministries (Health, Environment, Agriculture) complicate analysis and interpretation of available data. Improved communication and collaboration between the different relevant ministries/agencies dealing with various aspects of chemical risk assessment is required. The issue was further compounded in the Czech Republic by significant organisational changes in a system of public health institutes and related services (lack of necessary capacities). This resulted in the closure of some reference laboratories and a need to subcontract external partners for the chemical analyses. The HBM samples were collected annually during the earlier years, however, collection is now reduced to once in several years and in smaller sample sets.

Many countries also mentioned the lack of regional, national, and European infrastructure for analyses and storage of samples collected within the HBM programme.

Another challenge relates to the recruitment and active involvement of participants. It is becoming increasingly challenging to engage participants to gather a multitude of data and information. In Belgium, active engagement of participants in the evaluation of the FLEHS studies is crucial to improve participants recruitment and involvement and stimulates their understanding and use of personal HBM-results (Morrens et al., 2021). A decreasing participation rate was also mentioned by Sweden. An underrepresentation of populations groups with lower socioeconomic status is likely. Recruitment of socially vulnerable groups is still difficult and requires extra efforts to include them in a representative manner. By investing in direct, person-to-person contact with trusted buddies and supported by practical advice about cultural and linguistic sensitivity, it was possible in FLEHS III to increase study participation of socially disadvantaged people (Morrens et al., 2017).

Financial and recruitment issues are compounded by the statistical power (of the study) often being too low for detailed statistical analyses due to the limited sizes of the study groups. The connection between HBM and health effects requires large population groups and access to medical documentation. The issues on statistical power can partially be addressed and improved by combining and aligning HBM studies across Europe. The same holds true for combining HBM and health studies and in bringing existing cohorts together to increase the power of statistical analyses between exposure to chemicals under study in HBM4EU and selected health effects.

Another hurdle relates to the fact that the level of chemicals in the body is a result of the total exposure from all sources. More information on specific sources is needed to inform the use of the correct measures to reduce the exposure level. In addition, identifying new threats is a complex task as banned chemicals are replaced by new ones with similar properties, making it challenging to target investigations. Chemicals are mostly studied individually, but we are exposed to mixtures, and we need more knowledge to understand that synergistic effect on human health.

Many countries cited HBM4EU as being crucial in addressing a number of these barriers and, the continuity of HBM at European level in the frame of PARC is also important in the sustainability of their existing HBM programmes. The HBM4EU co-financing to regional or national programmes has been of utmost importance in overcoming decreased budgets and has created a mutually beneficial situation for both the national/regional and the European level as results are useful for policy on both levels. In France, it is expected that the national and European programmes (such as PARC) will be further aligned. In Sweden for example, HBM4EU was supported by the government, which resulted in an extended budget for the national programme health related environmental monitoring since 2017.

HBM4EU provided better communication of HBM-related issues across the consorts and more collaboration within countries between the departments, competent entities and research institutes involved in different aspects of HBM. The concept of NH under HBM4EU has been helpful for national players to join their efforts and mobilize capacities to make the regional or national HBM programmes more sustainable. For instance, the RECETOX's open-access research infrastructure in Czech Republic listed in the national Roadmap mobilised sufficient capacities for the analyses of samples from the alignment studies at the national and European levels and also those collected in predecessor DEMOCOPHES project. Countries in this group anticipated that the PARC partnership will play an important role in strengthening collaborations at the national and European level, setting up standards and establishing processes and motivating the project partners to participate in surveys.

The EIRENE RI - research infrastructure for environmental exposure assessment in Europe which was recently added to the 2021 Update of the European (ESFRI) Roadmap could be another important gateway and pillar of sustainability of the HBM programmes in Europe in providing technical solutions and supporting pan-European collaborations.

On open policy questions such as action on new emerging chemicals, mixture effects or identification of sources of exposure, national programmes benefit from the large network of experts in HBM4EU. For example, the German system as a longstanding and well-established tool has proven itself at the national level and is also increasingly benefiting from the international activities and networks, as the many open questions can be solved more quickly and precisely by large number of actors. This is demonstrated by the Europe-wide quality assurance of analytical methods and laboratories driven by the linked third parties as well as the harmonised SOPs for study preparation, sampling and storage of human samples, the knowledge gain regarding adverse outcome pathways and non-target screening do not only provide valuable results for the HBM4EU project but will also be important for the further work of the German National Hub, including the HBM Commission and the UBA.

Countries in Group 3 have indicated that they actively use HBM4EU products in their national settings. In the Czech Republic for example, materials were not only used at the regional and national levels to promote awareness, but also to get stakeholders to prioritise issues and phase out certain chemical uses - i.e dental amalgam etc. Germany indicated that the monthly newsletter 'HBM4EU Science Digest', is particularly appreciated for a quick overview of specialist topics. HBM4EU results and conclusions are continuously used to inform the general population, stakeholders and other players concerned via the

Table 5
Use of HBM data for policy purposes in countries from Group 3.

Country	Issue/Concern	Population (local/ regional/national)	Chemical	Ministry or Institute undertaking the study/other	Outcome	Reference
Austria	Regular monitoring of POPs in breast milk	National	POPs	BMK	Assess time trends to feed the health target "securing sustainable natural resources such as air, water and soil and healthy environments for future generations"	Bundesministerium für Klimaschutz (2019)
	Dietary exposure to HCB	Local (hot spot)	Hexachlorobenzene		Support for risk management measures and monitoring of the success of the minimisation measures	Steinwider et al. (2019)
Belgium	Elevated plasma levels of POPs in rural areas and hot spot Menen	Regional (Flemish population)	Dioxines/PCBs/DDT	CEH, DOMG	POPs action plan with measures on source, monitoring and sensibilization to further reduce POPs body burdens and meet the objective of the Stockholm convention	(Reynders et al., 2017; Keune et al., 2009; Colles et al., 2021)
	Increased asthma prevalence in urban areas	Regional	(indoor) air pollution	CEH, DOMG, AZG	Action plan to reduce asthma prevalence	
	Local increased chemical exposure in the industrial hotspots Menen, Genk-Zuid and the harbour of Ghent	Local	Multiple pollutants	CEH, DOMG, AZG, VMM	Local policy action plans	Colles et al. (2021)
	Exposure to endocrine disruptors in the general population and associations with bio-markers of ED effects	Regional (Flemish population)	Recent pesticides, Phthalates, PFAS, BPA	CEH, DOMG	Contribution to the Flemish strategy on endocrine disruption, to the national action plan endocrine disruptors (2021) and to the development of a Flemish PFAS action plan (generic and hot spot related actions) which is regularly updated.	
Czech Republic	HBM data on POPs		POPs		Evaluate effectiveness of measures in the Stockholm convention on POPs on the basis of trends and baselines	
France	Exposure to endocrine disruptors in the general population	Population surveys	Endocrine disruptors	SpF, ANSES,	Contributed to the launching of the French strategy on endocrine disruptors which led to stricter regulation of some of these compounds. Report on high priority EDCs.	www.ecologie.gouv.fr and 2nd National strategy on (2019)
	HBM study with focus on chlordecone	In the French West Indies	Chlordecone	INSERM, SpF	Contribution to the Chlordecone Action Plans (e.g. awareness and protection of the population, support of impacted professionals but also the improvement of knowledge on this substance)	(Dereumeaux et al., 2016; Guldner et al., 2010)
	Sources and modes of exposure for soil pollution (lead, arsenic and cadmium)	Local: in the surroundings of closed metal mines	Arsenic, lead, cadmium	SpF, Occitanie ARS (Agence Régionale de Santé)	The study has led to a series of appropriate operational measures.	Cochet et al. (2020)
Germany	Provision of HBM-I and -II values for PFOA and PFOS	National	PFAS (PFOS, PFOA)	UBA	Initiate and control minimisation measures. Support for the initiative to propose a ban for the whole substance group.	(Hölzer et al., 2021; Schümann et al., 2021)
	Provision of representative and time trend HBM data on PFAS	National and regional	PFAS	UBA	Support EFSA with data compilation, support Federal States to evaluate HBM results from populations in hot spot areas	Göckener et al. (2020)
	Provision of representative and time trend HBM data on Phthalates	National and regional	Phthalates	UBA	Initiate and control minimisation measures. Support of REACH activities	(Schwedler et al., 2020; Koch et al., 2017)
	Annual routine measurement of selected substances in human samples from the environmental specimen bank	Regional	Arsenic, Lead, Cadmium, Copper, Mercury, Organohalogen compounds, Polycyclic aromatic hydrocarbons	UBA	Continuous control	(Bartel-Steinbach et al., 2022; Lermen et al., 2021)
Sweden	POPs in mothers' milk and blood	Local	PCBs, PFAS, PNDE, DDE, PCDD/F	SFA and financed by SEPA	Indicators in the follow-up of the Swedish environmental quality objective "Non-toxic environment".	se (2019)
	Time trends for PFAS in breast milk of first time mothers	Local	PFAS	SFA and financed by SEPA	Increasing levels of PFHxS led to the discovery of exposure from the municipal drinking water. As a result, measures were taken for treatment of the water	(Gyllenhammar et al., 2015, 2016)
	Exposure to PFAS contaminated drinking water	Employers at an airport	PFAS	Dep. of Occupational and Environmental Medicine, Sahlgrenska University Hospital	Sampling of blood was initiated among employers	(Xu et al., 2020)

(*continued on next page*)

Table 5 (*continued*)

Country	Issue/Concern	Population (local/ regional/national)	Chemical	Ministry or Institute undertaking the study/other	Outcome	Reference
	Time trend and snap-shot studies for PFAS		PFAS		PFAS results were used in the REACH Annex XV restriction reports for PFHxS and C9-C14 PFCAs	(ECHA, 2020; Reade and Pelch, 2020)
	Bisphenols in urine from first-time mothers		BPF	SFA and financed by SEPA	Used to demonstrate human exposure in the risk management option analysis (RMOA) of bisphenol	Bjermo et al. (2019)
	Time trend studies of lead in blood of children and adults		Lead	Performed by Lund University and financed by SEPA	Used in the REACH Annex XV report to identify lead as a Substance of Very High Concern (SVHC)	(Skerfving et al., 2015; Lundh et al., 2020; Wennberg et al., 2017)
	PFAS in drinking water		PFAS	Performed by SFA and financed by SEPA	Results showed that low levels in drinking water may be an important source of exposure among children	Glynn et al. (2020a)
	POPs in HBM and aquatic monitoring program		POPs	Performed by Swedish University of Agricultural Sciences and financed by SEPA	The importance of chemical regulation was clearly shown using temporal trends.	Glynn et al. (2020b)

UBA website, social media and other communication products. In Belgium, the HBM4EU videos and factsheets were used in communication to the press in support of their reports.

3.6.4. *Overview of all 3 groups*

Overall, the primary obstacle cited by countries for establishing and sustaining national HBM programmes is funding; due to the high costs associated with the collection and chemical analysis of human samples. Although it is included in national strategies in some countries in Group 2, there is no associated ring-fenced funding. Those in Group 3 have acknowledged the usefulness of HBM programmes for chemicals policy but recognise that there are barriers and challenges that could affect the sustainability of such initiatives. These barriers include the lack of governmental support and long-term funding for the national/regional programmes due to the high associated costs.

A summary of the major barriers and challenges cited by countries in the three groups include:

- All groups mentioned competing priorities as a major barrier. For example, currently in most countries Covid-19 is a priority and resources are being targeted in that area. Furthermore, developing a national programme may never become a priority in some countries who may choose to adopt a different approach to investigating population exposure to chemicals.
- Organisational changes, and decommissioning/closure of some required infrastructure e.g. reference laboratories, to support the programmes (Group 3).
- Recruitment of participants and the collection of vast amounts of data are hugely onerous. Recruitment of vulnerable groups and those from lower socio-economic status (SES) is particularly evident in these programmes (Group 3).
- Lack of required competencies at ministerial level stifles the discussions needed to develop and implement HBM programmes (Groups 1 and 2).
- Fragmentation/dispersal of competencies, fieldwork, chemical analysis and associated databases between different ministries/ agencies makes data analysis and interpretation more challenging (Group 3).
- When the sample size is too small the statistical power of the study is reduced thereby reducing data analyses to brief descriptive statistics (Group 3).
- The level of chemicals in the body is a result of the total exposure from all sources therefore more information on specific sources is needed to inform the use of the correct measures to reduce the exposure level. Often, this information is not available because it is difficult to make an informed decision on an individual route of exposure as HBM generally assesses exposure by quantitating a biomarker of exposure in either blood, urine or other biological media thus, making communication with policy makers difficult.

The drivers tended to be varied among the countries in the three groups. The most noteworthy drivers cited include:

- Investigating exposures at hotspots (industrial, traffic etc.).
- Investigation of elevated levels of chemicals found in the population (from food, water, air, consumer products).
- Strengthening relevant capacity with the aim of integrating epidemiological data with environmental and human exposure data.
- Participation in HBM projects such as COPHES/DEMOCOPHES and HBM4EU has been cited by many as a positive way to raise public and political awareness and engage with stakeholders.
- Establishing and strengthening national hubs through HBM4EU has proved especially useful in raising the profile of HBM and getting it on the national/regional agenda in some cases.

The opportunities of an HBM programme are similar for most

countries and primarily include:

- Knowledge on the internal exposure of the population to chemicals.
- The information/data gleaned can be used to influence relevant stakeholders, policy makers and politicians to prioritise chemical issue(s) identified.
- These programmes can be used to monitor the impact of new and emerging chemicals.
- On open policy questions such as new emerging chemicals, mixture effects or identification of sources of exposure, national programmes benefit from the large network of experts in HBM4EU.

3.7. Potential useful non-governmental groups/players for the promotion of HBM

The suggestions of players who would benefit in some way from HBM were varied as highlighted in only Group 1. Hungary suggested that science communication experts and behavioral scientists will add to the success in developing policy and communication materials. Industry, NGOs, and universities were also highlighted as potential players. The UK suggested the UK Chemicals Stakeholder Forum (UKCSF) which enables discussion between stakeholders, government, and regulators in support of effective chemicals and waste management. Members of this forum were receptive when UKHSA (formerly PHE) presented the work of HBM4EU in June 2019. Portugal highlighted the Chemical industry, trade unions, industrial associations, and citizens collaboration. Lithuania cited private institutions would be beneficial in linking different exposure data/experimental results to the source of exposure. Switzerland suggested that the link between environmental monitoring and HBM will be strengthened by collaborating with environmental monitoring networks. Iceland mentioned journalists as important players for creating awareness.

3.8. The future of HBM in european countries

Countries in Group 1 have highlighted different ways that HBM will be considered nationally/regionally. In Lithuania, the government has included human biomonitoring in its preventative measure objectives and intend to use it as a tool for further investigation and health surveillance. The UK (led by UKHSA) will continue to develop HBM for public health protection and chemical management. The initial step will be through the HSfE and input into PARC. Additionally, there are calls from government to continue the establishment of a sustained HBM programme. Engaging the wider stakeholder through the UK Chemical Stakeholder Forum will continue and data from HBM4EU will be used as a comparator for that produced in the UK.

The Ministry of Health in Norway had presented an opinion to the Parliament in 2019 describing their visions for public health. The government wants to continue the collaboration with other European countries to survey the general population's exposure to environmental pollutants and increase the knowledge base on these exposures' potential impact on health. This was also mentioned in the chapter on "Green Health" in their International Strategy (2021–2025), as well as in the action plan for "a toxic free everyday life", just launched by the Ministry of Climate and Environment. The National Institute of Public Health (NIPH) has also funded a second collection of samples for the Norwegian Environmental Biobank (NEB; https://www.fhi.no/studier/miljobiobanken) to take place in 2022 which will be included in PARC. The aim is to implement sustainable surveillance of the populations' exposure to hazardous chemicals and changes in diet, as well as to generate a rich data source of exposures that can be linked to health information/registries and used in future risk assessments and research projects at European level.

In Finland, levels of PFAS in fish have been rising for the last 10 years; if this continues, given the governmental programme to promote the use of domestic fish, HBM in vulnerable subpopulations (women of childbearing age, 1 year old children) will be required.

Hungary is planning to investigate different group of substances (e.g. Bisphenols and biomarkers of air pollution) in previous sample collections (biobank samples) and to establish a regular HBM programme.

In the Netherlands concerns about exposure to chemicals, e.g. pesticides for residents living in close proximity to agricultural fields or PFAS and alternatives such as GenX, will promote the use of HBM for policy making. This may involve targeted HBM studies as well as suspect screening analyses.

Portugal's ambition is to develop better communication tools and to establish a national HBM-platform that will promote interaction between the relevant players particularly those with in-house analytical capacity, scientists, risk assessors, risk managers, citizens, and policy makers. HBM4EU output, including data generated from Portuguese samples collected in the national aligned studies, will be instrumental to show stakeholders and policy makers how useful the HBM activities are, in order to generate knowledge about citizens' exposure to chemicals and its potential impact on their quality of life and health. PARC will also be a driving force for the HBM activities in Portugal.

Switzerland urgently wants to establish reference values for the general population on chemicals of concern, notably metals, dioxins and other persistent legacy chemicals, as well as selected pesticides. The national study on health and bio surveillance will be launched in 2023 based on the results of a pilot phase. Another future endeavor is to establish a biobank and database that can be used in health-related matters.

Group 2 countries' vision for the future also includes HBM. Slovak Republic's National Action Plan for Environment and Health (NEHAP V) includes a long term goal of implementing a national HBM programme which will include prioritisation of chemicals included in HBM4EU. The first draft of the Slovakian national HBM programme was officially approved by the Ministry of Health of the Slovak Republic in May 2021.

The Ministry of Health in Cyprus recently approved HBM as a one of its priorities for public health & social rights protection and included it in the CY National strategy for EU affairs. European efforts to advance HBM have complemented those nationally and provided funding to promote and develop work and translate results into policy and communicaiton efforts.

Israel will incorporate the next round of the national programme into the PARC aligned studies, however, ensuring government funding remains a challenge.

In Spain, HBM is included in the Strategic Action Plan for Health and Environment. Current work is focused on implementation of the strategic plan and HBM at national level in the next years with campaigns designed to address exposure to chemical substances of interest in Spain (PFAS and mercury).

The countries in Group 3 cited their continuous support and promotion of HBM – with all countries except Sweden citing PARC as a core contributor to the sustainability of HBM.

Most of the countries projected that, in the future, environmental monitoring and HBM will be better linked to support the overarching goals of the European Commission (e.g. Zero Pollution ambition).

PARC will also provide the opportunity to utilise and build on the achievements of HBM4EU; further develop environmental and human monitoring and the continuous support for science-based policy making. The continuation of HBM activities at European level in the frame of PARC will be of great importance to sustain the national or regional programmes for countries in Group 3. In Germany, the GerES VI pilot study has been finalised and the main study will start mid-2023. Sampling and the analysis of samples of the German Environmental Specimen Bank is ongoing. The experiences from the different work areas within HBM4EU as well as from the coordination of the entire work programme are currently being evaluated for the successful implementation of planned PARC activities. In Belgium, the preparation of the 5th FLEHS study is ongoing, and the start is foreseen for 2022. The co-financing, knowledge exchange and participation in scientific research

in PARC is essential in the sustainability of the Flemish HBM programme. Moreover, the next FLEHS campaign will be embedded in a Flemish knowledge hub 'environment and health' with strong collaboration between the policy domains on environment and health. In addition, the growing acknowledgement of the importance of HBM for chemicals policy at EU level and the continuation of HBM4EU activities under PARC are important elements for the sustainability of both the Flemish and the Walloon HBM programmes in Belgium.

In Austria, great progress has been made regarding HBM. The national platform for human biomonitoring was able to successfully establish itself as a National Hub in HBM4EU. The sustainability of the activities is reflected in the fact that the National Hub in PARC unites more than twice as many partners as in HBM4EU. Also, for the Czech Republic, the PARC partnership will play a significant role in strengthening collaborations at the national and European level, setting up standards and establishing processes as well as motivating partnership members in participating in surveys.

The participatory organisational framework of HBM4EU, as well as in the French National Hub, is particularly inspiring for continued French National Biomonitoring programme as foreseen from January 2023 onwards. Santé publique France will be at the centre of the design and management of population surveys to produce future French biomonitoring data, that will help achieve the public health objectives set out in the agency, and also continue supporting French and European research, risk assessors and managers, occupational prevention experts, and policy makers (Rambaud1 et al., 2020). It will coordinate the contributions from other agencies and research institutes. PARC will ensure the continuation of the HBM4EU biomonitoring platform and this is essential for exposure assessments of the European population.

While Sweden mentions that PARC is not crucial for the sustainability of the Swedish national HBM-program, there is an ongoing discussion within the steering group about the need for an extended HBM-program where a health examination survey (HES) could also be included. A national infrastructure for data collection would be beneficial, leading to less fragmentation and a better way to join aligned studies in projects as HBM4EU and PARC.

Overall, the status and use of HBM in the future is varied for countries in Group 1; ranging from addressing specific environmental exposures to developing reference values. Many countries future plans include using the platform provided by HBM4EU to:

- develop and implement further HBM programmes.
- implement HBM in vulnerable sub-groups where an issue has been identified.
- establish biobanks and health related databases which can be utilised as required.
- utilise HBM results to develop population guideline reference values.
- include biomonitoring in government's objectives to develop/implement preventative public health measures.
- continue collaboration with EU countries to survey populations' exposure to environmental pollutants.
- develop better communication tools and establish national HBM platforms to promote interactions between relevant parties such as scientists, laboratory analysts, risk managers, policy makers etc.
- encourage engagement in PARC which can become the driving force for undertaking HBM activities.

The countries in Group 2 are focussed on including HBM in their various national action plans, using the prioritisation of chemicals in HBM4EU as a guide and implementing initiatives through the PARC project. In Group 3, the countries cited their continuous support and promotion of HBM and their belief that PARC will contribute to its longevity and sustainability. Given that they already have HBM programmes in place, their plans include linking environmental and human biomonitoring data to support the overarching goals of the European Commission. PARC is viewed as an initiative which can support this goal as well as the sustainability of their national programmes and the associated collaborations and partnerships.

The co-financing available through the PARC project is important and in cases essential for the sustainability of national initiatives. There is the appetite to further align national HBM programmes thereby strengthening the exposure assessment of European populations to chemicals component of the PARC project. Regardless of the status of HBM in countries in HBM4EU, all are unified in the desire to strengthen the scientific basis for chemical risk assessment and transition to the next phase (of evidence-based RA) through PARC.

4. Summary

4.1. How HBM can be used to raise awareness

The utilisation of HBM data to raise awareness is mostly affiliated with countries in Groups 1 and 2. Most countries in all the groups stated that environmental and public health issues/disasters resulted in either raising awareness for HBM or getting the attention of policy makers.

The Netherlands and Italy cited the industrial emission of PFAS, the issue of higher levels of dioxin in Baltic fish and fly ash from incinerators in Finland and England, the health impact in oil shale sector in Estonia and disasters like the collapse of the giant wall reservoir of an aluminium factory in Hungary all raised the awareness of HBM and its value in risk assessment and public health protection.

Similar claims of using environmental and public health issues for HBM awareness were also made by countries in Group 2. In Cyprus and Israel, cotinine measurement in children was used to assess the exposure to environmental tobacco smoke followed by intervention campaign and media coverage. It was also stated that HBM data was used for crisis management in Cyprus.

Environmental/public health issue or minor disaster should not be the only driver to gain the attention of policy makers. Other methods of raising awareness can be explored, such as in the case of Portugal where human biomonitoring workshops and focus groups were organised to promote networking possibilities and knowledge exchange and media appearances can be utilised.

4.2. Factors that influence the establishment of HBM programmes for policy development

Some countries in all three groups highlighted the inclusion of HBM module into HES as a prerequisite for establishing a HBM programme. Countries in Group 3 stated that the inclusion of a HBM module into a national health survey progressed to a wider monitoring programme that included human health, nutritional behaviour and exposure. The combination of environmental and human health monitoring can also be a good foundation.

Other factors that influence the establishment of HBM programmes include:

- The use of HBM data for risk assessment.
- HBM studies focused on specific populations or pollutants to gain better understanding of exposure to environmental chemicals.
- The use of HBM data to support scientific evidence for human exposure in chemical restriction.
- Monitoring the public health impacts of new chemicals coming into the market daily to inform chemical regulations.

- Greater collaboration between ministries and government departments to facilitate coordinated data analysis and interpretation and the timelier publication of results to inform policy development/implementation.
- The inclusion of HBM in a national chemical strategy or action plan.
- Exploring the public interest in the environment via focus groups and media campaigns.
- Monitoring and publishing the success of using HBM data to minimize risks.
- The lag between exposure and evaluation of internal exposure using HBM means that the evaluation is always retrospective 'after the event'. Many studies create a biobank of human as well as environmental samples, these can be used for trend analysis or setting baselines.
- HBM data are an integration of all routes and sources of exposure, however, for policy development it is necessary to understand the source of exposure and the use of environmental matrices should not be ignored. There are many occasions where HBM data would or should not be recommended.

4.3. Strengths and limitations

Countries within HBM4EU that have used HBM data either for raising awareness, policy development or chemical management have been assessed using the multi-case qualitative analysis. Each NHCP self-selected their group: this means they chose to describe their national HBM structures as:

- No national programme of HBM to support policy but more *ad hoc* reacting to needs as they arise mainly for awareness raising to get the attention of policy makers or the public.
- National hubs in their infancy with the use of HBM data in policy development.
- Well established national structures which are used to support chemical management at a national level.

Review of the narratives using the templates (as published in National hubs page-HBM4EU), shows that the division between the groups is blurred, not unexpectedly. It is also important to note that progression from Group 1 to 2 or 2 to 3 is not implied, this was just a means of dividing the NHs to allow for some exploration of the issues.

The breadth of HBM related studies reported in Group 1 are reflected in Group 3 as well as Group 2, this means that it is not a prerequisite to have a national programme before studies can be carried out. However, there may be more opportunity to carryout repeat surveys by those in Group 3 as the infrastructure is already present. Those in Group 3 also emphasised that the continuation of these national programmes is still dependent on competitive justification for funding and is not guaranteed-thus they have similar challenges to those in Group 2 or 1.

Also, by describing and understanding the relevant factors that led each country to choose their different groups, we have uncovered common themes in each of the three groups and identified factors that could be beneficial to all countries.

4.4. Recommendation

Narrative research collects and reviews experiences by those writing the narrative. The aim of this paper was to describe the experience of the NHCPs focusing on the use of HBM data in policy development or awareness raising in the countries which took part in HBM4EU. It is not an exhaustive exploration but a means to show how different structures and levels of engagement can be used to further chemical management. As with most qualitative research, the information gathered and analysed are based on personal interpretations which may be subjective. One of the issues faced is the interpretation of the simple template used to gather the evidence. The templates could be tested in one or two countries before the main study. This may help reduce misinterpretation of the data required. Such a study should be planned early in conception of a multi-national study such as the one described in this paper.

Consequently, intrinsic methods/steps on the utilisation of HBM data may sometimes appear vague or inconsistent. However, the narratives provided a holistic view of the utilisation and benefits of HBM data for policy support or raising awareness. We also acknowledge that one-time, reflective summaries may not fully capture key information or issues that are required. Additional research methods are required to provide a more detailed/robust outline of the steps/process needed for policy support. For example, the incorporation of other qualitative research methods such as focus groups or interviews with key players and monitoring/evaluating the level of awareness of HBM for policy development among policy makers and the investigation of how HBM4EU has impacted on the behaviour of European citizens or Public Health professionals may give additional insight. These can be explored in subsequent projects e.g PARC.

5. Conclusion

The value of a HBM programme or use of HBM data in any country has been extensively demonstrated throughout this article. Although challenges and barriers still exist, most countries within Europe are already conversant with the benefits and opportunities of HBM.

Many HBM and other population-based studies report the difficulty in recruitment - this could be those at-risk groups, such as lower socio-economic status, young children or the elderly. Thus, it is imperative to interpret the data in line with the demography covered in the study. Additionally, sampling frames that focus on "hotspots" and at-risk groups are important. However, there is a need to have baselines for population levels of exposure biomarkers, these allow us to develop reference values. HBM reference values also support communication to the public of their exposure level and allows public health professional to monitor exposures reflecting on the potential effects of such exposures.

Unsurprisingly, funding is a major barrier to the establishment of national HBM programs. However, there are many other factors, from lack of appropriate expertise to (lack of) public engagement, that are important. HBM4EU has had a positive impact on the level of awareness crucially for the utility of HBM data. Understanding the true cost of *ad hoc* or national programmes balanced with the impact of reducing exposure to chemicals would be a powerful message to support the ever-present battle for funding.

To propel HBM onto political agendas, countries must continue to raise their profiles, strengthen and extend engagement with relevant ministries and groups as highlighted in this article. The NHs established in HBM4EU supply an excellent basis for their further development in PARC.

The HBM boat has definitely left the harbour armed with many provisions; however, the journey is a long one and requires resilience and tenacity to ensure that the full benefits are achieved at the end.

Appendix 1

Group 1 National Hub Template

Introduction:	
• Background information on the evolution and status of HBM in your country e.g COPHES/DEMOCOPHES and EU programs.	300 words maximum for only the Introduction.
Main text - Results and Discussion ENSURE YOUR NARRATIVES ARE REFERENCED AS FAR AS POSSIBLE • Description of issue(s) which have resulted in the raising of awareness. • Include brief description of sample population, substances of concern and whether local/regional/national. • Give example of cases and specific studies • Description of HBM programme if it exists e.g. implementation of a HBM module into HES or relevant other activities funded by the government. • Describe which ministries (Environment, Health etc.)/policy makers and stakeholders involved/steering/financing the HBM programme. • Give examples - specific chemicals or outcomes. • Steps/processes needed or used to get the attention of policy makers. • Describe barriers e.g funding; challenges e.g. participant recruitment; opportunities e.g. enhancing cross government working and linking of env data with exposure measurements currently at play in your country with regards to HBM. • Have any of these barriers been addressed by HBM4EU? If yes - describe. • Other players who would be beneficial in raising awareness and working together to promote HBM	
Future plans - • Are there plans to use HBM data in the future for policy or awareness - give clear examples. Will the data from HBM4EU be used?	

Appendix 2

Group 2 National Hub Template

Introduction:	
Background information on the evolution and status of HBM in your country.	300 words maximum for only the Introduction.
Main text - Results and Discussion ENSURE YOUR NARRATIVES ARE REFERENCED AS FAR AS POSSIBLE • Description of HBM programme if it exists e.g. implementation of a HBM module into HES or development of a standalone HBM programme. • Examples of HBM data for policy development. Please specify chemicals or chemical groups. • Describe which ministries (Environment, Health etc.)/policy makers and stakeholders involved in/steering/financing the HBM programme. • Describe steps/processes used in involving policy makers. • Is HBM included in their business/strategic/action plan. • State which ministry is HBM data reported to or its being utilised. • Describe barriers e.g funding; challenges e.g. participant recruitment; opportunities e.g. enhancing cross government working and linking of env data with exposure measurements currently at play in your country with regards to HBM. • Have any of these barriers been addressed by HBM4EU? If yes - describe. • Elaborate on issues which propelled the HBM data of choice e.g. disaster, pollution, incidence/prevalence of a health-related issue	
Future plans - • Are there plans to increase the use of HBM data in the future for policy give clear examples. • Will the data from HBM4EU be used? • Has HBM4EU re-enforced the need for a National programme? • What are your future plans? • Do you think PARC will be crucial to the development of your HBM programme?	

Appendix 3

Group 3 National Hub Template

Introduction:	
Background information on the evolution and status of your National HBM programme in your country. Include year of establishment -Who pays for the programme of work? Give web links.	300 words maximum for only the Introduction.
Main text - Results and Discussion ENSURE YOUR NARRATIVES ARE REFERENCED AS FAR AS POSSIBLE • Describe which ministries (Environment, Health etc.)/policy makers and stakeholders involved/steering/financing the HBM programme. • Involvement with HBM and Steps/processes used in involving policy makers. • Is HBM included in their business/strategic/action plan. • State which ministry is HBM data reported to or it is being utilised. • Describe barriers e.g funding; challenges e.g. participant recruitment; opportunities e.g. enhancing cross government working and linking of env data with exposure measurements currently at play in your country with regards to HBM or other things of note. • Have any of these barriers been addressed by HBM4EU? If yes - describe. • Elaborate on issues which propelled the establishment and sustainability of your HBM programme.	

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Introduction:
• Detailed information of HBM priority substance used for policy development e.g. disaster, pollution, incidence/prevalence of a health-related issue. • Give examples where the work has led to policy implementation, monitoring, or control of chemical exposures etc • Have HBM or other monitoring activities been linked or adapted. Give examples in detail. • Other players who would be beneficial in the continued support of HBM at a governmental level and working together to promote HBM in your country. • Have you used HBM4EU data e.g newsletter, videos to support policy? Future Plans • Ways/process used in maintaining the programme • What are your future plans? • Do you think PARC will be crucial to the sustainability of your HBM programme?

Appendix 4

Guideline for group 1 Lead:

NH narratives should include the following	Group Lead will then collate and include the following
Introduction:	
• Background information on the evolution and status of HBM in your country.	• State purpose of group 1 narrative. • On background information of HBM, you can use infographics to state how/when/why it evolved (maybe via HBM4EU) for each country.
Main text - results and discussion ENSURE YOUR NARRATIVES ARE REFERENCED AS FAR AS POSSIBLE	
• Description of issue(s) which have resulted in the raising of awareness. • **Include brief description of sample population, substances of concern and whether local/regional/national.**	• Write HBM data (chemical) of interest. Indicate if it is in the list of priority substances. Identify and compare case studies.
• Description of HBM programme **if it exists** e.g. implementation of a HBM module into HES	• State countries which have HBM programmes and countries which have implemented a HBM module into HES or countries which have done both.
• List ministries (Environment, Health etc.) and stakeholders advocating for HBM.	• Analyse if it is the same for all the countries or different. • Again, an infographic may be possible- % with ministries 5 with other stakeholders etc
• Policy makers involvement with HBM or level of awareness of HBM. • Give examples - specific chemicals or outcomes	• Analyse level of awareness, policy makers involvement (look at what they are mostly interested in) for each country. • Summarise with infographics if need be.
• Steps/processes needed or used to get the attention of policy makers.	• Analyse steps used by each country. Compare countries and see if a group of countries use the same method.
• Describe barriers e.g funding; challenges e.g. participant recruitment; opportunities e.g. enhancing cross government working and linking of env data with exposure measurements currently at play in your country with regards to HBM.	• List all barriers, challenges, and opportunities for all countries. • Compare and state which is more prominent. • See if you can detect why these challenges, opportunities and barriers arise.
• Have any of these barriers been addressed by HBM4EU? If yes - describe.	•See if there are any patterns emerging
• Other players who would be beneficial in raising awareness and working together to promote HBM	• List players and state why they were suggested.
Future plans -	• Evaluate the level of future work
• Are there plans to use HBM data in the future for policy or awareness - give clear examples. Will the data from HBM4EU be used?	• How may hubs will use HBM4EU data in the future?

Appendix 5

Guideline for group 2 Lead:

NH narratives should include the following	Group Lead will then collate and include the following:
Introduction:	
Background information on the evolution and status of HBM in your country.	• State purpose of group 2 narrative. • On background information of HBM, you can use infographics to state how/when/why it evolved (maybe via HBM4EU) for each country.
Main text - results and discussion ENSURE YOUR NARRATIVES ARE REFERENCED AS FAR AS POSSIBLE	
• Description of HBM programme if it exists e.g. implementation of a HBM module into HES or development of a standalone HBM programme. • Examples of HBM data for policy development.	• State countries which have HBM programmes and countries which have implemented a HBM module into HES or countries which have done both. • Compare HBM data of all countries and policies. Use infographics if needed. • These could be the case studies
• List ministries (Environment, Health etc.) and stakeholders advocating for HBM.	• Analyse if it is the same for all the countries or different. • Present the ministries and stakeholders in a pictorial format.

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(*continued*)

NH narratives should include the following	Group Lead will then collate and include the following:
• Describe barriers e.g funding; challenges e.g. participant recruitment; opportunities e.g. enhancing cross government working and linking of env data with exposure measurements currently at play in your country with regards to HBM. • Have any of these barriers been addressed by HBM4EU? If yes - describe. • Elaborate on issues which propelled the HBM data of choice e.g. disaster, pollution, incidence/prevalence of a health-related issue Future plans - • Are there plans to increase the use of HBM data in the future for policy give clear examples. • Will the data from HBM4EU be used? • Has HBM4EU re-enforced the need for a National programme? • What are your future plans?	• List all barriers, challenges, and opportunities for all countries. • Compare and state which is more prominent. • See if you can detect why these challenges, opportunities and barriers arise. • See if there are any patterns emerging • Write HBM data (chemical) of interest. Indicate if it is in the list of HBM4EU priority substances. Identify and compare case studies. • Evaluate the level of future work • How may hubs will use HBM4EU data for policy in the future? • Will this build on current platforms or start from scratch

Appendix 6

Guideline for Group 3 Lead

NH narratives should include the following	Group Lead will collate and include the following
Introduction: Background information on the evolution and status of your National HBM programme in your country. Include year of establishment -Who pays for the programme of work? Give web links. Main text - Results and Discussion ENSURE YOUR NARRATIVES ARE REFERENCED AS FAR AS POSSIBLE	• State purpose of group 3 narrative. • On background information of HBM, you can use infographics to state how/when/why it evolved (maybe via HBM4EU or not) for each country.
• List ministries (Environment, Health etc.) and stakeholders involved in the HBM programme.	• Analyse if it is the same for all the countries or different. • Present the ministries and stakeholders in a pictorial format.
• Policy makers involvement with HBM and Steps/processes used in involving policy makers.	• Analyse policy makers involvement (look at what they are mostly interested in) access level of commitment for each country. • List the steps/processes used for their involvement and compare similarities with other countries. • Summarise with infographics if need be.
• Describe barriers e.g funding; challenges e.g. participant recruitment; opportunities e.g. enhancing cross government working and linking of env data with exposure measurements currently at play in your country with regards to HBM. • Have any of these barriers been addressed by HBM4EU? If yes - describe. • Elaborate on issues which propelled the establishment and sustainability of your HBM programme. • Detailed information of HBM priority substance used for policy development e.g. disaster, pollution, incidence/prevalence of a health-related issue. • Give examples where the work has led to policy implementation, monitoring, or control of chemical exposures etc	• List all barriers, challenges, and opportunities for all countries. • Compare and state which is more prominent. • See if you can detect why these challenges, opportunities and barriers arise. • See if there are any patterns emerging • Write HBM data (chemical) of interest. Indicate if it is in the list of priority substances. Identify and compare case studies. • Compare HBM data of all countries and policies. Use infographics if needed. • List if it was a health-related issue or environmental or occupational.
• Other players who would be beneficial in the continued support of HBM at a governmental level and working together to promote HBM in your country.	• List all and compare similarities.
Future Plans • Ways/process used in maintaining the programme • What are your future plans?	• List all and compare similarities.

Appendix 7

Activities of countries prior and within HBM4EU and ministries involved in HBM

Country	EU HBM activities prior to HBM4EU -Research	Cohorts/studies in HBM4EU	Ministries/agencies involved
Denmark	ESBIO/COPHES/DEMOCOPHES many EU projects related to genotoxicology, EDC, POPs and methods development	MiniPub, OCC, DEMOCOPHES	EPA, Food Agency; Labour Inspection, Research and Innovation; Board of Health
Estonia	Human Biomonitoring in the Oil Shale Industry Area in Estonia—Overview of Earlier Programmes and Future Perspectives		Ministry of Social Affairs, Health Board, Ministry of the Environment
Finland	EU project EDC Mix Risk Involvement in several EU-projects related to the risks of nanomaterials	FINRISK, FinHealth, Occupational	Ministry of Social Affairs and Health
Hungary	ESBIO/COPHES/DEMOCOPHES	DEMOCOPHES Specimen, In Air Quality	Ministry of Human Capacities and National Public Health Center
Iceland	Arctic monitoring program	Mercury, nutrition	Health and Environment
Ireland	ESBIO/COPHES/DEMOCOPHES		Environmental Health Service
Italy	National activities and EU projects	Analytics, statistics	Northern Adriatic cohort, occupational
Lithuania	Children		Ministry of Health
Luxembourg	ESBIO/COPHES/DEMOCOPHES	DEMOCOPHES Occupational	Ministry of Health
Netherlands	Metals, PFAS, Pesticides, occupational	Occupational, Specimen, Dutch Youth cohort	Ministry of Health, Welfare and Sports, occupational studies fall under the Ministry of Social Affairs
Norway	National platform since 1980 and EU projects. COPHES	MobA, IES, NEBII	Ministry of Health and Care

(*continued on next page*)

(*continued*)

Country	EU HBM activities prior to HBM4EU -Research	Cohorts/studies in HBM4EU	Ministries/agencies involved
Poland	ESBIO/COPHES/DEMOCOPHES	Occupational	Lodz
Portugal	ESBIO/COPHES/DEMOCOPHES Multiple national projects	Mercury, INSEF-ExpoQuim occupational	Ministries of Health, Environment and Science
Slovenia	ESBIO/COPHES/DEMOCOPHES EU Project: PHIME, CROME	DEMOCOPHES, HBM, SLOCRP	Chemicals Office (CORS) as part of Ministry of Health
Switzerland	DEMOCOPHES	Health Study	Swiss Federal Office of Public Health (FOPH), Food safety and vet (FSVO), Environment (FOEN)
United Kingdom	ESBIO/COPHES/DEMOCOPHES	Occupational	Health and Safety Executive, UKHSA, EA, Department of Health & Social Care (DHSC) and DEFRA

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Assessing the impact of coexposure on the measurement of biomarkers of exposure to the pyrethroid lambda-cyhalothrin in agricultural workers

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ABSTRACT

There are few published data on the impact of combined exposure to multiple pesticides (coexposure) on levels of biomarkers of exposure in workers, which may alter their toxicokinetics and thus the interpretation of biomonitoring data. This study aimed to assess the impact of coexposure to two pesticides with shared metabolism pathways on levels of biomarkers of exposure to pyrethroid pesticides in agricultural workers. The pyrethroid lambda-cyhalothrin (LCT) and the fungicide captan were used as sentinel pesticides, since they are widely sprayed concomitantly in agricultural crops. Eighty-seven (87) workers assigned to different tasks (application, weeding, picking) were recruited. The recruited workers provided two-consecutive 24-h urine collections following an episode of lambda-cyhalothrin application alone or in combination with captan or following tasks in the treated fields, as well as a control collection. Concentrations of lambda-cyhalothrin metabolites – 3-(2-chloro-3,3,3-trifluoroprop-1-en-1-yl)-2,2-dimethyl-cyclopropanecarboxylic acid (CFMP) and 3-phenoxybenzoic acid (3-PBA) – were measured in the samples. Potential determinants of exposure established in a previous study, including the task performed and personal factors were documented by questionnaire. Multivariate analyses showed that coexposure did not have a statistically significant effect on the observed urinary levels of 3-PBA (Exp (β) (95% confidence interval (95% CI)): 0.94 (0.78–1.13)) and CFMP (1.10 (0.93–1.30). The repeated biological measurements ("time variable") – defined as the within-subjects variable – was a significant predictor of observed biological levels of 3-PBA and CFMP; the within-subjects variance (Exp(β) (95% (95% CI)) for 3-PBA and CFMP was 1.11 (1.09–3.49) and 1.25 (1.20–1.31). Only the main occupational task was associated with urinary levels of 3-PBA and CFMP. Compared to the weeding or picking task, the pesticide application task was associated with higher urinary 3-PBA and CFMP concentrations. In sum, coexposure to agricultural pesticides in the strawberry fields did not increase pyrethroid biomarker concentrations at the exposure levels observed in the studied workers. The study also confirmed previous data suggesting that applicators were more exposed than workers assigned to field tasks such as weeding and picking.

1. Introduction

The assessment of risks associated with pyrethroid exposure is among the priorities of major government agencies such as Health Canada, the United States Environmental Protection Agency (U.S. EPA), and the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), as several *in vitro* cellular and *in vivo* animal studies have shown that exposure to repeated high doses of these chemicals induces early biological alterations, such as oxidative stress, immune alterations, and endocrine disruption (Barrón Cuenca et al., 2019; Costa et al., 2013; El Okda et al., 2017; Lee et al., 2020; Ravula and Yenugu, 2021; Shearer et al., 2019; Wang et al., 2016; Zepeda-Arce et al., 2017). Cases of acute intoxication or incidents in workers exposed to pyrethroids have also been reported, including respiratory and neurological symptoms (Amoatey et al., 2020; Curl et al., 2020; de Graaf et al., 2022; Ismail et al., 2017; Lucero and Muñoz-Quezada, 2021; Mattila et al., 2021; Ratanachina et al., 2020). The U.S. EPA has classified some pyrethroids, including permethrin, as possibly carcinogenic to humans, based on observations of (benign) lung and liver tumors in mice exposed to high doses, although these findings are not supported

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by available epidemiological studies (Burns and Juberg, 2021; De Roos et al., 2021; U.S. EPA 2018). It is therefore important to develop and apply tools to properly evaluate and control exposure to these contaminants, which are still insufficiently evaluated in some agricultural workplaces. To assess internal exposure to pesticides such as pyrethroids, urinary measurements of metabolites is considered a preferred tool (Arcury et al., 2018; Buchholz et al., 2021; Curl et al., 2021; Maule et al., 2019).

However, our latest work has raised the issue of multiple exposures to several pesticides and the impact that this coexposure (concomitant or combined exposure on the same day or on sequential days (OECD, 2018)) could have on the interpretation of biomonitoring data used to assess exposure to pyrethroids (Bossou et al., 2020; Bouchard et al., 2016; Khemiri et al., 2017; Ratelle et al., 2015a, 2015b). Currently, there is a lack of data on the impact of multiple pesticide coexposure on levels of exposure biomarkers to commonly used pyrethroids in agricultural settings.

Overall, the data published in the scientific literature on the impact of coexposure on the biological behavior of pyrethroids and their metabolites used as biomarkers of exposure are very limited in real-life context such as agricultural settings. Experimentally or in controlled studies, some studies reported decreased urinary excretion of pyrethroid exposure biomarkers in animals or volunteers coexposed with organophosphate insecticides (Hirosawa et al., 2011; Sams and Jones, 2011; Wielgomas and Krechniak, 2007). However, the doses administered were relatively high in relation to worker exposure levels and the exposure scenarios (pyrethroid/organophosphate coexposure) were not very representative of the exposure context of workers. Recently, our team experimentally evaluated the influence of coexposure to the fungicide captan on the kinetic profiles of the main metabolites of the pyrethroid lambda-cyhalothrin in a rat study, 3-(2-chloro-3,3,3-trifluoroprop-1-en-1-yl)-2,2-dimethyl-cyclopropanecarboxylic acid (CFMP), 3-phenoxybenzoic acid (3-PBA) and 4-hydroxy-3-phenoxybenzoic acid (4-OH3PBA) (Bossou et al., 2020). The *a priori* hypothesis was that captan could interfere with the CYP450 metabolism pathway of pyrethroids or the excretion mechanisms (Paolini et al., 1999) in a dose-dependent manner. More specifically, lambda-cyhalothrin metabolism to its main metabolites used as biomarkers of exposure (CFMP and 3-PBA) is catalyzed by CYP450 enzymes also implicated in the metabolism of captan (Kaneko, 2011; Paolini et al., 1999; Scollon et al., 2009). The results of the animal study of Bossou et al. (2020) showed that captan and lambda-cyhalothrin coexposure resulted in a trend toward lower levels of metabolite excretion, in particular on the benzyl metabolite pathway leading to 3-PBA formation. This was observed in the higher dose group exposed to the "Lowest-Observed Adverse Effect Level" (LOAEL) of 12.5 mg/kg bw/day reported by the U.S. EPA (2004) but not in the lower exposure group exposed to the "No-Observed Adverse Effect Level" (NOAEL) of 2.5 mg lambda-cyhalothrin/kg bw/day. Again, these animal results cannot be directly extrapolated to humans for interpretation of biomonitoring data because the experimental exposure doses were much higher than those estimated under human occupational exposure conditions (<1 μg/kg bw/day) (Chester et al., 1992). In addition, there may be interspecies differences in toxicokinetics.

In real-life exposure situations in workers, the impact of coexposure to different pesticides in workers on variability in levels of biomarkers of exposure, relative to other factors, remains to be verified. In order to properly interpret the significance of a measurement of these exposure biomarkers, it becomes necessary to fully understand the influence of parameters such as coexposure to other pesticides. We hypothesize that, at a certain exposure level, combined exposure to multiple pesticides may alter biomarker concentrations in urine used to assess internal exposure in agricultural workers and hence may have an impact on the interpretation of biomonitoring data. This research thus specifically aimed to evaluate the impact of coexposure on biomarkers of exposure to pyrethroid pesticides in agricultural workers and to identify the contribution of this factor to the variability in biological monitoring data. The pyrethroid lambda-cyhalothrin (LCT) and the fungicide captan were used as sentinel pesticides, since they are widely sprayed concomitantly on agricultural crops and share common metabolism pathways (Paolini et al., 1999).

2. Methods

2.1. Study population, crops and targeted pesticide active ingredients

A biomonitoring study was conducted in agricultural workers exposed to lambda-cyhalothrin alone or in combination with captan. Strawberry workers were targeted because this crop represents an important production in Quebec; it involves a large number of workers and pyrethroids and fungicides are widely used in these fields (MAPAQ, 2020, 2021). The workers were recruited using the Quebec Directory of Horticultural Producers obtained from the Quebec Fruit and Vegetable Growers' Association. From this list of farms organized by city and crop type, strawberry farm owners within a 100 km radius from the University of Montreal were contacted by telephone (using a standard text) to assess their willingness to solicit their field workers to participate in the study.

A total of 87 workers assigned to different tasks (application, weeding, strawberry picking) were recruited, and evaluated under their usual working conditions. The target workers were exposed to lambda-cyhalothrin alone, or alternatively, in combination with captan. In the case of combined exposure in applicators, lambda-cyhalothrin and captan were mixed and spayed at the same time; in the case of field workers, they entered an area previously treated with both chemicals. This sample size was based on the number of workers used in a previous study, which assessed the impact of various personal factors and exposure determinants on the kinetics of biomarkers of exposure to cypermethrin in vegetable crop workers by statistical multivariate analysis (Ratelle et al., 2016).

The recruitment strategy used for this study was the same as that described in Bossou et al. (2022). Eligibility criteria were: i) the worker anticipated being exposed to formulations containing the active ingredient lambda-cyhalothrin (Matador®, Silencer®, Demand CS®, Warrior®) alone or in combination with captan fungicide (Captan®, Supra Captan 80 WDG®, Captan 80-WP®, Maestro®) during the summer as part of their normal activities; ii) they were willing to provide a 24-h urine collection prior to application (-24 - 0 h prior to application) and two consecutive 24-h urine collections (0–24 h and 24–48 h) following the onset of an exposure episode (after spraying the pesticide or working in a treated field).

Subjects who participated in the study signed a free and informed consent form after receiving all necessary information about the project. Each participant was free to withdraw at any time. The study protocol, consent form, and other relevant documents were approved by the Clinical Research Ethics Committee (CERC) of the Université de Montréal. The anonymity of the subjects was also respected by coding the samples.

2.2. Urine collections and measurement of exposure biomarkers

Recruited workers were asked to provide a first full 24-h urine collection prior to an exposure episode to establish baseline exposure levels as well as two consecutive 24-h urine collections following an episode of lambda-cyhalothrin spaying alone or in combination with captan or tasks in treated fields (weeding and strawberry picking). Each 24-h samples were collected in 1.5 L polypropylene Nalgene® bottles. Workers were asked to write down the date and time of each micturition on the identification label affixed to the Nalgene collection bottles. The samples collected were kept in coolers with ice packs provided by our team. Samples were picked up on a daily basis at the workplace by a member of our research team and directly brought to the laboratory

where urine volumes were measured using graduated cylinders. On days when the team were not able to arrive before the end of the workday, workers transferred samples into the farm cold room until our team members arrived. Two aliquots of 120 mL per collection bottle were prepared and placed in polypropylene Sarstedt tubes for storage at – 20 °C until analysis.

All participants were well-informed of the importance of complete urine collections without omissions. They were also compensated for their time and efforts to ensure compliance to the protocol and limit the proportion of incomplete urine collection. However, one participant failed to collect all his 24 h-urine samples and was excluded in analyses.

The urine samples (5 mL) were subjected to an enzyme hydrolysis with β-glucuronidase/arylsulfatase enzyme to obtain the sum of free and glucurono- and sulfo-conjugated metabolites followed by solid-phase extraction where the metabolites were recovered in methanol (1 mL). The concentrations of lambda-cyhalothrin metabolites used as biomarkers of exposure, 3-(2-chloro-3,3,3-trifluoroprop-1-en-1-yl)-2,2-dimethyl-cyclopropanecarboxylic acid (CFMP; otherwise known as ClF_3CA) and 3-phenoxybenzoic acid (3-PBA), were then analyzed in the methanolic extracts using a high performance liquid chromatography coupled to triple quadripole (QQQ) mass spectrometry (UHPLC-MS/MS) method validated in our laboratory and published elsewhere (Bossou et al., 2022; Khemiri et al., 2018).

The limit of detection (LOD) was calculated to be 0.7–2.5 pmol/mL of methanolic extract for 3-PBA and 3.9–6 pmol/mL for CFMP. The limit of quantification (LOQ), which represents the lowest level of the calibration curve that is quantified with a less than 20% error, was 6 pmol/mL and 12 pmol/mL of methanolic extract for 3-PBA and CFMP, respectively. Corresponding LOQ values in urine are 1–2 pmol/mL of urine considering that 5 mL of urine were analyzed and the residue obtained after solid-phase extraction and evaporation was redissolved in 1 mL of methanol. This method allowed quantifying 3-PBA and CFMP metabolites in 88% and 43% of the analyzed samples, respectively.

Creatinine concentrations were also measured in urine using the Jaffé method, an alkaline picric acid method with deproteinization (PAP enzymatic colorimetric assay from Boehringer Mannheim, Germany). Concentrations of lambda-cyhalothrin metabolites corrected for creatinine (μmol/mol creatinine) were then established for each sample. For values below the LOQ, they were treated using a robust method called "regression on order statistics" (ROS) (Helsel, 2005). This method was used because it is less sensitive to small sample sizes, low censoring percentages and is more resistant to non-normality in the data (Huston and Juarez-Colunga, 2009).

2.3. Questionnaires and field observations

Potential determinants of metabolite levels used as biomarkers of exposure or potential confounders were assessed by questionnaire in addition to coexposure to lambda-cyhalothrin and captan. These included the main work tasks performed, work practices and hygiene, personal protective equipment as well as personal factors and lifestyle habits (see supplementary file S1). Main questions were used in a previous work on cypermethrin exposure in workers (Ratelle et al., 2016). Members of the team also conducted field observations during the first day of exposure. On the third day post-exposure, research team members also checked the daily questionnaire responses, and participants were asked to record any missing urine collection.

2.4. Data analysis

The impact of coexposure on variations in urinary levels of exposure biomarkers was established using *linear mixed effects models* (MIXM). The focus was on 3-PBA which is the most measured metabolite of pyrethroids and CFMP which is more specific. The potential determinants of exposure established in a previous study (Ratelle et al., 2016), including the task performed and personal factors, were considered in the models. Specifically, the subject variable was set as a *random effect* and a *compound symmetry* covariance *structure among repeated measurements* was considered. The levels of CFMP and 3-PBA metabolites expressed as concentrations (μmol/mol creat.) were considered as dependent variables in the models. Since the biomarker levels showed a log-normal distribution and not normal after analysis by the Kolmogorov-Smirnov test, the exposure biomarker levels (CFMP and 3-PBA) were expressed as log-transformed values to obtain a normal distribution with constant variance.

Potential determinants of biological levels of 3-PBA and CFMP were considered, including: 1) coexposure, *i.e.* exposure to lambda-cyhalothrin alone or lambda-cyhalothrin in combination with captan; 2) main occupational task performed (application including mix preparation and equipment cleaning, weeding, or picking); 3) time since the onset of lambda-cyhalothrin spraying alone or in combination with captan ($\leq$7 days and >7 days); and 4) farm size ($\leq$10 workers or >10 workers). Potential confounding variables considered in the models included age (log-transformed continuous variable in years), body mass index (log-transformed continuous BMI), ethnicity (Caucasian or Latino American), education (primary/high school or college/university), alcohol use (yes/no during the study period), cigarette smoking (yes/no), ibuprofen or acetaminophen use, other medication use.

Associations between biomarker levels (3-PBA or CFMP metabolites) in urine of farmworkers and potential determinants of exposure including the influence of coexposure or potential confounding factors were initially assessed in univariate models (explanatory variables considered one by one in the models). Multivariate models were then constructed by first inserting all variables and then sequentially subtracting those that did not contribute to the model according to the Akaike's information criterion (AIC) following the approach proposed by Zuur et al. (2009). Only predictors and confounders contributing to the fit of the multivariate models to the data were retained in the final models. Statistical analyses were performed using SPSS plus (SPSS Inc, Chicago). The level of statistical significance for the final multivariate models was set at $p \leq 0.05$.

3. Results

3.1. Study of the impact of coexposure versus other factors on the measurement of biomarkers of exposure in workers

After a screening telephone interview in early 2019 and 2020 to identify potentially eligible workers followed by a farm visit to recruit farmers, a total of 87 workers of strawberry fields – where lambda-cyhalothrin alone or in combination with captan was sprayed –signed informed consent to participate in the study. They provided urine samples during summer 2019 and 2020. Workers were recruited from 13 farms in three regions of the Province of Quebec (Montérégie, Laurentides, Lanaudière) and the main professionals tasks performed were spraying of pesticides (lambda-cyhalothrin or lambda-cyhalothrin combined with captan), weeding or strawberry picking in a treated area. In total, 70 of the recruited workers had an exposure episode to lambda-cyhalothrin alone and 49 had an exposure episode to lambda-cyhalothrin in combination with captan over the two-year study period. Some workers were thus monitored more than once since they performed different tasks in the fields (weeding versus picking) or were evaluated for more than one exposure scenario (exposure to lambda-cyhalothrin alone or in combination with captan) at different periods.

Table 1 shows the main personal characteristics of the 87 workers included in the study who provided serial urine collections following an exposure episode to lambda-cyhalothrin alone (70 workers) or in combination with captan (49 workers). A participant may have been evaluated for more than one task or more than one exposure scenario. Only 1% of all study participants were women and 82% of them were of Latin origin (from Guatemala, Honduras and Mexico); the median age was 33 years and their education level was low, with 84% reporting a high

Table 1
Characteristics of participants.

	All participants (n = 87[a])	LCT exposed (n = 70[b])	LCT + Captan exposed (n = 49[c])
Sex: n (%)			
Women	1 (1.1%)	0 (0%)	1 (2.0%)
Men	86 (98.9%)	70 (100%)	48 (98.0%)
Age: years			
Average (SD)	34.8 (10.2)	34.1 (9.91)	36.3 (10.2)
Median [Min, Max]	33.0 [20.0, 64.0]	31.5 [21.0, 64.0]	34.0 [20.0, 64.0]
Age categories: years (%)			
20–30	35 (40.2%)	31 (44.3%)	15 (30.6%)
31–40	30 (34.5%)	22 (31.4%)	22 (44.9%)
≥ 41	22 (25.3%)	17 (24.3%)	12 (24.5%)
Body weight: kg			
Average (SD)	72.3 (14.0)	71.3 (12.4)	74.3 (14.2)
Median [Min, Max]	70.0 [50.0, 127]	70.0 [50.0, 125]	71.2 [54.5, 127]
Height: cm			
Average (SD)	165 (9.38)	165 (9.34)	167 (9.19)
Median [Min, Max]	162 [150, 198]	162 [150, 198]	165 [155, 189]
BMI: kg/m^2			
Average (SD)	26.4 (4.11)	26.1 (2.97)	26.6 (4.60)
Median [Min, Max]	26.3 [17.9, 52.5]	26.2 [17.9, 34.1]	26.3 [21.2, 52.5]
BMI categories: kg/m^2 (%)			
< 24.9	30 (34.5%)	25 (35.7%)	17 (34.7%)
25–29.9	46 (52.9%)	38 (54.3%)	26 (53.1%)
≥ 30	11 (12.6%)	7 (10.0%)	6 (12.2%)
Country of birth: n (%)			
Bosnia	1 (1.1%)	0 (0%)	1 (2.0%)
Canada	15 (17.2%)	11 (15.7%)	10 (20.4%)
Guatemala	35 (40.2%)	33 (47.1%)	13 (26.5%)
Honduras	24 (27.6%)	16 (22.9%)	20 (40.8%)
Mexico	12 (13.8%)	10 (14.3%)	5 (10.2%)
Ethnicity: n (%)			
Caucasian	16 (18.4%)	11 (15.7%)	11 (22.4%)
Latino American	71 (81.6%)	59 (84.3%)	38 (77.6%)
Language: n (%)			
French	16 (18.4%)	11 (15.7%)	11 (22.4%)
Spanish	71 (81.6%)	59 (84.3%)	38 (77.6%)
Education: n (%)			
High school or less	73 (83.9%)	59 (84.3%)	39 (79.6%)
College and University	14 (16.1%)	11 (15.7%)	10 (20.4%)

[a] Number of participants included in the study.
[b] Number of participants who provided biological samples following application of lambda-cyhalothrin alone. A participant may have been evaluated for more than one task or more than one exposure scenario.
[c] Number of participants who provided biological samples following application of lambda-cyhalothrin in combination with captan. A participant may have been evaluated for more than one task or more than one exposure scenario.

school level or less as the highest education level. In addition, during the biological sampling period, 13% of the workers (11 workers out of 87) reported smoking tobacco; 16% (14 workers out of 87) reported consuming alcohol during at least one of their biological follow-ups; 13% (11 workers out of 87) mentioned taking ibuprofen or acetaminophen; and 9% (8 workers out of 87) reported taking other types of medication.

In terms of biological follow-up, considering that some workers provided more than one set of urine collections, tobacco use was reported for 10% of the biological follow-ups (i.e., for 14 of the 139 biological follow-ups); alcohol use was indicated for 12% of the biological follow-ups (16 out of 139 biological follow-ups); ibuprofen or acetaminophen use was reported in 10% of the follow-ups (14 out of 139 follow-ups) and other types of medication were reported in 7% of the follow-ups (10 out of 139 follow-ups).

The consumption of fruits, vegetables and cereals (number of servings according to Canada's Food Guide (2011)) was documented by questionnaire, but this variable was not considered in the results, as workers did not appear to be able to adequately answer these questions. Only one person reported the use of lice treatment and no participants reported the use of animal treatment or the use of pesticides for residential purposes. None of the participants reported signs or symptoms (as mentioned in supplementary file S1) that, although not specific, could be associated with exposure to this type of pesticide.

While most workers wore long pants and long-sleeved shirts and boots, only a proportion of workers wore gloves, goggles, and a hat (Table 2). Although only 45% of workers wore gloves, no significant effect of glove wearing on urinary levels of CFMP and 3-PBA was observed ($p > 0.05$). Wearing goggles and hats was also not significantly associated with urinary levels of CFMP and 3-PBA. Other personal protective clothing or equipment (mask, raincoat, scarf) were worn by only a small number of workers such that the association between wearing the latter and urinary levels of CFMP and 3-PBA was not tested.

Table 3 presents the distribution of urinary concentrations of CFMP, the more specific metabolite of lambda-cyhalothrin, for all participants and for participants stratified by exposure group, either exposure to lambda-cyhalothrin alone or in combination with captan. The results show that the distribution of CFMP values was similar for all groups in

Table 2
Protective equipment (PPE) for all participants as well as for participants stratified by exposure group (exposure to lambda-cyhalothrin alone or combination with captan).

Type of PPE	Population			P-value
	All[a]	Exposure to the LCT[b]	Exposure to LCT + Captan[b]	
Long pants: n (%)				
Yes	125 (89)	78 (90)	47 (89)	0.857
No	15 (11)	9 (10)	6 (11)	
Long sleeve shirt: n (%)				
Yes	121 (86)	72 (83)	49 (92)	0.106
No	19 (14)	15 (17)	4 (8)	
Hat: n (%)				
Yes	94 (67)	60 (69)	34 (64)	0.558
No	46 (33)	27 (31)	19 (36)	
Goggles: n (%)				
Yes	22 (16)	8 (9)	14 (26)	0.007
No	118 (84)	79 (91)	39 (74)	
Scarf: n (%)				
Yes	2 (1)	1 (1)	1 (2)	NA
No	138 (99)	86 (99)	52 (98)	
Raincoat: n (%)				
Yes	15 (11)	10 (11)	5 (9)	0.703
No	125 (89)	77 (89)	48 (91)	
Gloves: n (%)				
Yes	63 (45)	27 (31)	36 (68)	0.00002
No	77 (55)	60 (69)	17 (32)	
Boots: n (%)				
Yes	111 (79)	70 (80)	41 (77)	0.662
No	29 (21)	17 (20)	12 (23)	

[a] The n is the number of biomonitoring for all participants across all tasks (application, weeding, and harvesting) and all exposure scenarios (lambda-cyhalothrin alone versus lambda-cyhalothrin in combination with captan). A participant may have been evaluated for more than one task or more than one exposure scenario. The percentage represents the proportion of workers who reported wearing the equipment over the entire biological monitoring.
[b] The n is the number of biomonitoring for all participants across all tasks (application, weeding and harvesting) but for a given exposure scenario (lambda-cyhalothrin alone or in combination with captan). A participant may have been assessed for more than one task. The percentage represents the reported proportion of workers who reported wearing the equipment for all biological monitoring but for a given exposure scenario.

Table 3
Distribution of CFMP concentrations in urine for all participants as well as for participants stratified by exposure group (exposure to lambda-cyhalothrin alone or in combination with captan).

Time since the onset of an exposure episode (h)	Exposure group	N of samples[a]	CFMP concentration (μmol/mol creat.)						
			Geometric mean	Percentile					
				5th	10th	25th	50th	75th	95th
−24–0[b]	All	134	0.132	0.011	0.018	0.067	0.180	0.290	0.776
	LCT	85	0.141	0.012	0.025	0.076	0.182	0.309	0.799
	LCT + Captan	49	0.117	0.010	0.015	0.053	0.173	0.259	0.691
0–24	All population	138	0.146	0.010	0.023	0.056	0.147	0.389	1.598
	LCT	85	0.132	0.012	0.024	0.061	0.129	0.295	1.417
	LCT + Captan	53	0.173	0.008	0.018	0.039	0.180	0.780	2.829
24–48	All	135	0.122	0.019	0.031	0.063	0.133	0.281	0.582
	LCT	84	0.115	0.013	0.028	0.061	0.139	0.267	0.554
	LCT + Captan	53	0.136	0.028	0.035	0.064	0.131	0.309	0.620

[a] Some workers performed biological monitoring for more than one exposure scenario. This number represents the number of biological samples per exposure period and exposure scenario.
[b] Control urine collection in workers to be assessed for exposure to LCT or LCT + captan.

control urine as well as in 24–48 h urine collections post-exposure (for all participants, participants exposed to lambda-cyhalothrin alone and participants exposed to lambda-cyhalothrin plus captan). In 0–24 h collections post-exposure, CFMP metabolite values for the upper extremes of the distribution (75th and 95th percentiles in the table) were higher than in control urine or in urine collected 24–48 h after the onset of an exposure period (application or working in a field treated with lambda-cyhalothrin alone or in combination with captan). For the extremes of the distribution (75th and 95th percentiles), CFMP values in urine collected after the onset of an exposure episode to lambda-cyhalothrin combined with captan were also higher than in urine collected after exposure to lambda-cyhalothrin alone. Table 4 shows that the trend was the same for 3-PBA metabolite, which is not specific to lambda-cyhalothrin as it is a common metabolite of several pyrethroids. However, exceptionally, a high 3-PBA value was obtained for the 95th percentile of the distribution in control urine of workers to be later assessed for exposure to lambda-cyhalothrin plus captan.

Tables 5 and 6 present the potential determinants of urinary 3-PBA and CFMP levels in exposed strawberry workers, in particular the variable "exposure group" or so called "coexposure" (exposure to lambda-cyhalothrin alone or in combination with captan) but also potential confounding factors documented by questionnaire. Urinary metabolite levels showed a log-normal distribution as did age and body mass index (BMI); univariate and multivariate statistical analyses were therefore performed on the log-transformed values for these variables. When considered individually in the univariate model, age, ethnicity, education, farm size, main occupational task, and time since pesticide application showed a statistically significant association ($p < 0.05$) with 3-PBA levels and, in the case of CFMP, ibuprofen or acetaminophen use. For the 3-PBA, using the linear mixed effects model (MIXM), farm size, main occupational task and time since pesticide application as well as alcohol consumption, ibuprofen or acetaminophen use, and other medication use were retained for adjustment of the multivariate model, according to the Akaike's information criterion (AIC). These variables were considered as variables contributing to the final multivariate model. The variable "time," representing repeated measurements and defined as a within-subjects variable, was a significant predictor of observed biological levels of 3-PBA; the within-subjects variance (95% confidence interval (95% CI)) was 1.11 (1.09–3.49), $p < 0.001$. However, coexposure did not have any statistically significant effect on observed urinary 3-PBA levels (0.94 (0.78–1.13); $p = 0.48$). Table 5 shows that the main occupational task (pesticide application, weed control, or picking), time since exposure, and farm size were the main three predictors of observed biological levels in the final model. Compared to the picking task, the weeding and pesticide spraying tasks were overall associated with higher urinary 3-PBA concentrations ($p < 0.05$). However, the pesticide application task had a greater effect than the weeding task. No statistically significant associations ($p < 0.05$) were detected with the other factors assessed in the multivariate model (alcohol consumption, ibuprofen or acetaminophen use, or other

Table 4
Distribution of 3-PBA concentrations in urine for all participants as well as for participants stratified by exposure group (exposure to lambda-cyhalothrin alone or in combination with captan).

Time since the beginning of the exposure (h)	Exposure group	N of samples[a]	3-PBA concentration (μmol/mol creat.)						
			Geometric mean	Percentile					
				5th	10th	25th	50th	75th	95th
−24–0[b]	All	134	0.125	0.016	0.034	0.059	0.129	0.231	0.670
	LCT	85	0.136	0.015	0.029	0.082	0.151	0.263	0.589
	LCT + Captan	49	0.107	0.018	0.034	0.044	0.101	0.175	1.979
0–24	All population	138	0.151	0.021	0.032	0.081	0.160	0.260	1.418
	LCT	85	0.146	0.022	0.040	0.084	0.161	0.247	0.998
	LCT + Captan	53	0.159	0.015	0.031	0.067	0.148	0.322	3.020
24–48	All	135	0.129	0.014	0.029	0.057	0.133	0.305	0.729
	LCT	84	0.122	0.015	0.025	0.058	0.138	0.263	0.647
	LCT + Captan	51	0.140	0.010	0.030	0.057	0.104	0.408	2.064

[a] Some workers performed biological monitoring for more than one exposure scenario. This number represents the number of biological samples per exposure period and exposure scenario.
[b] Control urine collection in workers to be assessed for exposure to LCT or LCT + captan.

Table 5
Predictors of 3-PBA levels in workers' urine using a linear mixed effects model (MIXM).

Predictors		3-PBA concentration (µmol/mol creat.) (n = 139)[a]		
		Univariate Analysis [b,c]	Multivariate analysis [c,d,e]	
		Exp(β) (CI95%)	Exp(β) (CI95%)	P-value
Coexposure	LCT	1.02 (0.87–1.18)	0.94 (0.78–1.13)	0.48
	LCT + captan	Reference	Reference	
Age	Years (log)	1.93 (1.03–3.61)		
BMI	(kg/m^2) (log)	0.49 (0.13–1.89)		
Ethnicity	Caucasian	1.36 (1.12–1.65)		
	Latino American	Reference		
Education	Primary or High school	0.74 (0.62–0.90)		
	College or University	Reference		
Alcohol consumption	No	0.97 (0.76–1.23)	1.15 (0.90–1.45)	0.26
	Yes	Reference	Reference	
Cigarette consumption	No	0.81 (0.63–1.05)		
	Yes	Reference		
Ibuprofen or acetaminophen	No	1.18 (0.92–1.53)	1.14 (0.82–1.60)	0.43
	Yes	Reference	Reference	
Other medications	No	0.84 (0.62–1.13)	0.82 (0.60–1.12)	0.21
	Yes	Reference	Reference	
Size of the farm	≤10 workers	1.23 (1.01–1.51)	0.69 (0.50–0.95)	0.02
	>10 workers	Reference	Reference	
Main professional task	Pesticide application	1.38 (1.13–1.67)	2.49 (1.74–3.56)	<0.001
	Weeding	0.89 (0.76–1.04)	1.29 (1.00–1.67)	0.053
	Picking	Reference	Reference	
Time since pesticide application	≤7 days	0.87 (0.75–1.01)	0.56 (0.44–0.71)	<0.001
	>7 days	Reference	Reference	

[a] This number represents the number of biological monitoring for all participants across all tasks (application, weeding, and harvesting) and all exposure scenarios (lambda-cyhalothrin alone or in combination with captan). A participant may have been evaluated for more than one task or more than one exposure scenario.
[b] All variables were tested individually in the model. The variable "time" (urine collections at -24-0, 0–24, and 24–48 h following an exposure episode) was considered a repeated measure.
[c] The β estimates and 95% CIs were exponentially transformed from log values.
[d] The variable "time" was considered a repeated measure and a within-subject variable in the multivariate model. The within-subject variance (95% CI) was: 1.11 (1.09–3.49); $p < 0.001$.
[e] Intra-class correlation coefficient (ICC) was 0.54.

Table 6
Predictors of CFMP levels in workers' urine using a linear mixed effects model (MIXM).

Predictors		CFMP concentration (µmol/mol creat.) (n = 139)[a]		
		Univariate Analysis[b,c]	Multivariate analysis[c,d,e]	
		Exp(β) (CI95%)	Exp(β) (CI95%)	P-value
Coexposure	LCT	0.966 (0.84–1.10)	1.10 (0.93–1.30)	0.26
	LCT + captan	Reference	Reference	
Age	Years (log)	0.964 (0.55–1.69)		
BMI	(kg/m^2) (log)	1.837 (0.55–6.10)		
Ethnicity	Caucasian	1.050 (0.88–1.26)		
	Latino American	Reference		
Education	Primary or high school	0.875 (0.88–1.26)		
	College or University	Reference		
Alcohol consumption	No	1.038 (0.85–1.27)	1.13 (0.91–1.40)	0.26
	Yes	Reference	Reference	
Cigarette consumption	No	1.133 (0.91–1.41)		
	Yes	Reference		
Ibuprofen or acetaminophen	No	0.771 (0.62–0.96)	0.80 (0.59–1.08)	0.14
	Yes	Reference	Reference	
Other medications	No	1.018 (0.79–1.30)	1.22 (0.92–1.63)	0.16
	Yes	Reference	Reference	
Size of the farm	≤10 workers	1.041 (0.87–1.25)	0.94 (0.70–1.27)	0.69
	>10 workers	Reference	Reference	
Main professional task	Pesticide application	1.17 (0.98–1.40)	1.41 (1.01–1.96)	0.04
	Weeding	1.04 (0.91–1.21)	1.13 (0.89–1.43)	0.30
	Picking	Reference	Reference	
Time since pesticide application	<7 days	1.09 (0.95–1.24)	1.04 (0.84–1.30)	0.70
	>7 days	Reference	Reference	

[a] This number represents the number of biological monitoring for all participants across all tasks (application, weeding and harvesting) and all exposure scenarios (lambda-cyhalothrin alone or in combination with captan). A participant may have been evaluated for more than one task or more than one exposure scenario.
[b] All variables were tested individually in the model. The variable "time" (urine collections at -24-0, 0–24 and 24–48 h) was considered a repeated measure.
[c] The β estimates and 95% CIs were exponentially transformed from log values.
[d] The variable "time" was considered a repeated measure and a within-subject variable in the multivariate model. The within-subject variance (95% CI) was: 1.25 (1.20–1.31); $p < 0.001$.
[e] Intra-class correlation coefficient (ICC) was 0.20.

medications).

For CFMP, farm size, main occupational task, and time since pesticide application as well as alcohol consumption, ibuprofen or acetaminophen use, and other medication use were also included in the multivariate MIXM model, using Akaike's information criterion (AIC). The variable "time", representing repeated measurements and defined as a within-subject variable, was a significant predictor of the observed urinary CFMP levels; the within-subject variance (95% CI) was 1.25 (1.20–1.31), $p < 0.001$. However, coexposure had no significant effect on observed urinary CFMP levels (1.10 (0.93–1.30); $p = 0.26$) (Table 6).

In the final model, only the main occupational task (pesticide application, weeding or picking), was significantly associated with observed urinary CFMP levels. Compared to the weeding or picking task, the pesticide application task was overall associated with higher urinary CFMP concentrations. No statistically significant associations ($p < 0.05$) were detected with the other factors assessed in the multivariate model (farm size, time since pesticide application, alcohol consumption, ibuprofen or acetaminophen use, or other medications).

4. Discussion

4.1. Impact of coexposure and other factors on urinary 3-PBA and CFMP levels

This study is the first to assess the impact of pyrethroid-fungicide (lambda-cyhalothrin-captan) coexposure on levels of urinary biomarkers of exposure in workers, while accounting for other determinants. It is also the first to assess the impact of the work tasks on levels of biomarkers of exposure to lambda-cyhalothrin. Multivariate statistical analyses showed that coexposure to lambda-cyhalothrin and captan was not a significant contributor to the variability in CFMP or 3-PBA concentrations in urine, used as biomarkers of exposure. Only the main task was consistently associated with variations in urinary levels of CFMP and 3-PBA; the pesticide application task was associated with higher levels of metabolites in urine compared to the weeding or picking tasks. These results are very similar to those reported in a previous study on exposure to another pyrethroid, cypermethrin, which is metabolized to 3-PBA, as is lambda-cyhalothrin (Bouchard et al., 2019; Ratelle et al., 2016). In the latter study, some individually assessed personal or occupational characteristics were associated with the excretion of the metabolite 3-PBA after exposure to cypermethrin, but only the main occupational task was associated with the excretion of exposure biomarkers in the multivariate MIXM model. Similar to the present study, cypermethrin pesticide applicators had higher overall urinary levels of 3-PBA than workers performing tasks such as weeding, harvesting, or inspecting fields in an area treated with this pesticide.

In a German study, Hardt and Angerer (2003) also reported higher levels of the pyrethroid metabolites DCCA and 3-PBA in the urine of pesticide sprayers compared to the overall workers assessed (farmers, greenhouse workers or building exterminators). In a Japanese biological monitoring study of pyrethroid exposure in pesticide sprayers, geometric mean concentrations of 3-PBA in the urine of operators who sprayed in the two days preceding the survey were significantly higher than those who did not perform any spraying (5.4 μg/g creat. vs. 0.9 μg/g creat.) (Wang et al., 2007). However, a significant association between urinary 3-PBA levels and pyrethroid spraying was observed only in winter and not in summer. Hence, the fact that all biomonitoring data were collected during summer in our study excludes to some extent the seasonal variability.

In the present study, time since pesticide application (lambda-cyhalothrin alone or mixed with captan) as well as farm size were also associated with urinary 3-PBA levels in the multivariate MIXM model (but the result did not come out significant for CFMP). In the study by Ratelle et al. (2016), farm size – when considered individually in the MIXM model – showed an association with urinary 3-PBA excretion, but this association was not significant in the multivariate model (*i.e.* in combination with other factors).

Also, in the present study, when considered individually in the MIXM model, ethnicity, age, and education were associated with urinary 3-PBA levels, but these variables did not contribute significantly to the fit of the final multivariate model. On the other hand, alcohol consumption and the use of ibuprofen, acetaminophen or other medications contributed to the fit of the multivariate model (according to the AIC criterion), but did not show a statistically significant association with urinary 3-PBA or CFMP levels in the multivariate model.

In an exposure biomonitoring study conducted by our group in the general population of Montreal, Québec, Canada, prescription and over-the-counter drug use was associated with higher urinary excretion of pyrethroid metabolites, including 3-PBA (Fortin et al., 2008). In addition, Barr et al. (2010) reported that urinary excretion of 3-PBA in individuals from the general U.S. population was significantly associated with ethnicity and age in a multivariate model. In our study, the sample size was relatively small, and the group evaluated was composed mainly of healthy Latino workers.

Lopez-Galvez et al. (2018) evaluated pesticide exposure in 20 migrant farmers in a study in Sonora, Mexico and the impact of different factors, based on urine metabolite measurements including 3-PBA. Farmworkers age, language, personal protective equipment, time spent on the farm and season were important determinants of exposure. In a biomonitoring study of 50 textile workers in eastern China, Lu et al. (2013) also reported an effect of age and task on biological levels of pyrethroid metabolites (*trans*-DCCA, *cis*-DCCA and 3-PBA). In addition, in a study among farmers and their families, Trunnelle et al. (2014) reported a positive association between poor housing conditions and levels of urinary 3-PBA metabolites. They reported that poor housing conditions was a contributing factor to the higher levels of 3-PBA observed in the urine of these farmworker families.

Furthermore, in the present study, information on clothing worn was fairly consistent among workers (long pants (89%) and long-sleeved shirt or sweater (86%) and boots (79%) for the majority of workers); the small number of workers who reported wearing half-masks or helmets with filters (only nine pesticide sprayers) did not allow for a specific assessment of the impact of this protective equipment on biological levels of CFMP and 3-PBA. Only 45% of workers wore gloves, but there was no effect of glove wearing on urinary levels of CFMP and 3-PBA. Wearing goggles and hats was also not significantly associated with urinary levels of CFMP and 3-PBA. In contrast, in a recent biomonitoring study in which the metabolites 3-PBA and 4F-3PBA were measured in the urine of 45 farmers in northwestern Catalonia, Spain (Bravo et al., 2022), the use of specific personal protective equipment among farm workers, such as the use of gloves and masks during mixing, was associated with lower biological levels, although the differences were not statistically significant. However, a positive association was found between the use of a cap during mixing and during application. The authors reported that these caps were primarily used for sun protection, and when not cleaned after handling pesticides, they could represent a continuous source of exposure through dermal contact. In the same study, farm workers using tractors with cabin also had statistically lower concentrations of the metabolite of another pesticide, 2-diethylamino-6-methyl pyrimidin-4-ol, than those using tractors without cabin. In the present study, most applicators used a tractor for pesticide spraying, with cabin in eight cases and without cabin in four cases; only one applicator used a backpack sprayer. Because there was little difference in application conditions in our study, it was not possible to associate spraying equipment with biological levels.

Furthermore, in our study, there was no significant difference between the wearing of personal protective equipment (PPE) for the group exposed to lambda-cyhalothrin alone or in combination with captan, except for wearing gloves and goggles, which was more worn in the group coexposed to lambda-cyhalothrin and captan. Occupational hygiene practices in each exposure group (lambda-cyhalothrin alone or in combination with captan) were therefore relatively homogeneous, and the variability that could be associated with PPE between exposure groups was low.

4.2. Comparison of 3-PBA and CFMP levels with reference values in the general population or in exposed volunteers

The study also provided an overall indication of the magnitude of exposure of strawberry workers relative to the general population. In order to assess the importance of this exposure of all workers in the study, concentrations of 3-PBA metabolites in urine (24-h) from workers in our study were compared with those reported (in spot urine collections) in the general Canadian population and collected in the CHMS Cycle 6 (Health Canada, 2021). The 95th percentiles of the distribution of 3-PBA concentrations in the urine of our study workers reached values (3.02 μmol/mol creat. in urine collections performed 0–24 h after spraying lambda-cyhalothrin in combination with captan; seeTable 4) similar to the 95th percentile (CI) of 3.1 (1.06–5.02) μmol/mol creat. reported in the CHMS (Health Canada, 2021). The CHMS sample may have included workers exposed to pesticides. CFMP was not measured in

the CHMS study but lower 95th percentile values (0.145 and 0.261 μmol/mol creat.) were reported in spot urine samples collected from Sweden adolescents (Norén et al., 2020) and German individuals from the general population (Schettgen et al., 2016). Conversely, the 95th percentile concentration value of CFMP reported in the urine of the UK general population (0.839 μmol/mol creat.) (Bevan et al., 2013) was close to the values reported in our study (0.554–0.799 μmol/mol creat.) at -24-0 h prior to exposure and 24–48 h post-exposure.

Urinary levels of 3-PBA in the present study were comparable to those of Ratelle et al. (2016) where 34 patterns of 3-PBA excretion were observed in vegetable crop workers. Depending on the profile, median urinary 3-PBA values for a given individual ranged from 0.073 to 1.28 μmol/mol creat. and the 95e percentiles were as high as 9.07 μmol/mol creat. In the latter study, 16 profiles had geometric mean urinary 3-PBA concentrations above those reported in the CHMS at that time (Health Canada, 2013), and five profiles had 95th percentile values of 3-PBA higher than those reported in that survey. The observed levels of 3-PBA in our study were also comparable to those reported in German and Japanese studies where spot urine measurements (Panuwet et al., 2008; Wang et al., 2007) or 24-h collections were made (Hardt and Angerer, 2003). Pesticide exposure and uptake, and thus observed biological levels, may vary depending on several factors, including work habits, protective equipment, climate (heat and humidity) (Havenith, 1999).

The levels of 3-PBA and CFMP observed in the urine of workers can also be compared to the maximum urine values obtained from eight volunteers for whom the temporal profiles of CFMP and 3-PBA in urine were determined over a period of 84 h following the administration of an acute oral dose of 0.0025 mg lambda-cyhalothrin/kg bw, which is the EFSA acceptable daily intake (ADI) value (2014) or 0.025 mg lambda-cyhalothrin/kg bw (Khemiri et al., 2017). In the study of Khemiri et al. (2017), the maximum concentration of 3-PBA in urine was 10 μmol/mol creat. in the volunteer exposed to the ADI and ranged from 60 to 211 μmol/mol creat. in the other seven volunteers exposed to 0.025 mg/kg bw. The corresponding values for CFMP were 41 and 63–431 μmol/mol creat. In the present study, the 95th percentiles of the distribution of 3-PBA and CFMP concentrations in urine reached 2.8 and 3.0 μmol/mol creat. respectively; these values are 3.6 and 14 times lower than the urinary levels observed in the ADI-exposed volunteer.

4.3. Limitations and interest of the current biomonitoring study

In multivariate analyses, the levels of CFMP and 3-PBA between the group exposed to lambda-cyhalothrin alone and that coexposed to lambda-cyhalothrin and captan were compared while controlling for other factors that may influence urinary levels. The contribution of personal factors (age, ethnicity, BMI) and lifestyle habits (smoking and alcohol consumption or use of medication), as well as occupational exposure conditions (task, time since application, size of the farm in terms of number of employees) were therefore tested. One limitation was that some factors documented by questionnaire were not reported much so that their impact on exposure biomarker levels could not be tested (such as the impact of the "sex" variable since the farmers were mostly men, or the impact of domestic treatments or residential pesticide use since it was reported only in one case). In addition, although the questionnaires were translated in Spanish and a Spanish-speaking member of our team was available to assist and verify the participants' answers, the questions on the consumption of foods that may contain pesticide residues (consumption of cereals, fruits and vegetables, and number of servings) were not answered adequately or were poorly answered, so that they were not considered in the analyses. Also, the clothing and PPE worn by the workers were not considered in the multivariate analyses because, as mentioned above, the information on the clothing worn was fairly uniform among the workers (mostly long pants, long-sleeved shirts and boots), and the wearing of specific PPE (wearing half-masks or helmets with filters) was rarely reported. In addition, wearing gloves, goggles and a hat did not show a significant association with urinary levels of CFMP and 3-PBA.

Another limitation related to biological exposure monitoring concerns the fact that it involves the measurement of 3-PBA, the metabolite common to several pyrethroids, in addition to the more specific CFMP metabolite. As noted in a previous study on cypermethrin (Bouchard et al., 2016), the measurement of specific metabolites is important to confirm the source of exposure. It is only recently that the measurement of CFMP metabolites was conducted in large surveys (*e.g.*, Apel et al. (2023)). Furthermore, although it was confirmed that both captan and lambda-cyhalothrin were sprayed concomitantly by the applicators in our study and that field workers entered areas previously treated with both compounds, the metabolites of captan were not specifically measured in the urine of the workers under study to confirm internal exposure to both pyrethroids and captan.

Despite these limitations, this study showed that coexposure did not significantly impact biological levels of lambda-cyhalothrin metabolites, while controlling for other factors such as the main task performed, time since application, age, ethnicity, education level, medication, which may contribute to biological variability. In the study of Khemiri et al. (2017, 2018) in volunteers exposed to lambda-cyhalothrin orally and dermally under controlled conditions, variability in urine levels of CFMP and 3-PBA was significant despite identical exposure doses and the absence of coexposure. This indicates that physiological factors, related to absorption, distribution, metabolism and excretion, contribute significantly to inter-individual variability in the biological levels of metabolites used as biomarkers of exposure. The results of the present study thus suggest that at the levels of pesticide exposure observed in the targeted workers, coexposure does not contribute significantly to increasing this variability in the biological levels of CFMP and 3-PBA. At these exposure levels, coexposure therefore had no significant impact on the kinetics of lambda-cyhalothrin and its assessed metabolites used as biomarkers of exposure.

Furthermore, while the measurement of 3-PBA is not specific to lambda-cyhalothrin, the analyses carried out on the basis of this metabolite allow *a contrario* to assume that the results on the impact of coexposure to lambda-cyhalothrin and captan can be extrapolated and therefore generalized to other pyrethroids. Considering that the workers in the study were also exposed to other pesticides in their workplace (e.g. application of Roundup reported in some cases or other fungicides), the observed results indirectly point to a lack of impact of exposure to other pesticides used in the workplace on the levels of biomarkers of exposure to pyrethroids at the observed levels, although this remains to be confirmed.

Overall, this study provided novel data confirming that at the levels observed in the agricultural workers under study, biomarker concentrations in urine used to assess exposure to pyrethroids should not be influenced by coexposure to captan. As concluded from the animal results of Bossou et al. (2020), the present study thus suggests that the pyrethroid metabolites CFMP and 3-PBA, mostly measured in biomonitoring studies, remain useful as biomarkers of exposure in mixtures, when pesticide exposure levels are in the range of the sampled workers or at the general populational levels. Future perspectives include the use of a toxicokinetic model specific to lambda-cyhalothrin and Monte Carlo simulations to obtain the reconstructed absorbed dose possibilities for each worker, based on the amounts of the more specific metabolite CFMP measured in urine, throughout the biological monitoring period. The reconstructed daily dose results for applicators and other agricultural weed control and harvest workers can then be compared to limit values such as the *Acceptable Operator Exposure Level* (AOEL) reference value established by the European Commission (EFSA, 2014).

Author contributions

Yélian Marc Bossou: Methodology, Formal analysis, Investigation,

Writing – Original draft preparation. **Jonathan Côté:** Formal analysis, Investigation, Writing – Reviewing and Editing. **Étienne Dumais:** Formal analysis, Investigation, Writing – Reviewing and Editing. **Éloïse Morin:** Formal analysis, Investigation, Writing – Reviewing and Editing. **Clara Bianci:** Formal analysis, Investigation, Writing – Reviewing and Editing. **Michèle Bouchard:** Conceptualization, Methodology, Formal analysis, Supervision, Project Management, Writing – Original draft preparation, Writing – Reviewing and Editing, Funding acquisition.

Data availability

All data generated during this study are included in this article or are available on reasonable request from the corresponding author.

Ethics approval

The study protocol, the information and consent form, and other relevant documents were approved by Research Ethics Committee of the University of Montreal (*Comité d'éthique de la recherche Clinique de Université de Montréal* #CERC-19-007-D).

Consent to participate

The study was based on a voluntary participation. Participants received a slight financial compensation for their involvement and time. Subjects wishing to participate in the study signed an informed consent form, after receiving all necessary information about the project. Each participant was free to withdraw from the study at any time, without any prejudice.

Consent for publication

This manuscript has not been published or presented elsewhere and is not under consideration by another journal. All authors read and approved the final manuscript and consent for publication.

Declaration of competing interest

The authors have no competing interests to declare that are relevant to the content of this article.

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Appendix A. Supplementary data

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Coordination of chemical analyses under the European Human Biomonitoring Initiative (HBM4EU): Concepts, procedures and lessons learnt

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ABSTRACT

The European Human Biomonitoring Initiative (HBM4EU) ran from 2017 to 2022 with the aim of advancing and harmonizing human biomonitoring in Europe. More than 40,000 analyses were performed on human samples in different human biomonitoring studies in HBM4EU, addressing the chemical exposure of the general population, temporal developments, occupational exposure and a public health intervention on mercury in populations with high fish consumption. The analyses covered 15 priority groups of organic chemicals and metals and were carried out by a network of laboratories meeting the requirements of a comprehensive quality assurance and control system. The coordination of the chemical analyses included establishing contacts between sample owners and qualified laboratories and monitoring the progress of the chemical analyses during the analytical phase, also addressing status and consequences of Covid-19 measures. Other challenges were related to the novelty and complexity of HBM4EU, including administrative and financial matters and implementation of standardized procedures. Many individual contacts were necessary in the initial phase of HBM4EU. However, there is a potential to develop more streamlined and standardized communication and coordination in the analytical phase of a consolidated European HBM programme.

1. Introduction

The European Human Biomonitoring Initiative (HBM4EU) was launched in 2017 to develop and establish a coordinated and harmonized approach to human biomonitoring (HBM) across Europe. It built on the previous European projects *Expert Team to Support Biomonitoring in Europe (ESBIO)*, *European Coordination Action on Human Biomonitoring (COPHES)* and its demonstration project *DEMOCOPHES*, and on national or regional HBM programmes of some European countries (Kolossa-Gehring et al., 2012; Schindler et al., 2014; Den Hond et al., 2015; Joas et al., 2015). One of the characteristics of HBM4EU was a high degree of diversity, as it encompassed partners from 30 countries with different levels of HBM experience. Furthermore, it supported national as well as European authorities in chemical risk assessment, and it addressed a variety of chemicals, exposure scenarios and health outcomes (Ganzleben et al., 2017; Kolossa-Gehring et al., 2023). Consequently, coordination points had a vital role in HBM4EU in advancing the initiative from distinct activities to a coherent programme.

The chemical analyses in HBM4EU included human samples from four complementary approaches: Aligned national and regional HBM

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studies, connections to previous analyses in *DEMOCOPHES*, occupational exposure monitoring and an intervention study focussing on mercury (Table 1). These studies addressed two groups of priority substances. The first group had been selected in the preparation of HBM4EU according to policy-relevant questions defined by HBM4EU partner countries and European authorities, and the second group was defined in a prioritization process developed in HBM4EU (Ougier et al., 2021) (Table 1). An additional study on pesticide exposure using non-target and suspect screening, abbreviated SPECIMEn, was conducted in HBM4EU as well (Vitale et al., 2022), but not included in the coordination of analyses of the priority compounds. Except for some existing data from *DEMOCOPHES* or national and regional HBM programmes, new chemical analyses of the priority compounds were conducted in HBM4EU, based on the prerequisite of coordinated and harmonized approaches (Ganzleben et al., 2017).

For that purpose, a comprehensive quality assurance and control (QA/QC) programme was designed in HBM4EU, open to all European laboratories with an interest in performing HBM analyses of exposure biomarkers of the priority substances (Esteban López et al., 2021). It was organized and coordinated by the Quality Assurance Unit (QAU) established in HBM4EU and involved different HBM4EU partners taking responsibility for interlaboratory comparison investigations (ICIs) and/or external quality assurance schemes (EQUAS) in their field of expertise. This approach ensured greatest scientific expertise for the priority chemicals, substance-tailored ICI and EQUAS approaches with a common design and optimized timelines in parallel ICIs/EQUAS. The programme specified the criterion of at least two successful rounds of ICI/EQUAS participation to qualify for the analysis of specific biomarkers in HBM4EU.

The QA/QC programme resulted in the qualification of 75 laboratories from 25 countries for HBM analyses of different biomarkers related to the priority substances in Table 1. It was up to the sample owner, providing the samples to HBM4EU, to select a laboratory for the chemical analysis, on an informed basis. Collecting and conveying this information was part of the coordination of the analytical phase in HBM4EU, under the responsibility of Aarhus University (AU). The coordination also included progress monitoring of the chemical analyses. The Covid-19 pandemic required communication efforts beyond regular updates as well as adjustments of work plans that affected the overall HBM4EU timelines.

The objective of this article is to present and discuss the coordination of the chemical analyses in HBM4EU, starting from the conceptual approach and subsequently detailing the main activities. The article also includes the challenges encountered in the process and possible solutions for future projects.

2. Conceptual approach

The first step in the coordination of the analytical phase was to connect the qualified laboratories, i.e. laboratories with successful participation in the HBM4EU QA/QC programme, and the sample owners, with the purpose of providing the sample owners with the necessary information to select laboratories for the planned analyses (Fig. 1). In addition, this connection should give the laboratories the possibility to prepare for potential analytical tasks in their work plans. This first step required inputs from other tasks and work packages (WPs) in HBM4EU, including lists of qualified laboratories (Esteban López et al., 2021) and of the sample owners with their specific analytical interests (Gilles et al., 2021).

Once the sample owners had selected a laboratory, AU assisted with potential questions about administrative and technical issues. When the samples had been shipped to the selected laboratory, the monitoring phase began, i.e. the second part of the coordination work (Fig. 1). It involved regular contacts to each laboratory to enquire about progress and potential difficulties, which was intensified during the Covid-19 pandemic when work conditions became unpredictable as laboratories were affected by lockdowns and/or reduction of activities. AU regularly summarized the status of the analytical work in internal progress reports for the attention of task and WP leaders.

Although consecutive in the conceptual approach, the connecting efforts and the progress monitoring proceeded in parallel and overlapped in their timing. As the chemical analyses in HBM4EU included two groups of prioritized substances (Table 1) and both were covered by the QA/QC programme, the process in Fig. 1 was applied twice. However, fewer laboratories were involved in the second round of analyses than in the first one. In addition, the chemical analyses included a comparison of concentrations at different time points and samples from occupational studies, although the number of analyses was considerably lower than in the HBM4EU Aligned Studies (Table 1).

In the HBM4EU-MOM study and the HBM4EU occupational study on exposure in e-waste management, the coordination requirements were reduced to the progress monitoring. In both studies, one central laboratory was pre-selected for each type of analysis. The occupational studies on diisocyanates further included analyses of hemoglobin adducts and urine lysine adducts, which were of exploratory nature and thus not included in the HBM4EU QA/QC programme or the coordination of the analytical phase (Jones et al., 2022).

The outputs of the analytical phase were HBM data on the priority substances in the individual studies in Table 1, accompanied by contextual QA/QC information. These data were further processed and analysed in other WPs in HBM4EU and not part of the coordination of the analytical phase (Fig. 1). However, it meant that the analytical phase

Table 1
Summary of chemical analyses in studies on priority substances under HBM4EU.

Study	First group of priority substances	Second group of priority substances	Number of analyses	Reference
HBM4EU Aligned Studies: Alignment of national and regional HBM studies	Phthalates and 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH), bisphenols, per- and polyfluoroalkyl substances (PFAS), organophosphorous flame retardants (OPFRs), halogenated flame retardants (HFRs), polycyclic aromatic hydrocarbons (PAHs), cadmium	Acrylamide, mycotoxins, pesticides, UV filters, arsenic	29,074	Gilles et al. (2021); Gilles et al. (2022); Govarts et al. (2023)
Comparisons of different time points, including *DEMOCOPHES* samples	Phthalates and DINCH, bisphenols, OPFRs, PAHs, cadmium	–	4863	Vogel et al. (2023)
Occupational exposure studies	Chromium[a], PFAS[a], OPFRs[b], HFRs[b], phthalates[b] and DINCH[b], cadmium[b]	Diisocyanates[b], mercury[b], lead[b]	8574	Galea et al. (2021); Jones et al. (2022); Santonen et al. (2019, 2022); Scheepers et al. (2021)
HBM4EU-MOM[c]: Intervention study on mercury	–	Mercury	1305	Namorado et al. (2021); Katsonouri et al. (2023)

[a] First occupational study: Exposure to chromium.
[b] Second occupational studies: Exposure to diisocyanates and exposure in e-waste management, respectively.
[c] Methylmercury-control in expectant mothers through suitable dietary advice for pregnancy.

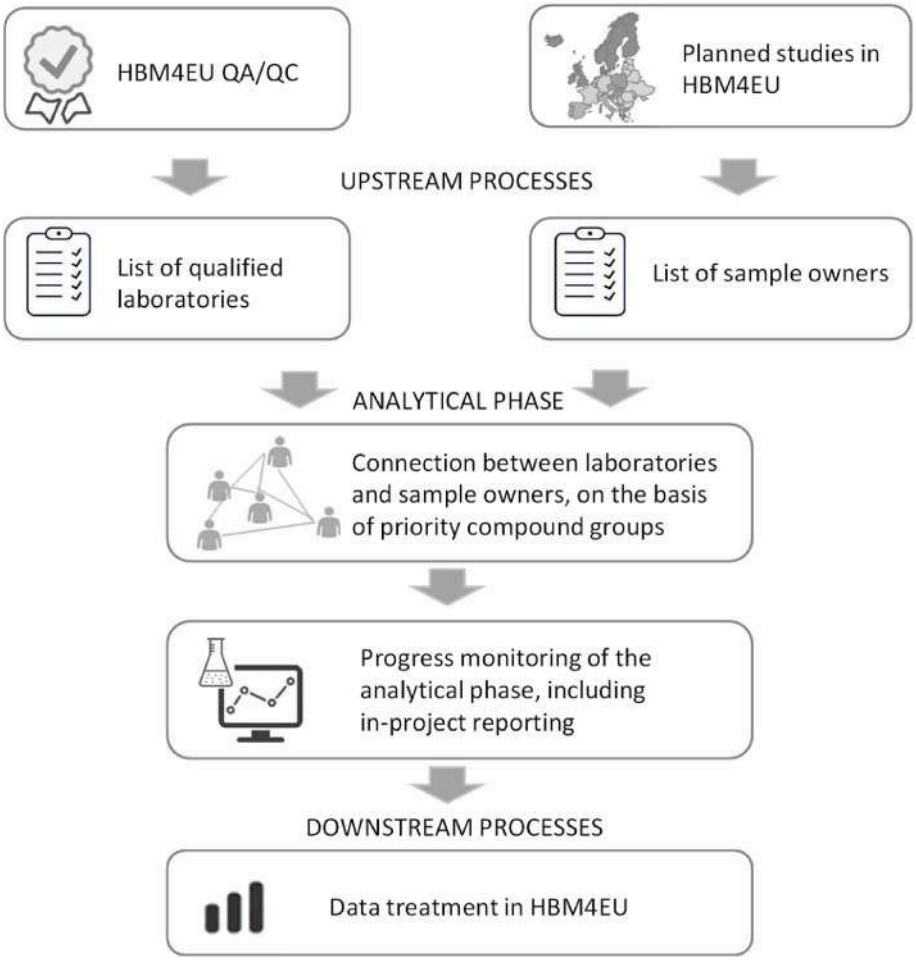


Fig. 1. Concept of coordination of the chemical analyses in HBM4EU.

had to be closely connected to upstream processes (lists of qualified laboratories, planned studies) and downstream processes (data processing and interpretation).

3. Connecting qualified laboratories and sample owners

Fig. 2 lists the activities included in the first step of the coordination work, i.e. the establishment of connections between qualified laboratories and sample owners. In order to collect information on analyses,

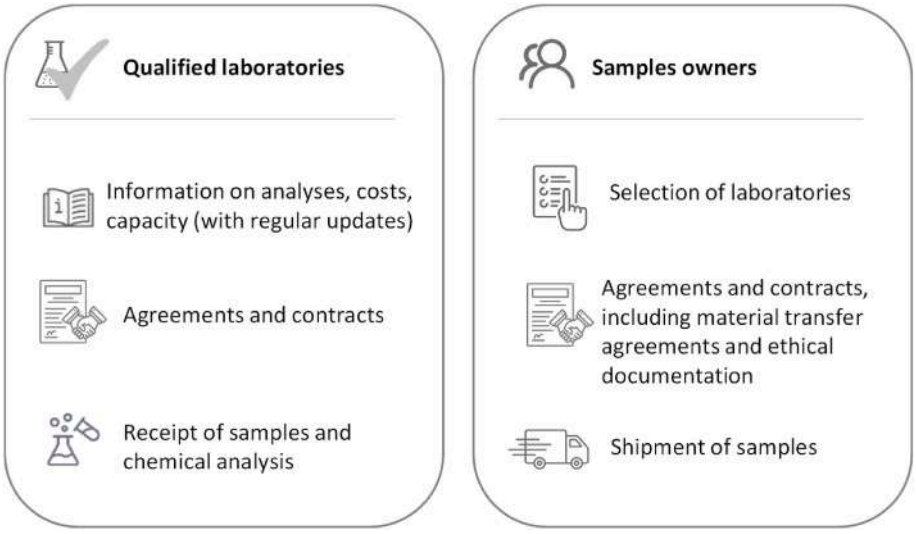


Fig. 2. Topics covered in the first step of the coordination of the chemical analyses in HBM4EU, i.e. the establishment of contacts between qualified laboratories and sample owners.

costs and capacity, a questionnaire was prepared for the qualified laboratories, including questions on the laboratory itself (e.g. use of a quality management system, involvement in HBM4EU and contact details), the specific biomarkers that the laboratory offered to analyze, the analytical methods (e.g. sample volume required, limit of quantification (LOQ), extraction, clean-up and instrumental techniques), the price and time frames for the specific analysis and whether or not the laboratory required any information from the sample owner. The questionnaire for bisphenols is shown as an example in Fig. 3. Questionnaires for the other priority substances are available in the Supporting Information (Fig. S1 - Fig. S13). The questionnaires were adapted to each group of substances to account for different biomarkers, matrices (urine, serum) and analytical methods.

Membership in the HBM4EU consortium was not a prerequisite to be selected for analysis. However, whether or not a laboratory was a partner in HBM4EU had administrative implications for the invoicing, as further discussed in Section 5. The only criterion for conducting analyses in HBM4EU was the successful participation in the QA/QC programme. The candidate list for the QA/QC programme was open for non-HBM4EU as well as HBM4EU laboratories (Esteban López et al., 2021). All laboratories were informed, as part of the questionnaire, that the analytical method had to be identical with that applied in the ICIs and EQUASs. Potential changes had to be disclosed in the questionnaire and would lead to an expert assessment of eligibility. However, no laboratory reported any changes.

Price for analyzing bisphenol biomarkers in urine

HBM4EU will investigate the human exposure to a list of prioritized substances in the European population. We kindly request your offer for analyses of bisphenol biomarkers in urine samples, to be performed in the HBM4EU project. The information in this form will be used in subsequent deliberations and negotiations between sample owners and laboratories.

Please fill in the requested information, sign the form and return it to hbm4eu@envs.au.dk.

Laboratory information	
Laboratory name	
Head of the laboratory	
Number of staff and qualifications	
Address	
Contact person	
Phone	
E-mail	
Is the laboratory member of the HBM4EU consortium?	
Is the laboratory ISO/IEC 17025 accredited?	
If not, does it work under a quality management system?	
Biomarkers	
Please indicate which biomarkers are included in the analytical method	
Bisphenol A (BPA)	
Bisphenol F (BPF)	
Bisphenol S (BPS)	
Analytical method	
Sample volume required (ml)	
LOQ (ng/ml)	
Will the analytical method be identical with that used in the HBM4EU ICI/EQUAS?	
If not, please specify any deviations, subject to evaluation by the HBM4EU Quality Assurance Unit. Please note that inconsistencies with the HBM4EU ICI/EQUAS might affect the eligibility of the laboratory for analyses in HBM4EU.	
In-house quality assurance/quality control	
Internal standards used for analysis	
Calibration method	
Extraction and clean-up	
Sample pre-treatment	
Type of deconjugation	
Enzyme used (ref number)	
SPE offline including type of column	
SPE online including type of column	
Other extraction and clean-up methods	
Derivatization (reagent)	
Instrumental analysis	
GC-MS (model, brand, column, ionisation)	
GC-MS/MS (model, brand, column, ionisation)	
LC-MS/MS (model, brand, column, ionisation)	
Other (please specify)	
Price	
Price, in Euro, for 300 samples	
Please specify person months (PM) for the analysis of 300 samples, costs of PM for different categories of staff involved in the analyses, and direct costs (consumables).	
Does the price include the determination of creatinine?	
If not, what is the price for creatinine determination in 300 samples? If possible, please specify PM and direct costs.	
Capacity and timeframe for analysis	
Time frame for 300 samples (assuming that they will be available in approximately one month).	
How much time does the laboratory need in advance to plan and prepare the analysis of 300 samples?	
Are there variations in the laboratory's capacity (due to holidays etc.)? If so, please specify.	
Does the laboratory have the capacity to analyse several sets of 300 samples in parallel?	
If yes, what is the maximum capacity?	
If no, what would be the time frame for several sets of 300 samples?	
Information required from the sample owners	
What information does the laboratory need from the sample owner for a potential contract for analyses?	
Other comments	
Validity of this document until	
Please note any additional comments.	

Date and signature

Fig. 3. Example of a questionnaire sent to laboratories qualified in HBM4EU, here for the analysis of bisphenols.

It should be noted that the design of the QA/QC programme, including the criterion of two successful rounds of participation, meant that the laboratories qualified for specific biomarker analyses at different points in time. Consequently, the collection of information from the laboratories was a rolling process, repeated after each update of the list of qualified laboratories in relation to completed rounds of ICIs and EQUAS. Furthermore, as the laboratories indicated in the questionnaires for how long this information was valid (Fig. 3), updates were requested from the laboratories when this time had passed. The information received from the laboratories was compiled in a table, with updates marked, and circulated to the sample owners on a weekly basis. The qualified laboratories, but no details on prices, capacities or methods, were also published on the HBM4EU website.

Parallel to the regular contacts with the qualified laboratories, inventories of all planned analyses were established and kept up-to-date, mainly based on information provided by the task leaders responsible for the studies in Table 1. This overview is shown in Table S1 of the Supporting Information, details are also given by Gilles et al. (2021, 2022), Govarts et al. (2023) and Santonen et al. (2019, 2022). In collaboration with the task leaders responsible for the studies in Table 1, questions were prepared for the sample owners, including information on the status of the sampling campaign, preparatory steps, such as ethical approval (Knudsen et al., 2023), and availability of auxiliary data. The selected laboratories were regularly added to the inventory, for internal use in HBM4EU. If a lack of progress was noted, the sample owners were contacted and asked if a decision had been reached on the choice of laboratory.

The sample owners were encouraged to contact AU as coordinators for the analytical phase to request information and updates according to their work plans. In some cases, sample owners had pre-selected a qualified laboratory or chosen to analyze the samples in-house, which they were also asked to communicate to AU's coordinating team. It is also worth noting that in this process of establishing contacts and exchanging information of relevance to the analytical work, including analytical costs, AU as the coordinator of the analytical phase was not involved in any deliberations of financial matters between the sample owners and the laboratories. AU and others assisted with guidance on the technicalities of budget transfer, as further specified in Section 5, but price negotiations between sample owners and laboratories or any involvement in the actual selection process were not part of the coordinating activities in the analytical phase.

Shipment of samples to the laboratories followed Standard Operating Procedures (SOPs) developed in HBM4EU (Pack et al., 2023). It was accompanied by Material and Data Transfer Agreements, which were also filed in a central HBM4EU database under the auspices of the HBM4EU ethics coordinator (Lermen et al., 2020; Knudsen et al., 2023). Thus, AU as coordinator for the chemical analyses was in close contact with the ethics coordinator to ensure correct documentation in accordance with rules for ethics and data protection. The HBM4EU coordinator, holding the main responsibility for ethics and data management in HBM4EU, was also copied on correspondence in this field.

4. Progress monitoring of the chemical analyses

In order to know the status of the HBM analyses and to assist with

potential difficulties on a one-on-one basis, close contacts to the sample owners and the selected laboratories were established. As discussed in Section 3, agreements between the sample owners and the selected laboratories were reached at different time points in HBM4EU. Consequently, sample shipment and the analyses in each laboratory had their own timelines. At a given point in time, the individual analyses in the studies of Table 1 had progressed very differently. The status was described in progress reports for internal use in HBM4EU, providing leaders and colleagues in other WPs with regular updates relevant for their work in HBM4EU. The first laboratories had passed the qualification criteria in July 2019, whereas the last analyses were completed with the end of the project in June 2022.

Monitoring the progress of the chemical analyses proceeded via e-mail communication, mainly with the responsible project leaders as the first contacts, but also, with their agreement and information, directly with the selected laboratories or sample owners. This communication was not standardized in any way, beyond carbon copying to the institutions involved in the respective study. In hindsight, standardized progress forms could have been circulated at regular intervals, but a more informal and individualized approach was chosen in HBM4EU, reflective of the close collaboration amongst most partners as well as the wish to create possibilities for open discussion and solution-oriented dialogue in case of problems or delays. Thus, a small team formed around each study, which proved efficient in finding solutions and conveying relevant information to other groups in HBM4EU.

Table 2 summarizes the analyses in HBM4EU according to the prioritized substance group, also including the number of laboratories qualified and eventually selected for the chemical analyses. As mentioned in Section 1, the total number of qualified laboratories was 75, but as one laboratory could be qualified for multiple biomarkers, the total number in Table 2 is higher. Of the 75 qualified laboratories, 34 laboratories (45%) were selected for analysis in HBM4EU, some of them for multiple analyses. Their geographical distribution is summarized in Fig. 4. A corresponding figure, stratified by priority group, is shown in the Supporting Information (Fig. S14).

The number of qualified laboratories is highest in the larger European countries as well as in those with existing HBM programmes (Fig. 4). The differences are smaller for the number of selected laboratories. However, while a large number of laboratories was selected for the chemical analyses, the number of samples analysed per laboratory varied considerably, ranging between 60 and 5198. About 30% of all analyses were conducted by three of the 34 selected laboratories, located in Germany, the Czech Republic and Denmark.

Fig. 5 shows how many samples in percentage of the total number were analysed in their country of origin. A corresponding figure with absolute numbers is presented in the Supporting Information (Fig. S15). The high percentage of cadmium analyses conducted at the national level suggests that this analysis is well-established in many European countries. Cadmium was also the biomarker with the highest number of laboratories qualified for analyses in HBM4EU (Fig. S14). However, other factors may also influence how many samples are analysed in the same country: Samples might have been chosen for HBM4EU analyses because analytical capacities were available in the country. Likewise, participation in the HBM4EU QA/QC programme might have been prioritized because samples were to be analysed from the same country. For the second group of priority substances (Table 1), only few invited expert laboratories had qualified for HBM4EU analyses, limiting the possibility for national-based analyses.

5. Challenges

5.1. Standardization

One of the central objectives of HBM4EU was the standardization and harmonization of procedures, taking into account that a large number of European countries and institutions participated in HBM4EU. While some of them had no prior experience with HBM and had to establish new routines, others with some HBM experience had to adjust their procedures to meet the standards developed in HBM4EU (Pack et al., 2023). This transition towards standardized procedures covered

Table 2
Substance groups and qualified laboratories performing chemical analyses.

Priority substance (group)	Individual biomarkers[a]	Matrix	Qualified laboratories	Selected laboratories	Analyses	QA/QC programme
Acrylamide	2	Urine	5	2	1795	Esteban López et al. (2021)
Arsenic	6	Urine	2	1	900	Esteban López et al. (2021)
Bisphenols	3	Urine	25	8	3613	Vaccher et al. (2022)
Cadmium	1[b]	Urine, blood[c]	38	11	3967	Nübler et al. (2021)
Chromium	1[b]	Urine, plasma, blood[d]	28	11	2758	Nübler et al. (2022a)
Diisocyanates	3	Urine	3	3	356	Jones et al. (2022)
DINCH	2	Urine	8	8	6160	Mol et al. (2022)
Halogenated flame retardants	10	Serum	15	5	1178	Dvorakova et al. (2021)
Mercury[e]	1	Hair	–	1	1305	-[e]
Mycotoxins	1	Urine	4	3	1304	Esteban López et al. (2021)
Organophosphorous flame retardants	4	Urine	5	5	2856	Dvorakova et al. (2021)
Per- and polyfluoroalkyl substances (PFAS)	12	Serum	21	6	1663	Nübler et al. (2022b)
Pesticides	9	Urine	2	2	2188	Esteban López et al. (2021)
Phthalates	15	Urine	20	9	5949	Mol et al. (2022)
Polycyclic aromatic hydrocarbons (PAHs)	13	Urine	5	5	2856	Nübler et al. (2023)
UV filters	2	Urine	2	1	1975	Esteban López et al. (2021)

[a] For a full list of biomarkers, see Esteban López et al. (2021).
[b] One parameter, but several matrices.
[c] Blood analyses were included in the HBM4EU QA/QC programme, while the final HBM4EU studies only included urine samples.
[d] The HBM4EU studies analysed Cr in red blood cells and plasma. Blood was used as a surrogate in the QA/QC programme, see details in Nübler et al. (2022a).
[e] Not included in the QA/QC programme because of a pre-selected laboratory accredited for these analyses and with prior experience from *DEMOCOPHES*.

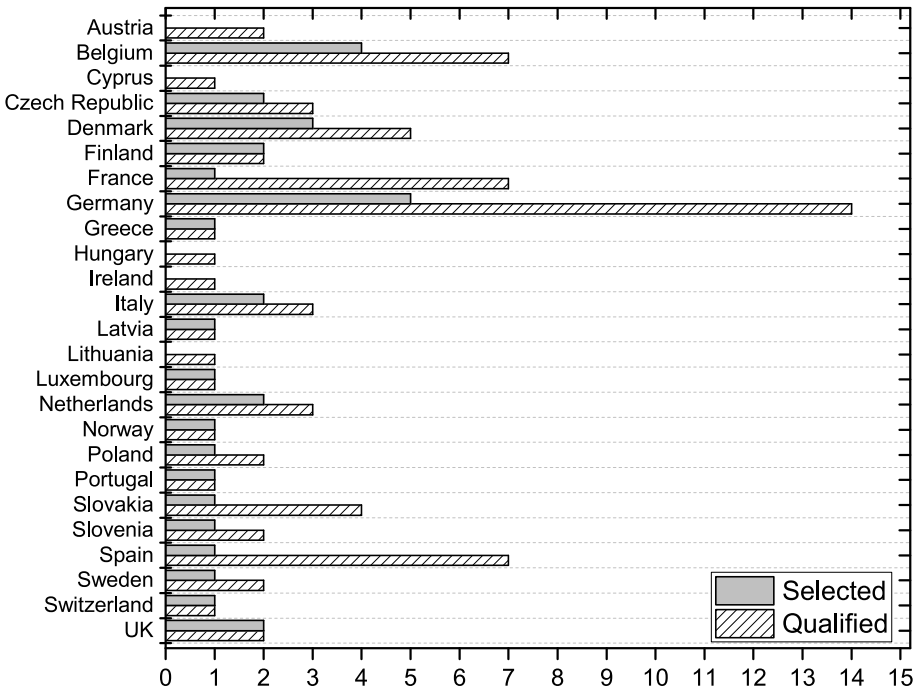


Fig. 4. Number of laboratories from different countries qualified and selected for the chemical analyses in HBM4EU.

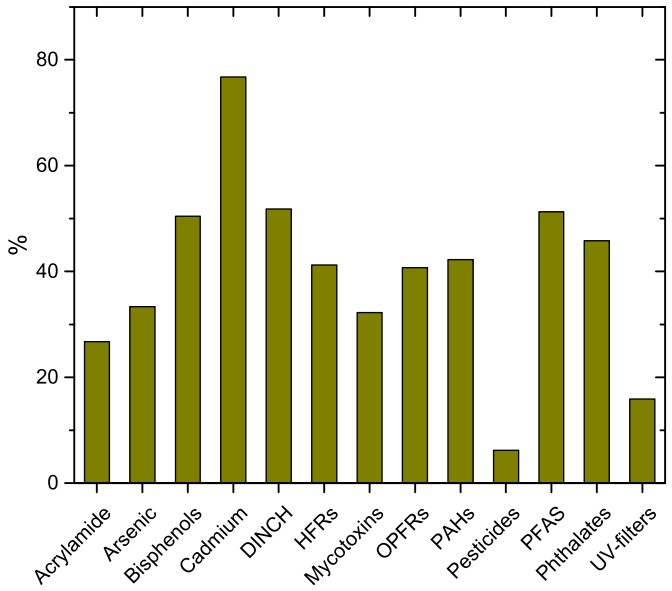


Fig. 5. Percentage of samples for different priority substances analysed by a laboratory of the same country as the sample owner, for the Aligned Studies and the time trend analyses. HFRs: Halogenated flame retardants. OPFRs: Organophosphorous flame retardants. PAHs: Polycyclic aromatic hydrocarbons. PFAS: Per- and polyfluoroalkyl substances.

many aspects of HBM4EU, including the material and data transfer. "Data" in this sense refers to (personal) data associated with the sample material, in contrast to (chemical) data as a result of the chemical analysis.

A detailed guidance document was developed in HBM4EU to ensure that procedures were in compliance with the General Data Protection Regulation (GDPR) of the European Union, also detailing terms and conditions for material transfer. This document contained a Material and Data Transfer Agreement form (Fig. S16 of the Supporting Information), to be completed by providers (sample owners) and recipients (qualified laboratories) and submitted to the ethics coordinator and HBM4EU coordinator (Knudsen et al., 2023). Establishing smooth workflows in this field proved challenging, probably reflective of an adaptation process, also including the translation of documents from national languages to English and vice versa, for example for the occupational exposure studies. An example of the difficulties encountered was the correct, standardized file naming, to ensure systematic entries in the database. Challenges related to ethics and GDPR were further discussed by Knudsen et al. (2023).

The extent of standardization in analytical chemistry is not a new question. As summarized in Table 3 and further discussed in Section 7,

Table 3
Details of analytical methods used by the qualified laboratories in HBM4EU.

Priority substance (group)	Matrix	Sample volume (mL)	LOQ (ng/mL)	Instrumental analysis
Acrylamide	Urine	0.1–2	1–5	LC-MS/MS
Arsenic	Urine	0.7–1	0.1–0.6	ICP-MS
Bisphenols	Urine	0.2–5	0.01–0.7	LC-MS/MS, GC-MS/MS
Cadmium	Urine	0.2–10	0.001–0.5	AAS, ICP-MS
Chromium	Blood, Urine	0.2–5	0.028–2.5	AAS, ICP-MS
DINCH	Urine	0.2–2	0.05–0.7	LC-MS/MS
Halogenated flame retardants	Serum	0.2–5	0.0001–2	GC-MS, GC-MS/MS, GC-HRMS
Mycotoxins	Urine	1–3	0.05–0.5	LC-MS/MS, LC-HRMS/MS
Organophosphorous flame retardants	Urine	0.3–5	0.02–0.5	LC-MS/MS, GC-MS/MS
Per- and polyfluoroalkyl substances (PFAS)	Serum	0.05–5	0.01–0.5	LC-MS/MS, LC-HRMS/MS
Pesticides	Urine	0.05–5	0.1–0.6	LC-MS/MS, GC-MS/MS
Phthalates	Urine	0.2–5	0.1–3.5	LC-MS/MS
Polycyclic aromatic hydrocarbons (PAHs)	Urine	0.5–11	0.001–6	LC-MS/MS, GC-MS, GC-MS/MS
UV filters	Urine	0.1–1	0.01–0.2	LC-MS/MS

LC: Liquid chromatography. MS: Mass spectrometry. ICP: Inductively coupled plasma. AAS: Atomic absorption spectroscopy. GC: Gas chromatography. HRMS: High resolution mass spectrometry.

different analytical methods were applied in HBM4EU. It is common practice in chemical monitoring programmes that laboratories follow general guidelines (e.g. OSPAR, 2016; EFSA, 2022), but keep some flexibility with regard to specific methods, as long as the quality of the data is ensured. Typically, laboratories document satisfactory performance in their chemical analyses by participation in externally organised proficiency testing schemes, in the same way as the ICIs and EQUAS organised in HBM4EU, and/or the analysis of certified reference materials (Arnaud et al., 2020; Göen et al., 2012a). This approach aims at the harmonization rather than the standardization of analytical methods and was the preferred approach in a multicentre HBM study like HBM4EU.

However, details in the analytical methods might require a higher degree of standardization to ensure comparability, for example the calculation of limits of detection (LODs) and LOQs, the use of either LODs or LOQs as well as the handling of concentrations below LOQs. For many chemicals, exposure levels of the general population are low or cover a relatively large range from low to higher concentrations (Göen et al., 2012b). How LOQs are defined and whether values below LOQs are considered as lower, medium or upper bound concentrations (EU, 2014; 2017), or assigned a different value, can therefore have an impact on the overall exposure level that is reported and assessed.

In addition, variability in LOQs can cause challenges in the comparability and aggregation of results (Table 3). This was experienced in the HBM4EU chromate study, where differences in LOQs in blood analyses of Cr led to considerable differences in detection frequencies between samples analysed in different laboratories (Galea et al., 2021; Ndaw et al., 2022). The variability was mainly a result of differences in the sensitivity of the analytical method, although differences in calculation methods also contributed to it.

5.2. Financial procedures

Since the chemical analyses were conducted in the framework of HBM4EU, an EU Horizon 2020 project, their invoicing followed the overall financial rules of HBM4EU. These were perceived as complex by many of the sample owners and qualified laboratories, especially the rules related to the difference in reimbursement rates between chemical analyses (50%) and other work in HBM4EU (70%). In addition, many laboratories were used to providing a total price for a service, but were now expected to differentiate person months and direct costs for the different chemical analyses. AU and other task leaders received many questions requesting clarifications on these matters. In response, the HBM4EU coordinator took the initiative, in collaboration with the relevant WP and task leaders, to prepare a guidance document that explained the administrative procedures of correct invoicing (Fig. S17 in the Supporting Information). It distinguishes three main cases:

- The sample owner and qualified laboratory are identical (i.e. in-house analysis)
- The qualified laboratory is a partner in HBM4EU
- The qualified laboratory is outside of HBM4EU (i.e subcontracting)

The second case occurred most frequently and was addressed by a budget transfer from the sample owner to the qualified laboratory, as approved by AU as part of the coordination process. However, this held the challenges that a) co-financing was necessary to cover the qualified laboratory's expenses, by either of the two partners, i.e. sample owner or laboratory, or another source, and b) a budget had to be allocated to the sample owner before the actual costs of analyses were known, as this information was collected as part of the analytical phase (Fig. 3). In order to work with realistic estimates, a survey was conducted in the first year of HBM4EU, to collect preliminary information on prices for chemical analyses. This led to a situation where some qualified laboratories felt that they were providing the same type of information repeatedly during the course of HBM4EU.

Clarifying the situation about invoicing and co-financing resulted in some delays in starting the chemical analyses, due to a combination of factors. The problems had to be understood in detail, several partners with leading functions in HBM4EU had to be involved, and a specific guidance document had to be prepared. Given the importance of this document, it passed several rounds of comments and adjustments, prior to broader communication to the sample owners and qualified laboratories.

5.3. Non-qualified laboratories

Although the prerequisite of passing the QA/QC programme to be eligible for analyses in HBM4EU and the associated criteria were clearly communicated at all levels of HBM4EU, a few analyses during HBM4EU were conducted by non-qualified laboratories (Table 4). These were usually laboratories that were qualified for other analyses in HBM4EU, possibly analysing the same samples for other priority substances, and adding more biomarkers from a cost-benefit perspective. Thus, these analyses were usually an "add-on" and did not result in a loss of information. As documented in Table 4, this was limited to very few analyses in the overall project, accounting for 2.4% of all analyses. Therefore, the main challenge was related to noticing this issue and communicating it efficiently to the downstream process (Fig. 1). These data were flagged as not quality assured through the HBM4EU QA/QC programme and disregarded in the calculations of European exposure values and geographical comparisons, as detailed by Govarts et al. (2023). This is different from the case of pre-HBM4EU data, for example for the time trend analyses, which were included if evaluated as being of acceptable quality. This was the case if the laboratory qualified in the HBM4EU QA/QC programme using the same method and documented continuous internal QA/QC measures (Govarts et al., 2023).

Furthermore, some biomarkers were novel and/or used on a more exploratory basis. In these cases, they were not covered by the full QA/QC programme and some pragmatic approaches had to be chosen to ensure analytical quality and comparability. This was the case for chromium analyses in exhaled breath condensate in the occupational studies, for which a small interlaboratory comparison was performed among the laboratories involved in these analyses (Leese et al., 2023).

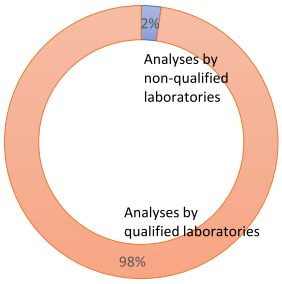
5.4. Capacity loss during the Covid-19 pandemic

The Covid-19 pandemic affected all partners in HBM4EU and caused delays in all project-related activities, in particular in sampling campaigns and laboratory work. Most research institutions and laboratories were shut down in spring 2020 and resumed work with varying capacity at different time points. However, as only few laboratories returned to full capacity immediately and all had to catch up with analyses that had been postponed in spring 2020, delays expanded. This situation required frequent contacts to sample owners and laboratories, to stay up-to-date with developments in each country and each laboratory and institution and to assess the implications for the overall work plan in HBM4EU. As Covid-19 countermeasures varied for each country and over time, these contacts and regular updates resulted in substantial additional work, which had not been foreseen in the planning of the analytical phase.

In addition to the regular progress reports, AU prepared "corona crises analysis" tables for the information of leaders in HBM4EU as well as the HBM4EU Management and Governing Boards. It soon became apparent that the Covid-19 related delays would have effects on the completion of the overall projects, as analytical results would be available later than anticipated. Based on updated information on the progress of the analytical phase, and on developments in the Covid-19 related effects on laboratory capacity, the HBM4EU Governing Board opted for a six months' extension of HBM4EU.

Table 4
Summary of analyses conducted by non-qualified laboratories during HBM4EU.

Study	Study group	Priority substances	Matrix	Analyses
HBM4EU Aligned Studies	Teenagers	PFAS[a]	Serum	300
	Teenagers	Arsenic	Urine	300
	Adults	Mycotoxins	Urine	200
Comparison of different time points, including *DEMOCOPHES* samples	Adults	OPFRs[b]	Urine	94
	Children	OPFRs[b]	Urine	99



[a] PFAS: Per- and polyfluoroalkyl substances
[b] OPFRs: Organophosphorous flame retardants

6. Lessons learnt

In general, the lessons learnt are connected to the fact that HBM4EU was a very large and ambitious project whose partners had different points of departures, in terms of previous experience. Reaching the stage of a harmonized and standardized HBM initiative across Europe was an ambitious goal and an achievement in itself.

6.1. Time buffers

Despite many years of experience in the field, a risk remains of underestimating the time required to implement certain steps in a new project. The size and diversity of HBM4EU amplified these usual time requirements. Changing established routines or building up new workflows in standardization attempts was more difficult and time-consuming than expected. It is an obvious and slightly banal lesson that time estimates should be conservative, including buffers that also allow newcomers in the field to catch up with experienced partners. However, it remains challenging to implement more generous timelines while keeping up with the rapid international development in research and monitoring, including ambitions of leading the development in some fields, as well as responding to urgent data needs for risk assessment and regulatory purposes.

6.2. Administrative guidance

The administrative and financial side of the analytical phase in HBM4EU was generally considered complex. To avoid confusions and delays, guidance should be developed and provided a priori. A help desk function was included in the WP for QA/QC and chemical analyses, which would probably benefit from an administrative counterpart, preferably staffed with administrative and financial experts rather than scientists. In general, the categorization of activities with different internal funding rates should be avoided. A uniform funding rate would have precluded the substantial additional administrative effort experienced in HBM4EU (Kolossa-Gehring et al., 2023).

6.3. Standardization

In addition to the standardized procedures around ethics and the standardization of technical elements such as LOQs, the coordination of the analytical phase could also be developed towards more standardization, provided that the HBM programme has a more permanent structure. While the same forms were used for regular updates in the phase connecting the sample owners and qualified laboratories (Fig. 3), also providing recognizability for the recipients, the monitoring of the chemical analyses was still mainly based on one-to-one correspondence. This was useful in the establishment of HBM4EU, but could be replaced by more standardized forms in a long-term perspective. Similarly, while progress reports had a recognizable format, they were prepared at varying intervals and would benefit from more regularity, perhaps aligned with HBM4EU Management Board meetings. Flexibility in the communication will still be important, to allow discussions of partner-specific questions and concerns, but developments towards SOPs in the coordination of the chemical analyses could be an option.

6.4. Connection to ethics

Although not included in the original concept (Fig. 1), it proved useful and efficient to collaborate with the ethics coordinator and to assist with the filing of Material and Data Transfer Agreements. As coordinator of the analytical phase, AU was in regular contact with sample owners and qualified laboratories and could use these communication channels to follow up on information required elsewhere in HBM4EU. In general, it is worth considering how to focus the communication, so partners do not feel that they receive uncoordinated and potentially duplicate requests. Shared sites for document exchange and communication could be an improvement to e-mail-based communication. It will be important to optimize communication both between different parts of the project and over time.

6.5. Capacity building

During HBM4EU, an increasing number of laboratories participated in the HBM4EU QA/QC scheme and obtained satisfactory results, documenting an increase in the HBM analytical capacity in Europe (Esteban López et al., 2021). However, approximately one third of the chemical analyses were conducted by only three European laboratories, leaving room for a wider implementation of high-quality HBM analyses. This extension may require a first analysis of existing obstacles. Training activities were included in HBM4EU (Kolossa-Gehring et al., 2023), but would benefit from more continuous and focused initiatives to improve technical capabilities and overcome potential obstacles. Capacity building could be linked to a set of minimum performance criteria for an HBM programme, including satisfactory results in regular proficiency testing and sufficiently low LOQs to avoid discrepancies in detection frequencies.

7. A network of laboratories – discussion of the HBM4EU experience

Different strategies exist for chemical analyses in HBM programmes around the world. In the US National Health and Nutrition Examination Survey (NHANES), for example, the analyses are centralised at the Environmental Health Laboratory of the Centers for Disease Control and Prevention (CDC) (CDC, 2022). HBM4EU has chosen a decentralized approach in its analytical phase, reflecting the European diversity as well as the wish to build transnational capacity in the field of HBM

analyses. In addition, an unprecedented high number of analyses had to be completed in a relatively short time frame, which was not possible for a single laboratory. Obviously, this strategy required a higher degree of coordination, in addition to the QA/QC programme, to ensure high-quality and comparable results as well as administrative clarity. However, many of the coordination efforts were related to the fact that HBM4EU was new and to the unexpected challenges of Covid-19 during the analytical phase. As discussed in Section 6, communication between partners could be more streamlined in an established and more permanent programme. This would reduce the correspondence that was necessary in coordinating the chemical analyses in HBM4EU.

Regarding efficiency, the network of laboratories carrying out the chemical analyses in HBM4EU has advantages and disadvantages. Laboratories that had successfully participated in the HBM4EU QA/QC programme could start the chemical analyses immediately without further method development. Distributing the work amongst several expert laboratories, according to their reported capacities, increased efficiency. On the other hand, the data analysis in HBM4EU was dependent on complete datasets, meaning that potential delays in one single laboratory carried the risk of delaying the whole downstream data analysis process. The interlinkages and inter-dependencies might need stronger emphasis in the communication with the participating laboratories. However, the different timelines between laboratories were also related to the upstream processes, which provided samples from the HBM4EU Aligned Studies at different points in the HBM4EU project.

The fact that HBM4EU only needed a six months' extension to complete its work plan, including the chemical analyses, indicates a robust design and an efficient steering that was not severely affected by Covid-19 restrictions. After the first wave in spring 2020, Covid-19 countermeasures began to vary between countries and over time, ranging from temporary lockdowns to near-normal work routines. This diversity made the decentralized analytical strategy more robust than the concentration on few expert laboratories would have been. Some progress was always possible with the chemical analyses, and the close contact between laboratories, sample owners and the coordinators for the analytical phase ensured regular updates and individually optimized solutions.

For some of the priority substances, a variety of methods was applied by the 75 laboratories qualified for the chemical analyses in HBM4EU (Table 3). Most suitable analytical methods had been discussed and recommended in HBM4EU (Vorkamp et al., 2021), but the laboratories were free to use a method of their choice provided it had generated satisfactory results in the HBM4EU QA/QC programme (Esteban López et al., 2021). This diversity of methods increased robustness and might also favour methodological developments as different methods are tested and optimized. In addition, it has a strong capacity building component since laboratories can learn from each-other and implement procedures needed for chemical monitoring. Laboratories with less experience in HBM analyses were given the opportunity to establish and improve their analytical capabilities. However, some method standardization may be advisable, for example in terms of minimum performance criteria, as discussed in Section 5. Furthermore, the harmonization of different methods requires external QC, in terms of regular proficiency testing exercises and certified reference materials. Given the large number of compounds and laboratories, this is a considerable effort, but with the obvious benefit of creating long-term structures for coordinated and harmonized HBM chemical analyses in Europe.

8. Conclusions and outlook

The analytical phase in HBM4EU included a large number of participants in terms of sample owners (providing samples to the HBM4EU Aligned Studies from national and regional cohorts and collections) and laboratories having passed a comprehensive QA/QC scheme to qualify for chemical analyses in HBM4EU. This required a high degree of coordination, also ensuring connections to upstream and downstream processes in HBM4EU, i.e. the preparation of the analytical phase and the data treatment, respectively. A central coordination point was essential in HBM4EU, also regarding the unexpected challenge of managing consequences of Covid-19 measures. Given the novelty and complexity of the HBM4EU project, it initially operated largely on an individualized communication basis. There is potential to further develop streamlining and standardization of the coordination process in a long-term and consolidated programme, in close collaboration with experts in administrative, financial, ethical as well as data-related questions.

The decentralized approach of chemical analyses involving a network of laboratories appears to be the best solution for a European HBM programme, generating high-quality and comparable data in a harmonized, efficient and robust framework. It has the potential to be consolidated in a group of national and European reference laboratories in the HBM field. Certain aspects of the chemical analyses, for example LOD and LOQ calculations, would benefit from more standardization, and a set of minimum performance criteria will ensure better comparability between laboratories. Thus, the coordination of the chemical analyses should be linked to general QA/QC questions, as addressed in the HBM4EU QAU. Regular proficiency testing and certified reference materials for HBM are points where more discussion has been initiated to overcome current lacks.

Combining and formalizing the chemistry-related structural elements of HBM4EU, such as the laboratory network, the QA/QC programme, the QAU and the coordination of the chemical analyses, would create a cornerstone of a European HBM programme. These structures should be sufficiently flexible to include possibilities of extensions, towards other chemical substances, novel biomarkers and emerging scientific questions. An obvious extension could be the connection to chemical analyses in exposure media and the environment, as envisaged in the Horizon Europe Partnership for the Assessment of Risks from Chemicals (PARC).

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Appendix A. Supplementary data

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Decay rate estimation of respiratory viruses in aerosols and on surfaces under different environmental conditions

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ABSTRACT

Majority of the viral outbreaks are super-spreading events established within 2–10 h, dependent on a critical time interval for successful transmission between humans, which is governed by the decay rates of viruses. To evaluate the decay rates of respiratory viruses over a short span, we calculated their decay rate values for various surfaces and aerosols. We applied Bayesian regression and ridge regression and determined the best estimation for respiratory viruses, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), severe acute respiratory syndrome coronavirus (SARS-CoV), middle east respiratory syndrome coronavirus (MERS-CoV), influenza viruses, and respiratory syncytial virus (RSV); the decay rate values in aerosols for these viruses were 4.83 ± 5.70, 0.40 ± 0.24, 0.11 ± 0.04, 2.43 ± 5.94, and 1.00 ± 0.50 h^{-1}, respectively. The highest decay rate values for each virus type differed according to the surface type. According to the model performance criteria, the Bayesian regression model was better for SARS-CoV-2 and influenza viruses, whereas ridge regression was better for SARS-CoV and MERS-CoV. A simulation using a better estimation will help us find effective non-pharmaceutical interventions to control virus transmissions.

1. Introduction

Emerging infectious diseases have a significant impact on public health and economies (Jones et al., 2008). Approximately 15 of 57 million (>25%) annual global deaths are related to infectious diseases (Morens et al. 2010). Out of these, the majority (3.96 million) are due to respiratory infections (Morens et al., 2010). Acute respiratory diseases are the most widely reported infections among individuals of all age groups (Monto 2002). Respiratory viruses replicate in the respiratory tract and are subsequently transmitted by respiratory secretions, causing infections ranging from asymptomatic to symptomatic (Kutter et al., 2018; To et al., 2020; Zhao et al., 2020). Respiratory tract infections are caused by various respiratory viruses, including severe acute respiratory virus coronavirus 2 (SARS-CoV-2), severe acute respiratory syndrome coronavirus (SARS-CoV) (Casanova et al., 2010; Darnell and Taylor 2006; Lai et al. 2005), Middle East respiratory syndrome (MERS-CoV) (Chu et al., 2020; van Doremalen et al., 2021; Gabutti et al., 2020; Su et al., 2016), influenza viruses (Harper, 1961; Hirose et al., 2022; Izumikawa, 2019; Sutton et al., 2013), and respiratory syncytial virus (RSV) (Moreira et al., 2018; Paynter 2015).

Most cases of outbreaks are associated with indoor environments (Chau et al., 2021; Qian et al., 2021). Superspreading events have been reported in indoor environments such as restaurants (Y. Li et al., 2020; Majra et al., 2021; Qian et al., 2021), households (W. Li et al., 2020; Qian et al., 2021), buses (Majra et al., 2021; Mangili and Gendreau 2005; Shen et al., 2020; Tsuchihashi et al., 2021), airplanes (Flight et al., 2020; Mangili and Gendreau 2005), trains (Pestre et al., 2012), classrooms (Charlotte 2020; Rothamer et al., 2020), and healthcare facilities (Bin et al., 2015). Most transmissions led to clusters within an exposure period of less than 12 h. For instance, during a 10-h nonstop commercial flight, a cluster of 16 infected individuals was reported from one probable index case (Flight et al., 2020). The above-mentioned studies concluded that clusters of infections were established within 2–10 h, highlighting that a critical time interval for successful transmission may

Abbreviations: SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; SARS-CoV, severe acute respiratory syndrome coronavirus; MERS-CoV, middle east respiratory syndrome coronavirus; RSV, respiratory syncytial virus; VIF, variance inflation factor; AIC, Akaike's information criterion; RH, Relative humidity.

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be governed by the decay rate of the viruses in indoor environments.

Infected individuals expel large droplets and aerosols into the air via expiratory activities such as coughing, sneezing, breathing, and speaking (Klompas et al., 2021; Yin et al., 2022). There are three dominant modes of respiratory virus transmission (1) droplet transmission: the direct inhalation of relatively larger droplets (>5 μm); (2) airborne transmission: the inhalation of tiny droplets, aerosols, and droplet nuclei floating in the air (<5 μm); and (3) fomite transmission: viruses can remain viable on inanimate surfaces for hours to even days and cause indirect contact through fomites (Castaño et al., 2021; Chaudhuri et al. 2020; Delikhoon et al., 2021; Kutter et al., 2018; Patel et al., 2020; Pease et al., 2021). A previous study reported that the sizes of 87% of exhaled particles are less than 1 μm, emphasizing the importance of considering aerosol transmission in long-range transmissions (Zhang et al., 2020). Recently, the World Health Organization and U.S. Centers for Disease Control and Prevention have issued scientific declarations on the importance of aerosols in SARS-CoV-2 transmission (Klompas et al., 2021). Meanwhile, droplet transmission causes short-range transmission (<1 m), and these droplets remains in the air for a short period (<17 min) (Kutter et al., 2018). Droplets travel directly from the mouth or nose of the infected individual to the nostrils or mouth of susceptible individuals and cause deposition on the upper respiratory tract and mucous membranes (Arslan et al., 2020; Biryukov et al., 2020a; Kutter et al., 2018; Miller et al., 2021). Fomite transmission occurs by the rapid deposition of larger droplets on inanimate surfaces (Castaño et al., 2021; Karia et al., 2020). Influenza virus is predominantly transmitted via fomites (Nicas and Jones 2009) and causes infection by gaining entry via hands and subsequently through facial membranes such as the nose, mouth, or eyes.

Environmental factors such as temperature, humidity, and solar radiation can affect the stability of viruses in aerosols and surfaces (Casanova et al., 2010; Gamble et al., 2021; Paynter 2015; Schuit et al., 2020; Wood et al., 2010). The relative humidity (RH) around the surface can affect the evaporation rate and concentration of compounds such as salts and proteins in the droplets, which influence the decay rate of viruses (Guo et al., 2021). At a low humidity, owing to the high evaporation rate, respiratory droplets reduce in size and form tiny droplets and droplet nuclei (Paynter 2015). Conversely, respiratory droplets are larger at a high humidity and settle faster on surfaces because of their lower evaporation rate (Paynter 2015). Higher temperature and higher humidity levels have a synergistic effect on virus decay compared to lower temperature and humidity (Chan et al., 2011). However, few studies have indicated an increased daily incidence at lower temperatures and humidity (Chan et al., 2011). Additionally, solar radiation affects the viability of viruses in the environment; ultra violet light is a natural environmental virucide which disrupts viral replication by causing the formation of photodimers (Sutton et al., 2013). Therefore, determining viral decay rates under different environments is vital for adopting interventional strategies and decisions initiated by policymakers and government health authorities (Dublineau et al., 2011).

Although we found a few prediction modeling approaches used in wastewater epidemiology (Kadoya et al., 2021; Zhu et al., 2022) and virus transmission dynamics (Vuorinen et al., 2020), there have been limited modeling studies that predict viral decay rates in indoor environments (Guillier et al., 2020). To the best of our knowledge, no study has focused on viral decay rates at shorter time intervals or on virus-specific or surface-specific decay rate estimation based on environmental conditions. Therefore, the objectives of our study were to predict the decay rates of respiratory viruses (SARS-CoV-2, SARS-CoV, MERS-CoV, influenza virus, and RSV) and develop estimation models for decay rates on different surfaces under diverse environmental conditions (temperature, relative humidity, and solar radiation), which would help determine effective control measures.

2. Materials and methods

2.1. Article screening

Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines used in clinical medicine were employed to identify the articles providing details about the decay rates of viruses (Fig. 1) (Moher et al., 2009). Here we focused on specific respiratory viruses, including SARS-CoV-2, SARS-CoV, influenza viruses, MERS-CoV, and RSV. Articles were screened using the Google Scholar and PubMed search engines. Articles collected from PubMed coincided with those collected from Google Scholar; therefore, the articles mentioned hereafter are based on the results from Google Scholar. Articles were screened between March 2021 and January 2022. The keywords used were "X" AND ("inactivation" OR "decay" OR "stability" OR "viability") AND ("surface" OR "environment") where X was each virus type ("SARS-CoV-2," "SARS-CoV," "Influenza," "MERS-CoV," or "RSV"). Several criteria were considered before selecting the eligible papers. The inclusion criteria were (1) not a review paper; (2) a peer-reviewed paper; (3) published in English; and (4) contained experimental data on the decay of the target virus and presentation of the extractable data related to the virus decay rate (containing data for at least two-time points).

During the first screening, non-English articles, dissertations, book chapters, and conference reports were eliminated from the collected articles. After the first screening, the titles of the selected papers were checked. Then, the abstracts of those articles were further evaluated to check whether the selected paper could specify the decay rate values of the target virus. The assembled articles were then subjected to a full-text review for data extraction.

The select eligible articles were then used to calculate viral decay rates using a first-order reaction. In a first-order reaction, the rate of the reaction is proportional to the concentration of the reacting substance (HIATT 1964). The exponential form of the decay is as follows:

$$\frac{C}{C_0} = e^{-kt} \tag{1}$$

since

$$\log\frac{C}{C_0} = -\frac{kt}{2.303} \tag{2}$$

where C_0 is the initial virus concentration ($TCID_{50}$/ml or PFU/ml), C is the virus concentration at time t, t is the duration of the virus on the surface or in the aerosol, and k is the virus decay rate on the surfaces or in the aerosols. The decay rate was calculated based on the ratio proportion of the virus concentration to the initial concentration. When the plot of log C/C_0 versus time is a straight line with a slope of $k/2.303$, the decay rate is given by the following equation:

$$k = -\frac{2.303\log C/C_0}{t} \tag{3}$$

The decay rate was calculated as the regression slope of ln (C/C_0) versus time (in hours) using linear least-squares regression. The R^2 and p values of each curve were reported separately. Out of the calculated decay rates from the eligible articles, decay rates with $p < 0.1$ were used in this study. If k was determined by the regression slope of log (C/C_0) versus time (in hours), it was transformed into ln (C/C_0). If a study presented first-order k values, they were directly extracted from the paper, along with any reported errors or R^2 values. Unit conversions were appropriately performed where needed. The calculated decay rates were then transformed into $\log_{10}$ values; this would enable readers to make intuitive comparisons of the decay rates, which differ by several orders of magnitude owing to differences in experimental conditions. Several methods, such as cell culture and PCR techniques (Bischoff et al., 2013; Johnson et al., 2021; Jones et al., 2020; Lindsley et al., 2015),

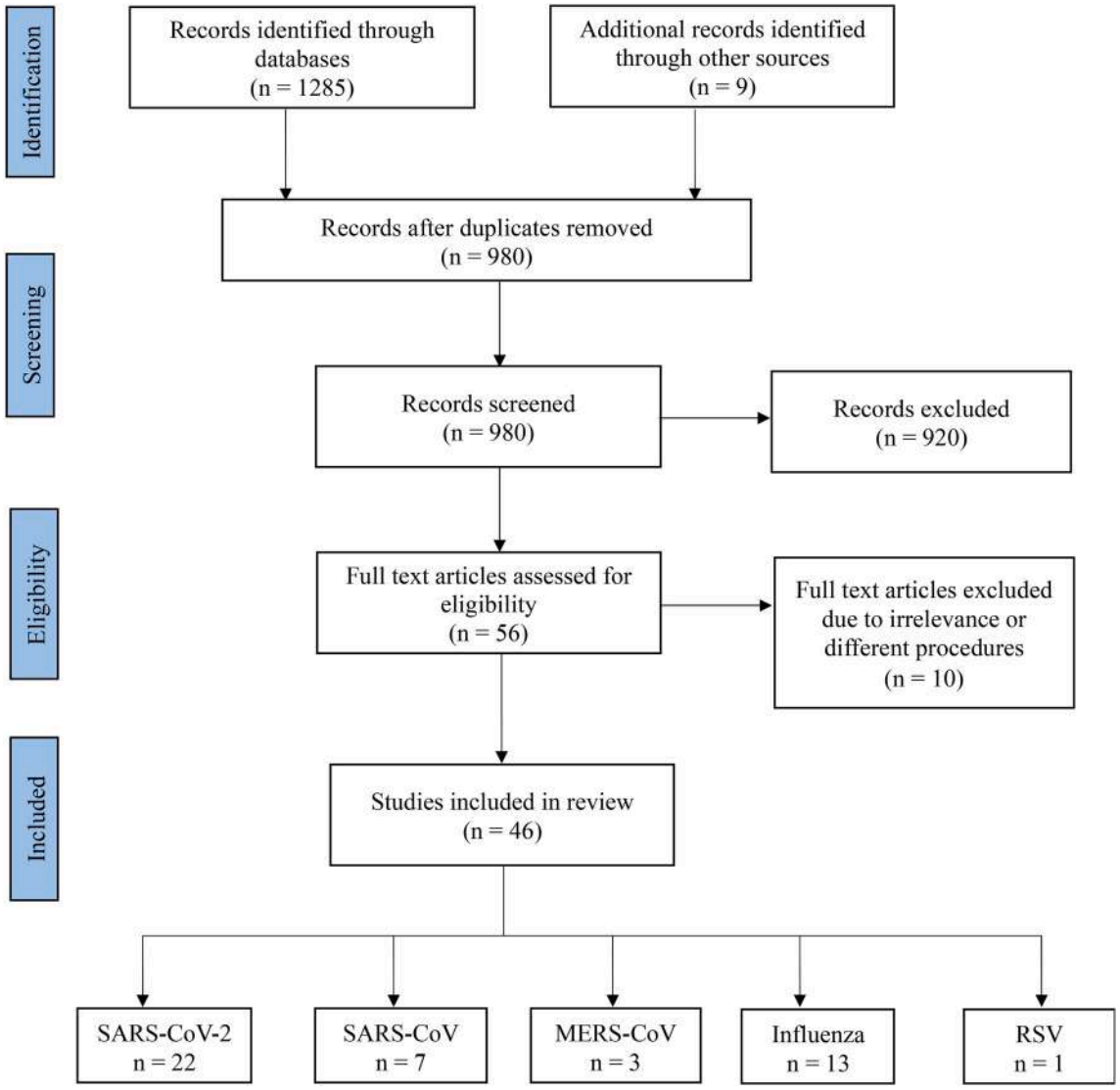

Fig. 1. Flowchart depicting the literature selection steps followed in this study.

have been used to determine viral concentrations in suspensions. In this study, we used experimental data generated from cell culture methods ($TCID_{50}$/mL or PFU/mL) to calculate decay rates using Equation (3).

The first-order decay rate (k) in units per hour (h^{-1}) was calculated for a maximum period of 12 h. If the values of the first-order decay rate were not provided in the papers, but the plots were available as the virus concentration changed over time, the virus concentration for each time point of the curve was digitized using the Web plot digitizer version (AssessmentUS EPA National Center, 2009).

In addition to the decay rate values of the target virus, information related to the surface type and environmental conditions (temperature, humidity, and solar radiation) were collected separately for each virus. For example, if a range of temperature or humidity range was provided, the given mean of the reported range was recorded. To address the environmental differences in each experiment (temperature, relative humidity, and solar radiation), we included temperature and humidity as continuous variables which can reflect the experimental conditions. In addition, due to the lack of quantitative information in the previous studies, we assumed solar radiation as a categorical variable to reflect the presence and absence of solar radiation for data input in the simulation and predictions. Before model development, the multicollinearity of the variables was evaluated using the variance inflation factor (VIF). In basic regularized regression analyses, the multicollinearity problem is solved so that all the explanatory variables can be used in the model (Kadoya et al., 2021).

Bayesian formulations are widely used in diverse environmental applications. There are several advantages over other data aggregation methods: Bayesian approach incorporates quantitative prior information about the distributions and range of model parameters or measurements. All parameters are treated as probability distributions. This approach uses whole dataset simultaneously to fit the model parameters, which share details across datasets and minimize the uncertainty by up to 95% (Britten et al., 2021). To address the multicollinearity, ridge regression shrinks the absolute values of the coefficients of the model variables, which all explanatory variables can be used (Kadoya et al., 2021). Thus, two modeling approaches—ridge regression and the Bayesian regression model —were used in this study.

2.2. Bayesian regression model

We used a Bayesian regression model based on the Markov Chain Monte Carlo method (MCMC) (Kadoya et al., 2021). Before developing the Bayesian regression model, probability distributions of decay rates were determined using Akaike's information criterion (AIC) using the "fitdistrplus" package in the R software. External factors affecting viral decay rates were considered explanatory variables: environmental

factors (temperature, relative humidity, and solar radiation) and surface type (Chan et al., 2020; Raiteux et al., 2021; Rockey et al., 2020). Categorical variables (solar radiation; surface type: aerosol, paper, steel, banknote, mask, skin, cloth, cardboard, plastic, glass, and metal) were assigned a value of 1 (with sunlight or with assigned surface type) or 0 (without sunlight or assigned surface type). The relationship between the variables was expressed as shown in equation (4). The model parameter coefficients were then estimated using an MCMC-based Bayesian regression model. The structure of the Bayesian regression model used in this study is shown in Fig. S1.

$$\text{Population parameter of decay rate} = a + b \cdot \text{Temperature} + c \cdot \text{Humidity} + d \cdot \text{Solar radiation} + e \cdot \text{Aerosol} + f \cdot \text{Paper} + g \cdot \text{Steel} + h \cdot \text{Bank note} + i \cdot \text{Mask} + j \cdot \text{Skin} + k \cdot \text{Cloth} + l \cdot \text{Cardboard} + m \cdot \text{Plastic} + n \cdot \text{Glass} \quad (4)$$

$$X(a\text{-}n) \sim \text{Normal}\ (\mu(a\text{-}n), \sigma(a\text{-}n)) \quad (5)$$

where X represents the intercept (a) and coefficients for each variable named from b to n, μ represents mean and σ represents standard deviation. Based on data availability, the number of explanatory variables (types of surfaces) was selected for the model. Therefore, the total number of explanatory variables used in the models varied for each virus type. We ran 4000 simulations to ensure the model convergence for all parameters. The statistical analysis and parameter estimation were performed using the statistical software R (version 3.6.1) and R Stan (Stan Development Team (2022). "RStan: the R interface to Stan." R package version 2.21.7 n.d.; Statistical and computing. R Foundation for Statistical Computing, Vienna n.d.). The R code for the Bayesian regression model is available in the supplementary materials (code S1).

2.3. Ridge regression model

There are three basic regularized regression analyses—lasso, elastic net, and ridge regression (Kadoya et al., 2020). Regularization helps us avoid multicollinearity and model overfitting, which ensures generalization of new data (Alkinani et al., 2021; Kadoya et al., 2020). Lasso and elastic net are sparse estimation methods that select essential variables in the model (Kadoya et al., 2020; Oishi et al., 2021); ridge regression, on the other hand, uses all explanatory variables. Furthermore, in contrast to ordinary least squares regression, which reduces the sum of the square residuals between observed and predicted values, ridge regression minimizes model overfitting by adding a penalty to the size of the coefficients (Alkinani et al., 2021).

$$L(\widehat{\mathscr{B}}) = \sum_{i=1}^{n} (y_i - x_i\widehat{\mathscr{B}})^2 + \lambda \sum_{j=1}^{m} \widehat{\mathscr{B}}_j^{\,2} = \left\| y - X\widehat{\mathscr{B}} \right\|^2 + \lambda \|\widehat{\mathscr{B}}\|^2 \quad (6)$$

λ parameter is the regularization penalty. $L(\widehat{\mathscr{B}})$ gives ridge regression estimates, where x is explanatory variables (temperature, humidity, solar radiation, and surface type: aerosol, paper, steel etc.) and y is objective variable decay rates. When λ is zero, it is same as ordinary least square method. While larger the λ is stronger the coefficients' size penalized. In this study, decay rate estimation was performed using the "glmnet" package in the R software (version 3.6.1). The R code for the ridge regression model is given in the Supplementary Materials (code S2).

2.4. Model validation

We randomly divided the datasets into training (80%) and test (20%) datasets for each virus type to evaluate the prediction: for SARS-CoV-2 65 training data and 17 test data, for influenza viruses 33 training data and 9 test data, for SARS-CoV 14 training data and 4 test data, and for MERS -CoV 28 training data and 7 test data respectively. Explanatory variables were scaled before splitting the datasets. K-fold cross-validation was used to obtain robust and accurate results. Root mean square error (RMSE), mean absolute error (MAE) and area under the curve (AUC) were calculated separately for each model to evaluate the goodness of fit. In addition, R^2 values of the train and test data of the better estimation model were calculated to check whether model overfitted data.

3. Results

3.1. Article screening

We identified 1285 papers using Google Scholar and nine from other sources; 1226 articles were removed during the first screening. After further screening (Figs. 1), 46 articles were identified as eligible for further analysis. We identified 197 decay-rate values in this study. There were 82 decay rate values for SARS-CoV-2 (from 24 publications), 22 decay rate values for SARS-CoV (from 7 publications), 35 decay rate values for MERS-CoV (from 3 publications), 42 decay rate values for influenza virus (from 13 publications), and 16 decay rate values for RSV (from 1 publication). The identified decay rates were classified based on the virus and surface type.

The selected literature used in this study is indicated in Table S1. Across all the 197 values collected, decay rate values ranged between −1.69 and 1.46 $\log_{10}$ (h^{-1}) for all surfaces and aerosol (Fig. 2). The decay rate values of SARS-CoV-2 varied between −1.49 and 1.20 $\log_{10}$ (h^{-1}) on all surfaces and aerosols. The decay rate values of SARS-CoV varied between −1.26 and 1.39 $\log_{10}$ (h^{-1}) on all surfaces and aerosols. The decay rate values of MERS-CoV varied between −1.19 and 0.74 $\log_{10}$ (h^{-1}). The decay rate values of the influenza virus varied between −1.69 and 1.46 $\log_{10}$ (h^{-1}). RSV on aerosols decay rate varied between −0.33 and 0.34 $\log_{10}$ (h^{-1}).

Higher decay rate values were observed for RSV, SARS-CoV-2, and influenza virus than for MERS-CoV and SARS-CoV. Among the decay rate values collected for influenza viruses, there were articles related to the decay rate values of influenza A (H1N1, H7N1, and H3N1), influenza B, avian influenza (H9N2 and H6N1), and bovine influenza viruses. Based on our inclusion criteria, a limited number of articles were identified for each type of influenza virus. According to the International Committee on Virus Taxonomy, influenza A, influenza B, avian influenza, and bovine influenza are classified under the *Orthomyxoviridae* family. Therefore, in this study, the calculated decay rate values for influenza A, influenza B, avian influenza, and bovine influenza were classified into "influenza viruses."

Among the identified decay rate values, the authors used different

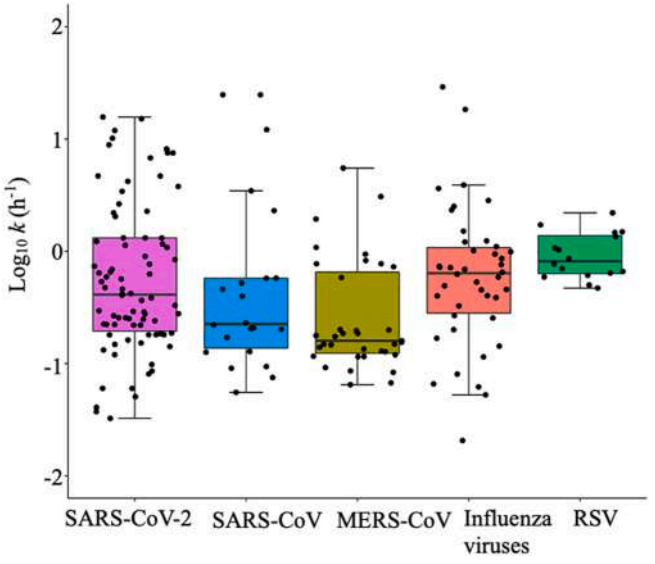

Fig. 2. $\log_{10}$ decay rates of different respiratory viruses from aerosols and other surfaces.

types of surfaces to determine the viability of viruses. Unfortunately, there were limited articles for each type of reported surface. Therefore, it is difficult to identify the surface-specific decay rate for each surface type used in these studies. Hence, we grouped the surfaces into different categories to use the maximum number of decay rates collected in this study. Plain paper, inkjet paper, inkjet photo paper, paper, magazine, and tissue paper were grouped into one category named "paper." Silver, copper, and metals were grouped into the category named "metals." Tyvek, polypropylene, soft toys, and plastic were grouped as "plastic." Surgical masks, N95 masks, and N100 masks were grouped as "masks." The $1 and $20 banknotes were grouped as "banknotes." Cotton, handkerchief, pajama, fabric, and clothing were grouped as "cloth." Accordingly, for this study, we considered 10 categories of surfaces in total (steel, plastic, cloth, glass, banknote, skin, metal, cardboard, paper, and mask).

Eighty-two decay rate values for SARS-CoV-2 were obtained using the Vero E6 cell lines. In addition to those for aerosols (n = 8), decay rate values were reported for a variety of surfaces, including paper (n = 4), steel (n = 32), plastic (n = 11), cloth (n = 2), glass (n = 8), banknotes (n = 4), skin (n = 3), metal (n = 1), cardboard (n = 2), ceramic (n = 1), and masks (n = 6). Twenty-two decay rate values for SARS-CoV were obtained using the Vero E6 and FRhK-4 cell lines. In addition to those for aerosols (n = 2), decay rate values were reported for metal (n = 2) and plastic (n = 18) surfaces. Forty-two decay rate values for influenza viruses were obtained using the Vero E6, MDCK, EBL, and HBE cell lines. In addition to those for aerosols (n = 32), decay rate values were reported for surfaces, including paper (n = 4), plastic (n = 3), and glass (n = 3). Thirty-five decay rate values for MERS-CoV were obtained using the Vero E6 cell lines. In addition to those for aerosols (n = 5), decay rate values were reported for various surfaces, including steel (n = 10), plastic (n = 7), and metal (n = 13). Sixteen decay rate values for RSV were obtained using the Vero E6 cell line for aerosols. Different culture mediums buffered solutions were used in experiments. Culture mediums were Dulbecco's modified Eagle's medium, Glascow minimum essential medium, tissue culture media, and simulated saliva. Buffered solution was phosphate buffered saline (PBS).

Fig. 2 illustrates the obtained distribution of all the decay rate values for each virus type under different environmental conditions (temperature, humidity, and solar radiation), matrices (aerosol, paper, steel, banknote, mask, skin, cloth, cardboard, plastic, glass, metal), enumeration methods (including $TCID_{50}$/ml and PFU/ml), and cell lines (Vero E6, Madin–Darby Canine Kidney, Human Bronchial Epithelial, and Embryonic Bovine Lung). It is important to note that there is a diverse distribution among the decay rate values for each type of virus and within a virus type.

3.2. Effects of environmental factors on virus decay rate

We represented the distribution of all collected decay rate values of respiratory viruses based on variations in temperature and relative humidity (Fig. 3).

Even within the same temperature/humidity range, there is a significant deviation in the decay rates by up to two orders of magnitude (Fig. 3), probably due to the coupling effect of different experimental conditions (Guo et al., 2021). In this study, we separately analyzed the effects of environmental factors on the coronaviruses SARS-CoV-2, SARS-CoV, and MERS-CoV. The effects of temperature and relative humidity on the decay rate of SARS-CoV-2 are shown in Fig. 4, and those of other coronaviruses (SARS-CoV and MERS-CoV) are shown in Figs. S2 and S3.

Based on the decay rate values collected in this study (Table S4), higher decay rate values for SARS-CoV-2 were reported for papers and aerosols. In contrast, the lowest decay rate values were reported for glass, plastic, and metal. For SARS-CoV, higher decay rate values were reported for glass and plastic, whereas the lowest decay rate values were reported for steel surfaces. For MERS-CoV, higher decay rate values were reported for aerosols, and the lowest decay rate values were reported for plastics and steel. In addition, higher decay rate values were reported for steel and paper for influenza viruses, whereas the lowest decay rate values were reported for aerosols and glass surfaces. For RSV, based on the identified decay rates for the aerosols in this study, decay rate values ranged between $-0.63\ h^{-1}$ and $0.34\ h^{-1}$ within a temperature range of 20.5–23.5 °C and a relative humidity range of 20–90%.

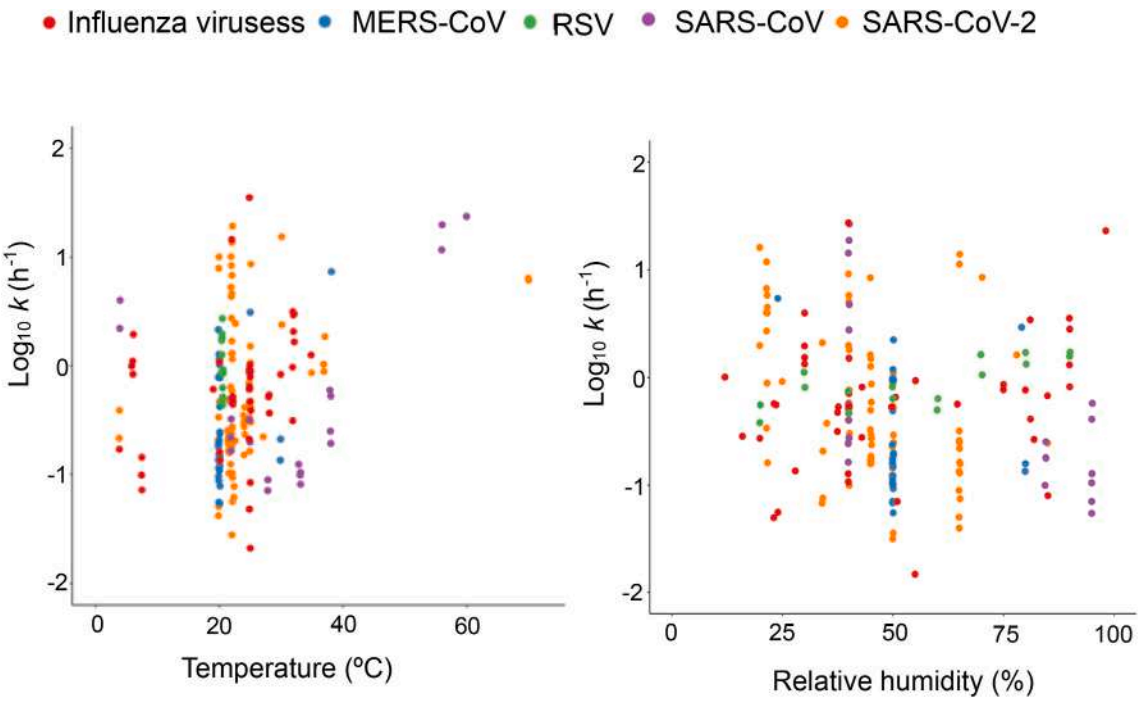

Fig. 3. Distribution of $\log_{10}$ viral decay rates based on environmental factors. (a) Temperature and (b) relative humidity.

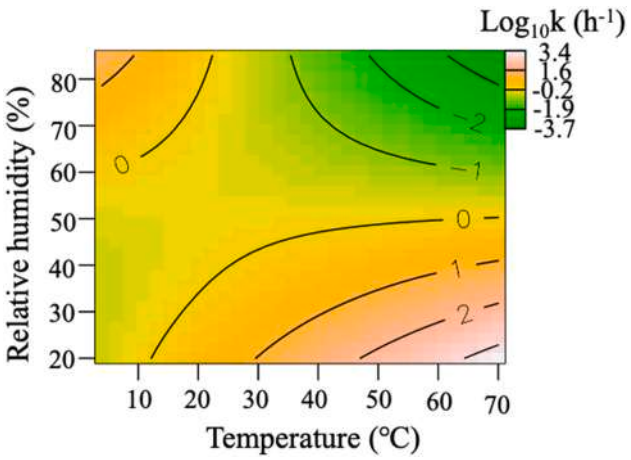

Fig. 4. Effects of environmental factors on the $\log_{10}$ decay rate values of SARS-CoV-2. Rate; contour plot illustrating the effects of relative humidity and temperature on the decay rate.

3.3. Bayesian regression model and ridge regression model estimation

We developed decay rate estimation models using Bayesian regression and ridge regression models. VIF values for the variables for each virus are presented in Table S3. AIC values of decay rates are presented in Table S2. Mean and standard deviation of the values are presented in Table S4. The observed and estimated values obtained using the Bayesian regression model are presented in Fig. 5, and the observed and estimated values obtained using the ridge regression are presented in Fig. 6. The model performance criteria values for the ridge regression and Bayesian regression models are presented in Table 1. R^2 values of the train and test data to check whether the model over-fitted data or not are presented in Table S11. Based on the model performance criteria values for SARS-CoV-2 and influenza viruses, the Bayesian regression model was the better estimation model; meanwhile, the ridge regression model could be considered the better estimation model for SARS-CoV and MERS-CoV. The coefficients of the best estimation models are shown in Fig. 7. Influenza viruses were not included, as there were no available decay rate values for a wide range of temperature and humidity coefficient values. Predicted decay rate values from the better estimation model and the relevant environmental conditions are presented in Table S5, S6 and S7 for SARS-CoV-2, SARS-CoV and MERS-CoV

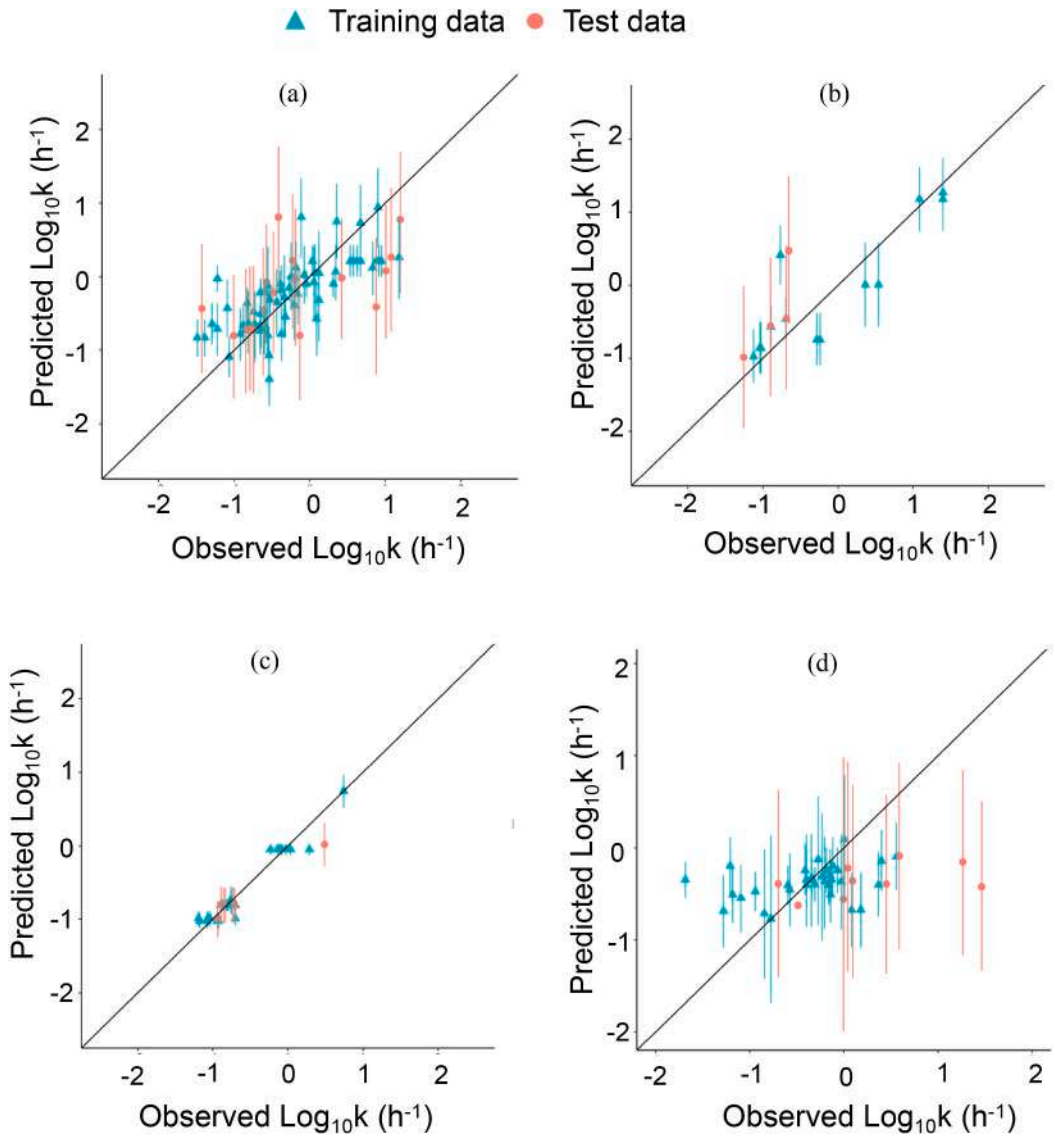

Fig. 5. Comparison of observed and predicted data using the Bayesian regression model. (a) SARS-CoV-2, (b) SARS-CoV, (c) MERS-CoV, and (d) influenza viruses.

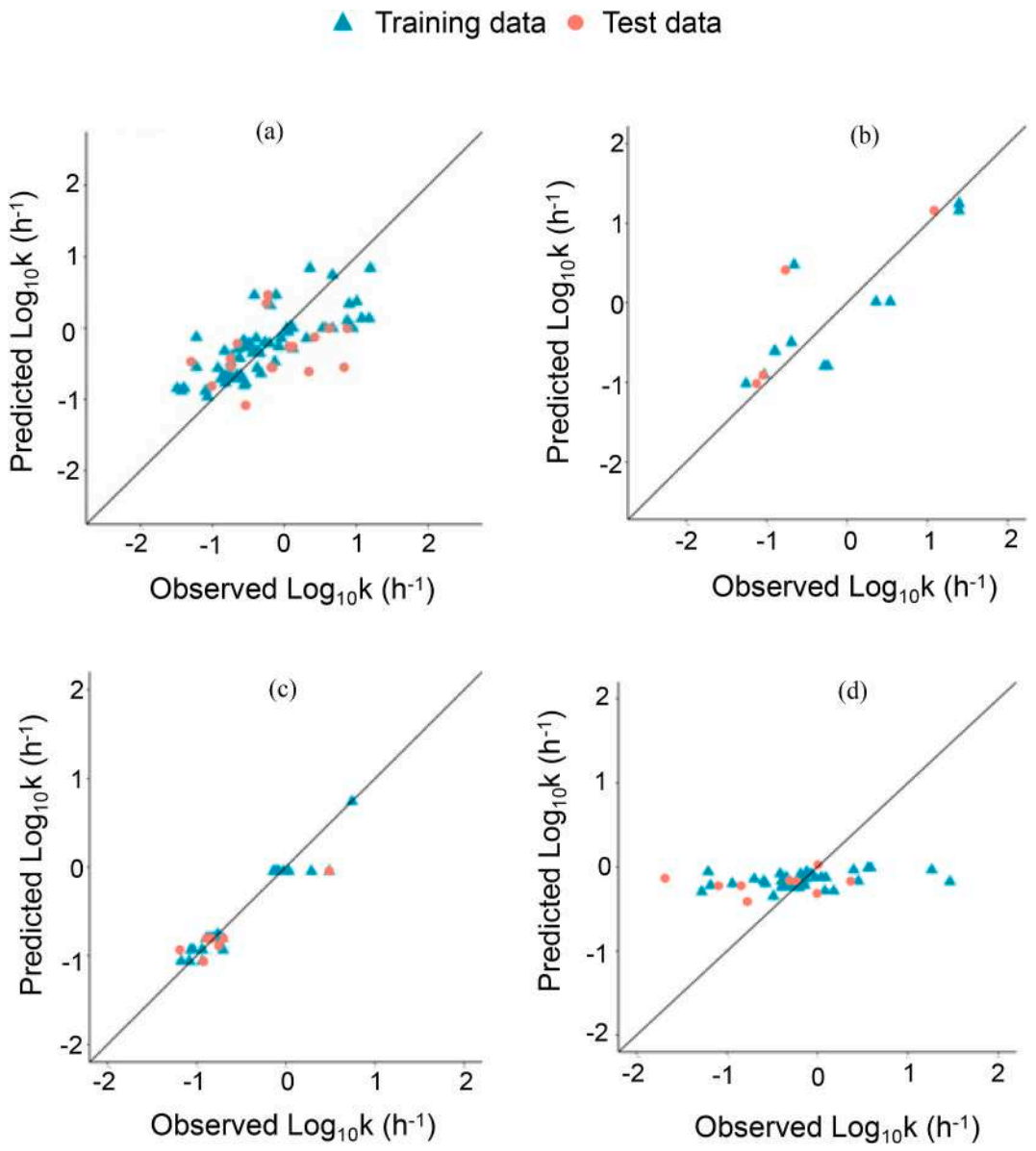

Fig. 6. Comparison of observed and predicted data using the ridge regression model. (a) SARS-CoV-2, (b) SARS-CoV, (c) MERS-CoV, and (d) influenza viruses.

Table 1
Comparison of goodness of fit between the ridge regression and Bayesian regression model based on RMSE, MAE and AUC.

Virus type	RMSE	MAE	AUC
Bayesian regression model			
SARS-CoV-2	0.41	0.31	1
SARS-CoV	0.40	0.37	1
MERS-CoV	0.12	0.08	0.5
Influenza viruses	0.50	0.35	1
Ridge regression model			
SARS-CoV-2	0.45	0.34	1
SARS-CoV	0.35	0.34	0.5
MERS-CoV	0.12	0.08	1
Influenza viruses	0.57	0.40	1

Bold virus type represents the better estimation model. Abbreviations: SAR-CoV-2: sever acute respiratory syndrome coronavirus 2; SAR-CoV: severe acute respiratory syndrome coronavirus; MERS-CoV: Middle East respiratory syndrome coronavirus; RMSE: Root mean square error, MAE: mean absolute error, AUC: area under the curve.

respectively.

We found that the decay rates were lower at lower temperatures (Fig. 7). The temperature in the available dataset ranged between 4 °C and 70 °C. Therefore, we assumed temperatures between 4 °C and 35 °C as a lower temperature range in the simulation. Using better estimation models, we simulated the decay rate values of the selected surfaces under different environmental conditions (Fig. 8). In addition, we estimated the virus decay rates during distinct seasons of the year, assuming two seasons—summer and winter (Fig. 9). We used 20 °C and 40% RH for summer and 28 °C and 70% RH for winter, assuming the indoor conditions.

According to the simulation (Fig. 8) log $_{10}$ decay rate of SARS-CoV-2 ranged from −0.345 to 0.104 h^{-1}, -0.839 to 0.197 h^{-1}, -0.484 to 0.095 h^{-1}, -0.698 to 0.212 h^{-1}, and -0.706 to −0.265 h^{-1} for aerosol, steel, masks, cloth, and plastic respectively. For SARS-CoV log $_{10}$ decay rates ranged from −0.911 to 0.911 h^{-1} for plastic. For MERS-CoV log $_{10}$ decay rates ranged from 0.122 to 0.512 h^{-1}, -0.678 to −0.735 h^{-1}, -0.543 h^{-1} to −0.605 h^{-1}, and -0.345 to −0.786 h^{-1} for steel, aerosol, plastic, and metal respectively (Table S9). The coefficients of the better estimation model are presented in Table S8. Estimated decay rate values for the

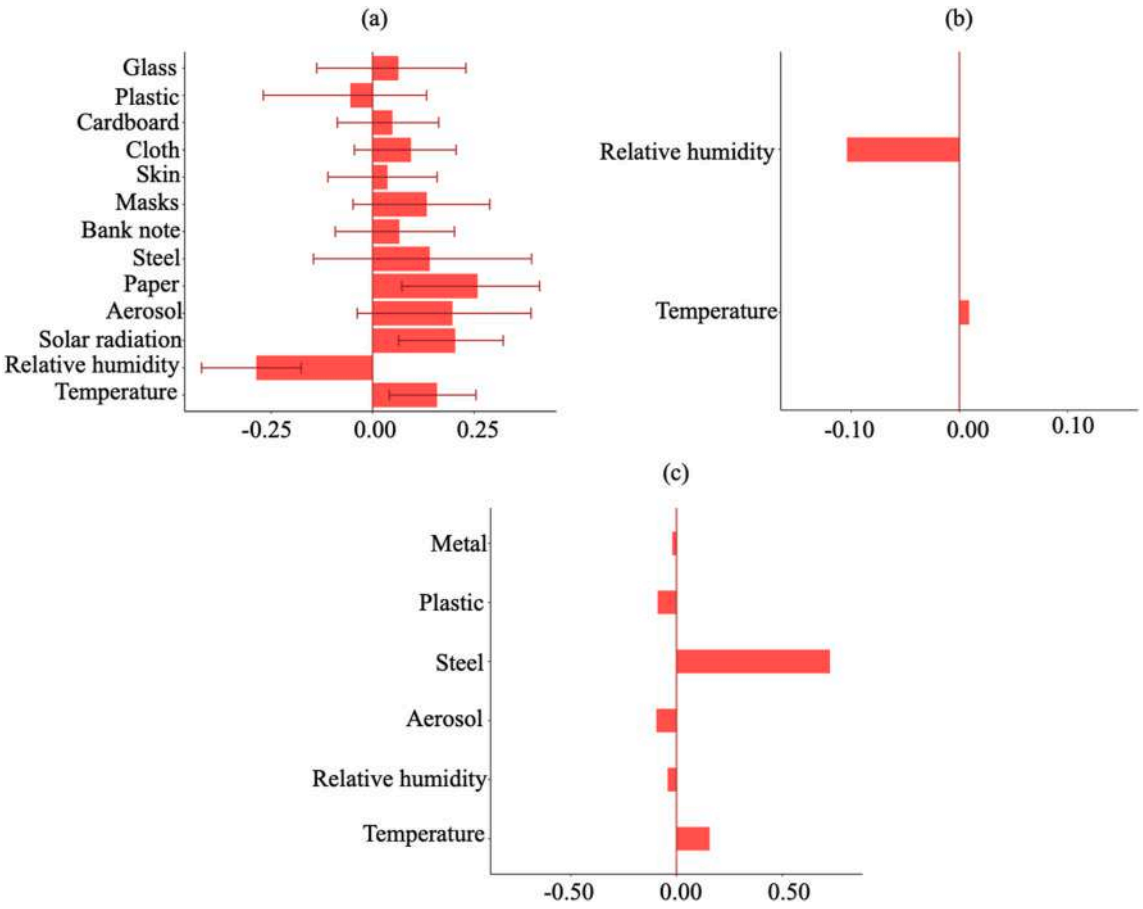

Fig. 7. Coefficient estimates from better estimation models with 2.5 and 95 percentiles (error bars) and posterior medians (bars). (a) SARS-CoV-2, (b) SARS-CoV, and (c) MERS-CoV.

seasons are presented in Table S10.

It is important to highlight that the simulated decay rates on each surface in summer were higher than those in winter. For aerosols, there was an approximately 1.7-fold difference in the decay rate of SARS-CoV-2 and MERS-CoV during summer and winter. For porous surfaces, including papers, there was an approximately 1.6-fold difference in the decay rate of SARS-CoV-2. For non-porous surfaces, including plastics, there was an approximately 1.7-fold difference in the decay rate of SARS-CoV-2, a 3.7-fold difference in that of SARS-CoV, and a 1.9-fold difference in the decay rate of MERS-CoV. The decay rate values for plastics were lowest for both SARS-CoV-2 and MERS-CoV.

4. Discussion

Viruses undergo rapid inactivation under conditions such as high temperatures and high relative humidity (Biryukov et al., 2020b, 2021); mechanisms of viral inactivation include thermal denaturation of proteins and nucleic acids (Marr et al., 2019). However, several studies have also indicated partial inactivation of viruses in aerosols and droplets at high relative humidity levels (Lin and Marr 2020; Yang and Marr 2011). This indicates a non-linear relationship between the decay rate values and environmental factors.

There has been a debate over the use of relative or absolute humidity to determine virus viability. Absolute humidity is the mass concentration that defines the amount of water vapor per air volume. In contrast, relative humidity is the water vapor pressure/concentration ratio to the saturation vapor pressure/concentration (Marr et al., 2019). A few studies have argued absolute humidity to be a more important predictor of viability of viruses than relative humidity (McDevitt et al., 2010; Shaman and Kohn 2009). However, most studies have concluded that relative humidity is important in determining virus viability (Hemmes et al. 1962; Marr et al., 2019). Marr et al., (2019) reevaluated the viability of influenza virus in aerosols and its transmission in animal models and concluded that the combination of relative humidity and temperature is equally valid as absolute humidity as a predictor. Hence, in our study, we included relative humidity to develop an estimation model for virus decay rate values. Relative humidity is an extrinsic factor that affects virus viability in two ways—(1) it can control the evaporation kinetics and change the solute concentration in the droplet, droplet size, and, physical fate; and (2) increasing the solute concentration and frequent exposure to higher solute concentrations can increase the virus inactivation (Lin and Marr 2020; Marr et al., 2019).

The type of deposition solution used in decay experiments tends to influence the activity of viruses, regardless of the environmental conditions. Dulbecco's modified Eagle's medium, when used as a deposition solution, caused a greater inactivation of bacteriophage phi6 than that achieved with PBS under the same relative humidity and temperature range; this highlights the importance of using the right type of deposition solution for enveloped viruses (Rockey et al., 2020). Additionally, salt and protein contents in the medium could further affect viral inactivation (Rockey et al., 2020; Yang et al. 2012); proteins affect the resistance of viruses to drying and hence influence the persistence of the viruses in the environment (Pastorino et al., 2020), whereas high salt concentrations affect the persistence of viruses owing to evaporation effects (Yang et al., 2012). Interactions between proteins and salts at different relative humidity values further complicate virus inactivation predictions (Yang et al., 2012). Yang et al. investigated the influenza virus decay rate with salt concentrations in different media. At 60% relative humidity, influenza virus decay rates increased linearly in the droplets in DMEM with NaCl concentrations up to 420 gL^{-1}; similarly, in

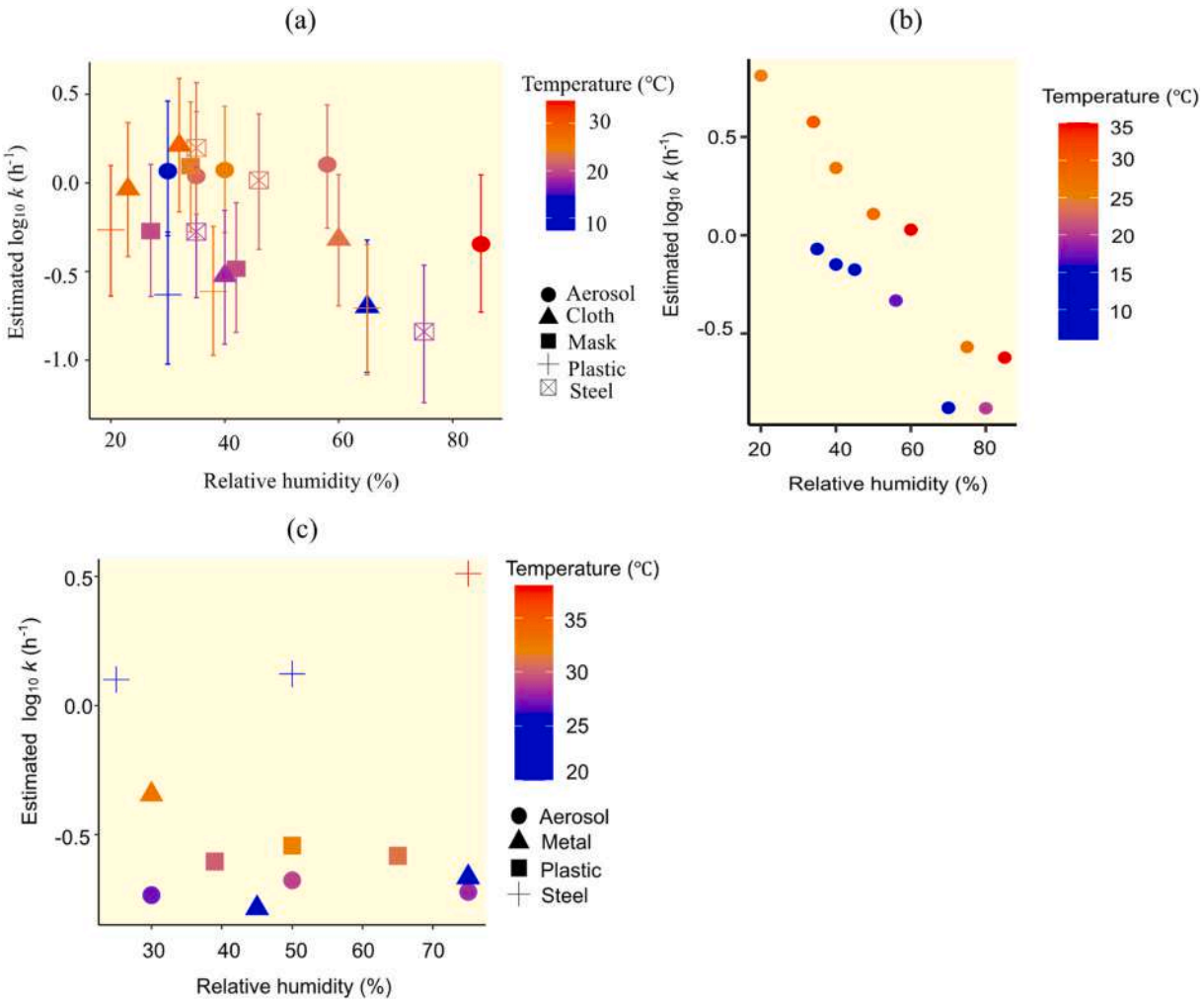

Fig. 8. Simulation of viral decay rates under different environmental conditions using better predictive model on select surfaces and aerosols from a randomly generated dataset. For (a) SARS-CoV-2 using Bayesian regression model. (b) SARS-CoV using ridge regression model for plastic surfaces. (c) MERS-CoV using ridge regression model.

the PBS medium, the decay rate increased linearly with NaCl concentrations up to 25–510 gL^{-1}. Yang et al. concluded that increasing salt concentration increases virus inactivation, and interactions among proteins, salts, and the virus can mitigate the adverse effects of high salt concentrations. However, the salt and protein contents of PBS and DMEM did not significantly affect SARS-CoV-2 inactivation (Rockey et al., 2020). Future work is necessary to test the effects of the ingredients in the culture medium on the virus decay rate under different humidity and elevated temperatures.

Sunlight has a significant effect on the decay rates of viruses. Simulated sunlight inactivates SARS-CoV-2 in aerosols with half-lives shorter than 6 min and a 90% inactivation within 20 min (Rothamer et al., 2020; Schuit et al., 2020). The decay rates of viruses exposed to solar radiation depend on the type (UVA, UVB, and UVC) and intensity (Rothamer et al., 2020; Sutton et al., 2013; Wood et al., 2010). A significant reduction in viral activity under high intensity solar radiation has been reported; UVC light at 254 nm causes rapid inactivation of SARS-CoV on surfaces (Darnell et al., 2004), while influenza virus (H7N1) decay rates increase with solar radiation (UVB and UVC), which causes a 1 $\log_{10}$ titer reduction within 69 min (Sutton et al., 2013).

Casanova et al. (2010) determined the effects of air temperature and humidity on coronavirus (SARS-CoV) survival on surfaces. To overcome the challenges associated with pathogenic viruses, two surrogate viruses, mouse hepatitis virus and transmissible gastroenteritis virus, were used in the study. At 4 °C, viruses persisted for 28 d on stainless steel and inactivation was faster at 20 °C than at 4 °C at all the tested humidity levels (Casanova et al., 2010). Furthermore, Casanova et al., 2010 reported that the relationship between relative humidity and inactivation was not monotonic and that a greater survival effect is exerted at low (20%) and high relative humidity (80%) levels than that at moderate humidity (50%) (Casanova et al., 2010). A similar relationship was observed in our collected decay rate values for SARS-CoV (Fig. S2). Pyankov et al. (2018) reported that the decay rate of MERS-CoV was higher under hot and dry climatic conditions (38 °C and 24% RH), with only 4.7% remaining infectious for over 60 min after aerosolization, while a survival rate of 63.5% was reported at 25 °C and 79% RH (Pyankov et al., 2018). Humidity affects both influenza virus transmissions and survival (Yang et al., 2012). Hemmes et al., (1962) and Harper, 1961 found higher decay rates at both medium and high relative humidities (Harper 1961; Hemmes et al., 1962). However, Schaffer et al., 1976 found moderate inactivation at higher temperatures and higher inactivation rates at medium temperature (Schaffer et al. 1976). The viability of influenza virus in aerosols and droplets is poorly understood (Yang et al., 2012). Based on previous studies on SARS-CoV-2, lower inactivation rate values were reported at low temperatures; however, sensitivity to heat occurs at a high temperature of approximately 70 °C (Aboubakr et al. 2021; Meyerowitz et al., 2021). Similarly, in our study, compared to those at low temperatures (0–40 °C), high decay rates were reported at higher temperatures (75–100 °C).

In addition to temperature, relative humidity, and solar radiation, we

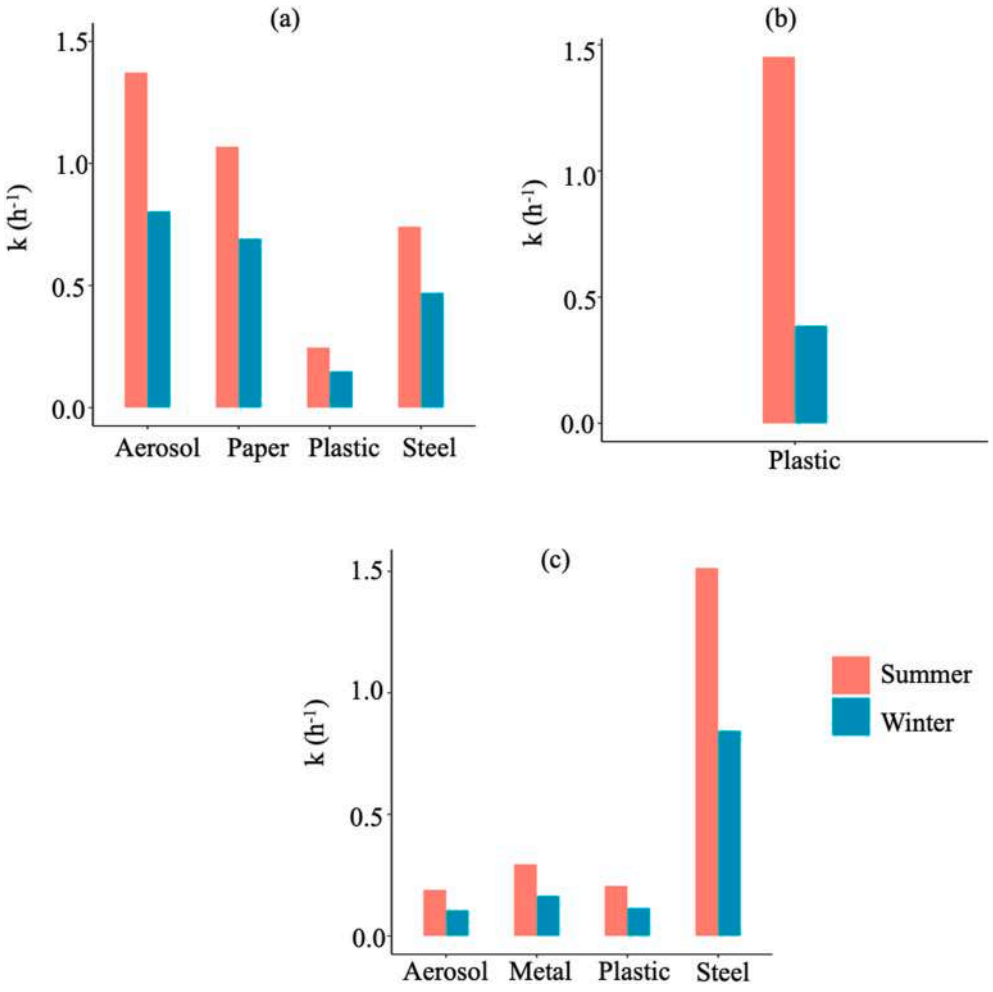

Fig. 9. Simulation of viral decay rates under different environmental conditions for selected surfaces: summer and winter. For (a) SARS-CoV-2 using the Bayesian regression model 50% credible interval of the predicted decay rate values used in the graph; (b) SARS-CoV using the ridge regression model; (c) MERS-CoV using the ridge regression model. The temperature and relative humidity used for the simulations were 20 °C and 40% RH for summer, and 28 °C and 70% RH for winter, respectively.

considered other important factors affecting the virus decay rate, which include particle size, the porosity of the object surface, water absorption degree, surface tension, aggregation state of the droplet on the surface, water absorption degree, surface tension, aggregation state of the droplet on the surface, and type of virus (Guo et al., 2021). However, there is limited information available related to the factors listed above. We highly recommend considering these factors in future research, which will help to determine the decay rate of viruses. Furthermore, the volume of the virus-containing droplet significantly influences virus decay, and rapid virus inactivation results in a lower droplet volume (Guo et al., 2021). There can be differences in the initial volume of the virus-containing droplets titrated in the experiments in the literature. This may have caused significant differences in the experimental data reported (Guo et al., 2021). Guo et al., (2021) analyzed the relationship between the virus decay rate and titration volume and concluded that there was a greater risk of transmission with a larger droplet volume (Guo et al., 2021). Previous literature includes decay rate values on surfaces on different experimental methods with scattered results, making comparisons difficult because of biased information (Guo et al., 2021). Guo et al. (2021) further evaluated the effect of the volume of viral suspensions on the decay rates of viruses used in previous studies. In future research, it is recommended to focus more on the droplet volume used on the surfaces in the viability experiments to avoid experimental bias.

SARS-CoV-2 exhibited lower decay rates on plastic, glass, banknotes, steel, and skin than on surfaces such as paper and masks. The decay rates on porous surfaces (paper and masks) were higher than those on non-porous surfaces (plastic and glass) within a shorter time interval (data not shown). Similarly, for MERS-CoV, lower decay rate values were found in metal and plastic, which are non-porous surfaces. In contrast, for SARS-CoV, higher decay rates were found in plastics, and lower decay rates were found in metals. This may be because our dataset for SARS-CoV included decay rate values at higher temperatures. However, the decay rate values for steel were higher for SARS-CoV-2 and MERS-CoV than for the other non-porous surfaces (data not shown). In future research, we recommend determining the virus decay rate on porous and non-porous surfaces under different environmental conditions. This will help elucidate precise decay rates based on the surface type.

When comparing the decay rates of SARS-CoV-2 based on surface type during summer, there is an approximately 5-fold difference in aerosols and plastics, which is higher than the seasonal difference. For MERS-CoV, there is an approximately 5-fold difference between metals and steel. Based on the strength of our simulation, we recommend that it is vital to reinforce the control measures based on the surface type compared to the seasonal difference. It is essential to disinfect surfaces frequently, such as plastics and metals. For the surfaces with higher decay rates, it is crucial to focus on the source to adopt control measures, such as wearing masks, promoting cough etiquettes, and social distancing. Proper ventilation will reduce the impact of aerosol transmissions by aerosols. The regular implementation of the control measures is recommended to control the transmission of virus.

There are several limitations of this study. First, a few data points are available for viruses and surface types separately for model validation. Limited studies are available on the decay rate estimation of viruses for a shorter time interval on different surfaces. The higher number of data

points improves the model validation and accuracy of the prediction. Thus, we recommend determining virus decay rates in aerosols and surfaces for a shorter time interval in future research. In addition, virus inactivation mechanisms in aerosols and surfaces: porous and non-porous, are different. With the lack of data, we considered both aerosols and surfaces together. In future research, these models can be developed separately for aerosols and surfaces: porous and non-porous, based on the inactivation mechanisms. Virus decay rates are affected by the salts in the buffer solution and culture medium. Therefore, we recommend considering culture medium, buffer solution, and salts in the buffer as variables in the model to predict virus decay. In this study majority of the selected studies used the VeroE6 cell line. Few studies used FRhK-4, MDCK, EBL, and HBE cell lines. Since differences in the cell line can affect virus decay, conducting virus decay rate experiments in different cell lines in addition to diverse environmental conditions is essential.

Surface types in the models were decided based on data availability. Therefore, we recommend using different surface types for decay rate estimations in the model. However, the surfaces used in this study can be used as a basis for the decay rates on other surfaces not included in the model. For instance: the decay rate of paper reflects the higher decay rates of viruses on porous surfaces, which provides an understanding of other porous surface types not provided in this study.

Our study would serve as a basis for decision-makers in determining effective non-pharmaceutical interventions against virus transmission based on the surface type and environmental conditions. Additionally, we identified data gaps in the studies on viral inactivation of surfaces under environmental conditions and the importance of their inactivation in indoor environments for effective control of virus transmission.

5. Conclusions

In this study, we developed models to estimate the decay rate of respiratory viruses in aerosols and on various environmental surfaces using Bayesian regression and ridge regression models. Simulated decay rates demonstrated that decay rates during summer were higher than those during winter. However, the variations in decay rates among different surfaces were greater than seasonal differences in decay rates. According to our simulations, it is vital to reinforce control measures based on surface types rather than seasonal differences. In addition, we recommend determining decay rates of viruses on diverse types of porous and non-porous surfaces under different environmental conditions: temperature, humidity, and solar radiation, and experimental conditions: culture medium, buffer solution, and salts in the buffer in future research.

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Appendix A. Supplementary data

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Effects of different laying periods on airborne bacterial diversity and antibiotic resistance genes in layer hen houses

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ABSTRACT

Poultry farms are a complex environment for close contact between humans and animals. Accumulating evidence has indicated that pathogens and drug resistance genes in chicken houses may pose a serious threat to public health and economic concerns. However, insufficient knowledge of the indoor aerosol microbiome and resistome profiles of layer hen houses hampers the understanding of their health effects. Environmental surveillance of antibiotic resistance may contribute to a better understanding and management of the human exposure risk of bioaerosols under the environmental conditions of chicken houses. In addition, the chicken house has a long operation cycle, and the bacterial diversity and antibiotic resistance genes of aerosols in different periods may be different. In this study, air samples were collected from 18 chicken houses on three farms, including the early laying period (EL), peak laying period (PL), and late laying period (LL). 16S rRNA gene sequencing and metagenomics were used to study the composition of the bacteria and resistome in aerosols of layer hen houses and the results showed that they varied with laying period. The highest alpha diversity of bacteria was observed in PL bioaerosols. The dominant bacterial phyla included Firmicutes, Bacteroidetes and Proteobacteria. Three potential pathogenic bacterial genera (*Bacteroides*, *Corynebacterium* and *Fusobacterium*) were found. The most abundant ARG type was aminoglycosides in all laying periods. In total, 22 possible ARG host genera were detected. ARG subtypes and abundance were both higher in LL. Network analysis also showed higher co-occurrence patterns between the bacteria and resistome in bioaerosols. The laying period plays an important role in the bacterial community and resistome in layer house aerosols.

1. Introduction

China is the largest egg producer, accounting for approximately 40% of the global share in 2020, and egg production continues to grow worldwide (Dai et al., 2022). Many frequent animal feeding operations may produce high concentrations of bioaerosols, which are generally defined as aerosolized particles containing microorganisms (Heederik et al., 2007; Jiang et al., 2022; Millner, 2009; Viegas et al., 2020; Wang et al., 2019). Antibiotics are often used to prevent animal diseases and improve the performance of layers (Bushen et al., 2021). Increasing antibiotic abuse may contribute to the evolution of antibiotic-resistant bacteria, which may significantly reduce the effects of antibiotics and cause an increased number of deaths each year due to aerosol pollution (Castro-Vargas et al., 2020; Gao, 2018; Zeineldin et al., 2019). Exposure can occur through inhalation or skin contact with this bioaerosol (Hoppin et al., 2014; Paton et al., 2015). Some studies have shown that agricultural workers and residents living near farms have a higher risk of respiratory diseases and bacterial infections (Audi et al., 2017; Hedelin et al., 2016; Viegas et al., 2013). The allergic immune reaction of broiler workers was higher than that of nonagricultural workers (Gautam et al.,

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2018). Specific microbial composition and accumulation of antibiotic resistance genes in the aerosols of poultry farms were two key factors that induced high exposure risks for agricultural workers. On the one hand, with the rapid development of the breeding industry, the development and spread of ARGs in breeding systems have received increasing attention. The levels of ARGs in the feces of chickens are higher than those of pig and cattle farms, and ARG contamination in the feces of layer hens is more severe than that of broilers (Gu et al., 2020; Hubbard et al., 2020). On the other hand, *Bacteroides*, *Corynebacterium* and *Fusobacterium*, which are widely distributed inside poultry farms, have been identified as risk pathogens (Brennan and Garrett, 2019; Hoefer et al., 2021b; Hong et al., 2012; Zafar and Saier, 2021). More importantly, the chicken house has a long operation cycle, and the bacterial diversity and antibiotic resistance genes in different periods may be different. Therefore, it is very important to understand the microbial composition and resistance gene contamination during different laying periods. The egg production of layer hens varies greatly during the laying period, which can be divided into three periods, including the early laying (EL) period, peak laying period (PL) and late laying (LL) period (Zhu et al., 2021). Although antibiotic use is not a sustainable strategy, farmers in some areas still use several antibiotics in the EL period to reduce the immune stress that may occur after repeated vaccination. Antibiotic use is reduced during PL periods, while it is increased again in LL periods due to the rapid decline in egg production caused by problems such as chronic inflammation. These antibiotics often included beta-lactams, tetracyclines, macrolides, and aminoglycosides. Farms that overuse antibiotics are more likely to have problems with resistant bacteria than those with normal usage, and resistant bacteria can persist in the farm environment even after selection pressures have dissipated(Xu et al., 2020).

It has been reported that the main source of microbial pollution in the air of closed livestock and poultry houses is feces (Chien et al., 2011; Clark et al., 1983; Cormier et al., 1990; Donham et al., 1986; Nehme et al., 2008). Until now, most studies of antibiotic resistance in chicken farming have focused mainly on feces (Qiu et al., 2021; Sergeant et al., 2014). Information on antibiotic resistance in the air of layer hen farming systems is still very limited. At the same time, there are also few studies on the microbial composition and potential zoonotic pathogens in the environment of layer houses during different laying periods (Zhu et al., 2021). Indoor air in layer houses is an important reservoir of ARGs (Wychodnik et al., 2020). There is an urgent need for risk assessment of microorganisms and antibiotics in the layer hen house environment to distinguish the environmental impacts of antibiotic use at different periods of eventual egg production.

In this study, bacterial community and resistance genomes in bioaerosols from layer houses at different laying periods were analyzed to obtain taxonomic and compositional changes, as well as their co-occurrence networks in different ecological niches. This can extend our understanding of the link between bioaerosol microbes and ARGs, the key driving forces shaping resistance genes in the air, and facilitate targeted interventions to avoid antibiotic residues and improve egg safety in layer hens.

2. Materials and methods

2.1. Layer hen farms

This study was carried out in three representative commercial layer hen farms in Hebei Province in the winter of 2019 that belong to the same company, and the data were analyzed anonymously. There were three types of layer hens on each farm: EL period (17–30 weeks), PL period (31–50 weeks), and LL period (50–70 weeks). Two samples were drawn from each house type per farm. The samples were taken consecutively but on different days in December. During our visits, none of the farms were dusted, and no disease outbreaks were reported. These farms cover an average area of about 60000 m^2, with 18 chicken houses. Each of the selected layer hen houses covers an average area of about 1020 m^2, with six workers and about 50000 layers. In addition, these workers were not wearing any personal protective equipment during our sampling period. In these chicken farms, Tetracyclines, Sulfonamides, Macrolides, and Aminoglycosides are the commonly-used antibiotics for veterinary purposes. Unfortunately, there is no specific record of the antibiotics used at different layer periods by the farms. Temperature and humidity were controlled with pipe heating and mechanical ventilation, and layers were raised in cages for egg production with numbers ranging from 50000 to 55000.

2.2. Sample collection and preparation

A total of 18 bioaerosol samples were obtained, as shown in Table S1. Each farm has 3 types of stables: 2 EL houses, 2 PL houses and 2 LL houses. Sample data for all farms are given in the table. Collection of air samples was performed according to our previously published method with slight modification (Cui et al., 2022). Briefly, a high-volume air sampler (HH02-LS120 of Beijing Huarui Hean Technology Co., Ltd., China) equipped with a 19.8 cm × 23.8 cm quartz fiber membrane (Cat No. 7204, Tissuquartz 2500 QAT-UP; PALL, NY, U.S.) was placed in the middle of each chicken house corridor at a height of 1.5 m above ground away from heating or cooling equipment to collect aerosol samples. For each sample, the device was run continuously for 12 h at a flow rate of 1000 L/min. Before sampling, the sampler was sterilized with 75% alcohol, and all filter membranes were sterilized by baking in a muffle furnace at 500 °C for 48 h. Samples were immediately transferred to the laboratory in ice boxes after collection. The membranes were stored at −80 °C before use.

2.3. DNA extraction

DNA extraction was performed as described in our previous publications (Cui et al., 2022; Guo et al., 2018). In brief, each membrane was cut into an average of 8 sections with sterile surgical scissors, with a weight error of ±1 mg per section(Gao et al., 2017). One-eighth of the membrane was cut into small pieces and placed in an Eppendorf tube preset to 1 mL ultrapure water (ST876, Yaji, Shanghai, China) and sterile steel beads. Samples were homogenized using a homogenizer to elute particles and then centrifuged (at 25000 g for 10 min at 4 °C). DNA was extracted using the MO-Bio PowerSoil DNA Isolation Kit (Carlsbad, CA, USA.). All DNA template concentrations were quantified by Q5000 UV/Vis spectrophotometry (Quawell, USA). “Blank” samples (sterilized water) were extracted to control for methodological contamination (Salter et al., 2014).

2.4. 16S rRNA amplicon and metagenomic sequencing

The V3/V4 region of the 16S rRNA gene was targeted for amplification using the previously published universal primers 515F and 806R (515F: 5′-TGTGCCAGCMGCCGCGGTAA-3′; 806R: 5′GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2012). Each sample was amplified in triplicate using the PCR conditions described previously (Yang et al., 2018). Samples were verified by 2% gel electrophoresis, and the target bands were excised and purified with the Qiagen QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). Samples were sent to Novogene Co., Ltd. (Beijing, China) for sequencing application on the Illumina MiSeqPE250 platform.

The raw 16S rRNA gene sequence was processed with Mothur software to retain high-quality reads (V1.36). Uparse software (Uparse v7.0.1001, http://www.drive5.com/uparse/) was used to cluster the sequences with 97% identity, which were clustered into operational taxonomic units (OTUs)(Li et al., 2020). OTUs were assigned to rRNA sequences of the SILVA (SSU132) 16S rRNA using an 80% confidence threshold. The DNA extracted for 16S rRNA was also used for metagenomic sequencing on the Illumina HiSeq 4000 platform.

2.5. Calculation of ARG abundance

ARG abundance was determined using ARGs-OAP (Yang et al., 2016). In brief, potential ARG readings and 16S rRNA genes were identified, and ARG-like readings were identified and annotated using BLASTX by applying CARD, the Comprehensive Antibiotic Resistance Database (Li et al., 2015; Yang et al., 2013). The abundance of ARG readings in the samples was calculated and normalized by the number of 16S rRNA genes, defined as relative abundance. ARG abundance was expressed as ARG copies per copy of the 16S rRNA gene (Xiong et al., 2018). ARG types and subtypes are counted automatically using the custom script package described earlier (Yang et al., 2013).

2.6. Data analysis

Alpha diversity was calculated using QIIME 2 (Quantitative Insights Into Microbial Ecology) (Bolyen et al., 2019). All statistical analyses were performed using Graph Pad Prism 9 (Graph Pad Software, USA.). A P value < 0.05 was considered statistically significant. The nonparametric Kruskal–Wallis test was used for statistical comparison. The composition differences of bacterial communities and ARGs in the air of layer hen houses during different periods were analyzed using the Bray–Curtis similarity index and visualized using nonmetric positions scaling (NMDS). The ARG and genus networks were analyzed based on Spearman's correlation matrix. The graph was visualized with Gephi software (Gephi Consortium, USA) to generate a network diagram (V 0.9.2).

2.7. Sequence submission

All sequence data obtained have been submitted to NCBI BioProject under accession numbers PRJNA875241 and PRJNA875383.

3. Results

3.1. Diversity of aerosol bacteria at different laying periods

An average of 88,559 raw reads were obtained from each sample. After quality control, 62,253 valid data points were obtained, and the quality control efficiency was 70.30%. Sequences were clustered into 23,501 OTUs at a 97% identity threshold (mean = 1306, Table S2). As shown in Fig. 1 and Fig. S1, the observed diversity was greatest in the PL period. The observed species, Shannon and ACE indexes of samples from the PL period were higher than those of samples from the EL or LL period ($p < 0.01$) (Table S2). NMDS analysis of the microbial OTU relative abundance data shown in Fig. 1D revealed clear separation of the samples from different periods ($p < 0.05$), and samples from the same period clustered more closely together.

3.2. Composition of abundant airborne bacterial phyla

The dominant phyla (abundance >1%) of all samples are shown in Fig. 2. The most abundant bacterial phyla were Firmicutes (46.00%–68.01%), followed by Bacteroidetes (7.50%–23.68%), Proteobacteria (2.04%–15.93%), Cyanobacteria (1.17%–14.67%), Fusobacteria (3.60%–6.62%) and Actinobacteria (1.25%–2.83%) (Fig. 2A, Table S3). The relative abundance of Firmicutes was significantly higher in the PL period than in the EL and LL periods ($p < 0.01$ Fig. S2A). The relative abundance of Bacteroidetes was significantly higher in LL than EL ($p < 0.05$, Fig. S2B). The relative abundance of Proteobacteria detected in EL was significantly higher than that in PL and LL ($p < 0.01$, Fig. S2C).

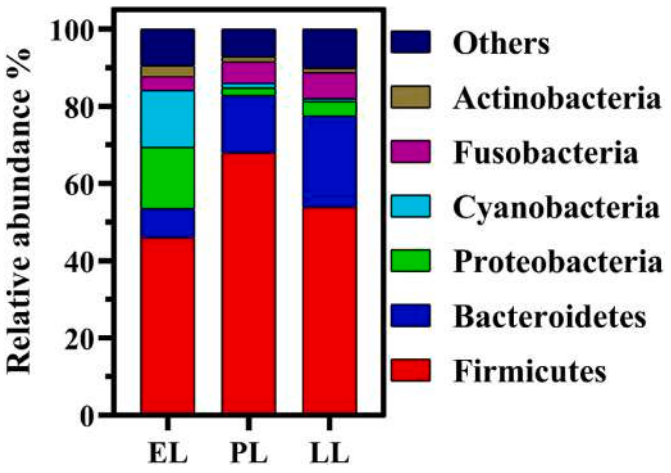

Fig. 2. Compositions of microbial communities at the phylum level among the three laying periods. Composition and relative abundance of different phyla.

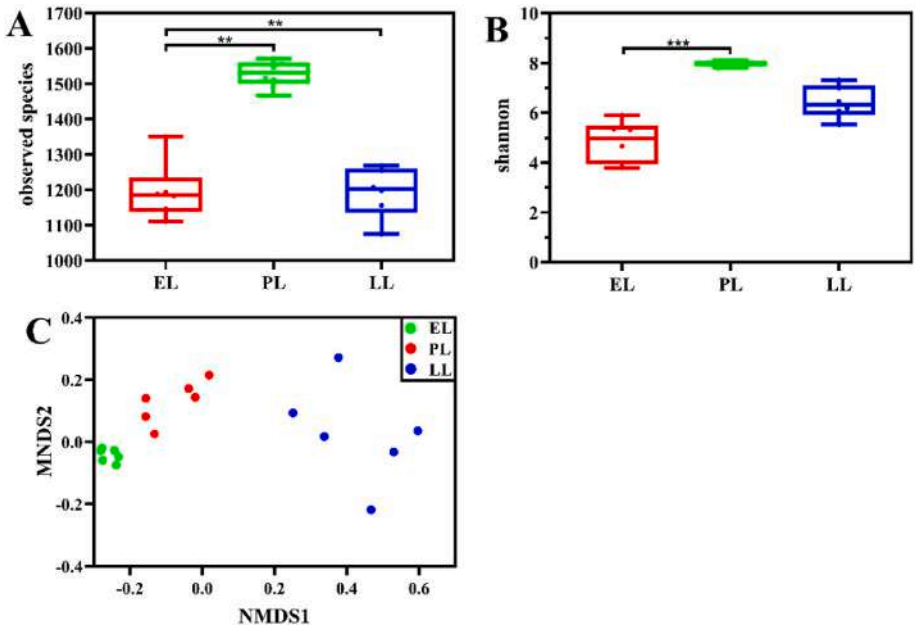

Fig. 1. Diversity analysis of microbial community profiles in air samples of three laying periods. (A) Observed species for each laying period; (B) Shannon index for each laying period; (C) nonmetric multidimensional scaling (NMDS; Bray–Curtis distance) plot based on OTU abundances demonstrating the differences in microbial community composition of air samples among the laying periods. Wilcoxon rank sum test: **($p < 0.01$) and ***($p < 0.001$). Abbreviations: EL, early laying period; PL, peak laying period; LL, late laying period.

3.3. Composition of abundant airborne bacterial genera

The dominant genera (average abundance >1%) within each period are shown in Fig. 3A. There were 6, 7 and 10 dominant genera in the EL, PL and LL periods, respectively (Table S4). *Lactobacillus* was the main genus in EL (55.96%) and PL (63.19%), while *Bacteroides* was the most abundant genus in LL (28.32%) (Fig. 3A). According to the list of human pathogens provided by the Ministry of Health of China (MOHC)(MOHC, 2006; Yan et al., 2021), three potential pathogen genera posing potential risks to humans (*Bacteroides*, *Corynebacterium* and *Fusobacterium*) were identified. The abundances of *Bacteroides*, *Corynebacterium* and *Fusobacterium* in LL buildings were significantly higher than those in EL buildings ($p < 0.01$). The abundance of *Bacteroides* and *Fusobacterium* in LL building was significantly higher than that in PL building ($p < 0.05$, Fig. 3B). There were also significant changes in nonpathogenic bacteria during different periods. The abundances of *Lactobacillus* and *Facklamia* at the EL period were significantly higher than those in PL ($p < 0.01$ Fig. 3B). The abundance of *Comamonas* in the LL and PL periods was significantly higher than that in EL ($p < 0.05$, Fig. 3C). The abundance of *Jeotgalicoccus* in PL was significantly higher than that in LL ($p < 0.01$, Fig. 3C). The abundance of *Stenotrophomonas* in the LL period was significantly higher than that in the EL ($p < 0.01$) and PL ($p < 0.05$) periods (Fig. 3C).

3.4. Airborne ARGs in layer houses

The total number and relative abundance of ARG subtypes were the lowest in the PL period and were significantly lower than those in the LL house ($p < 0.01$, Fig. 4A and B). There was a total of 18 ARG types in all samples, and 14 were shared across all laying periods.

Compared with LL, EL showed a higher abundance of aminoglycosides ($p < 0.05$). EL showed a higher abundance of beta-lactam, fosfomycin, tetracycline, and vancomycin ($p < 0.05$, Fig. 4C) and a lower abundance of multidrug and polymyxin ($p < 0.05$, Fig. 4C) than PL. In addition, PL showed a lower abundance of beta-lactam and macrolide-lincosamide-streptogramin (MLS) compared with LL ($p < 0.05$, Fig. 4C), while Chloramphenicol, Polymyxin and Sulfonamide ($p < 0.01$, Fig. 4C) were significantly higher than LL. The abundance of tetW and tetQ in the PL period was significantly higher than that in the LL period. The abundance of ermF in the EL period was significantly higher than that in the LL period. The abundance of APH (3″)-I in the EL period was significantly lower than that in the LL period ($p < 0.05$ Fig. S3). In addition, NMDS analysis was used to investigate the similarities between different periods based on the abundance of ARG subtypes. The ARG groups in the samples were clustered by different types of chicken houses, and there were significant differences ($p < 0.05$ Fig. 5).

3.5. Network co-occurrence analysis of ARGs and bacterial genera

Network analysis based on Spearman's rank correlations was applied to investigate the relationships between ARG subtypes and taxa. Firstly, $r > 0.8$ and $p < 0.01$ was selected, but there were too many related bacteria genera, which made it difficult to present and visualize. To narrow the research scope, $r > 0.9$ and $p < 0.001$ were selected for analysis. The co-occurrence network contained a total of 89 nodes (22 genera and 67 ARG subtypes) and 636 edges (Fig. 6). The co-occurrence relationships between ARGs and genera identified 22 bacterial genera as possible hosts of the 67 ARGs of eight types (Table S5). *Arcobacter*,

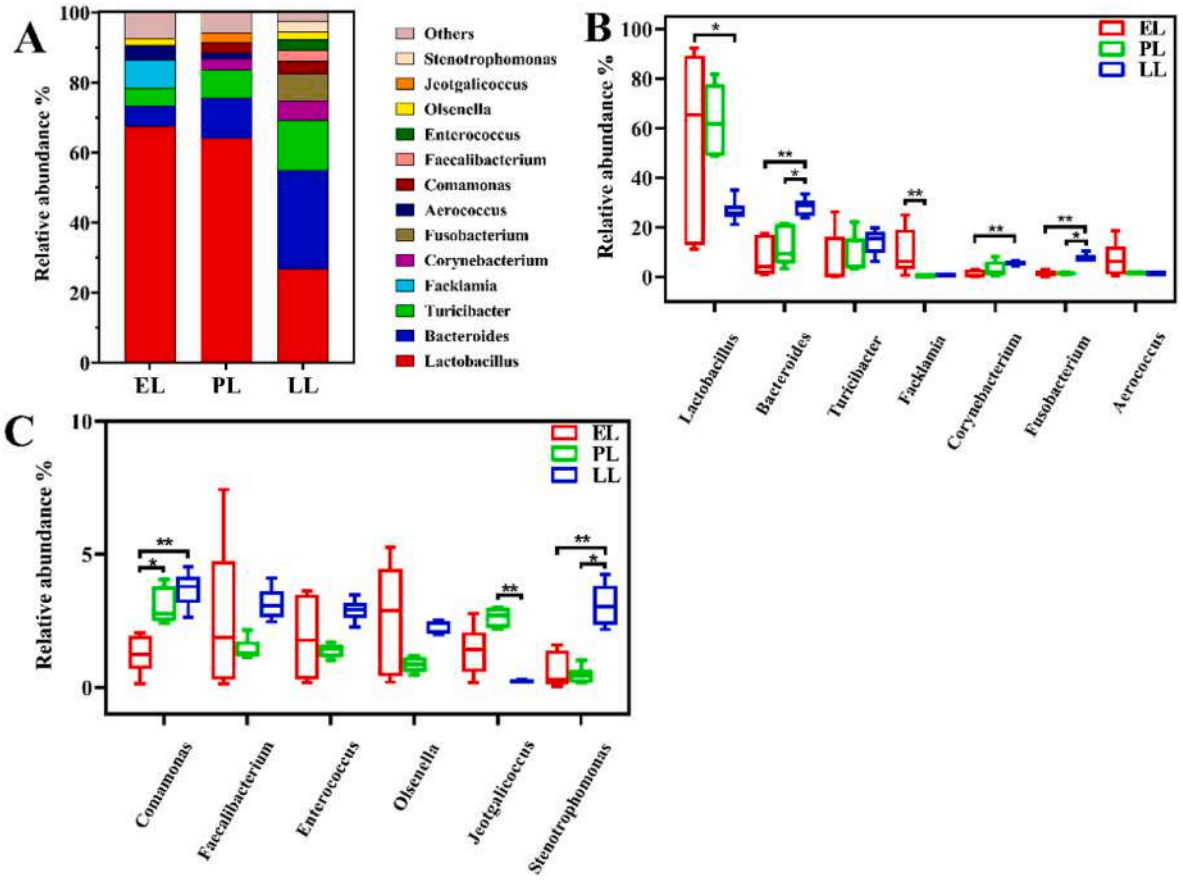

Fig. 3. Compositions of microbial communities at the genus level among the three laying periods. (A) Composition and relative abundance of dominant genera (average abundance >1.0%); (B and C) Comparison of the relative abundance of dominant genera. Wilcoxon rank sum test: *($p < 0.05$) and **($p < 0.01$). Abbreviations: EL, early laying period; PL, peak laying period; LL, late laying period.

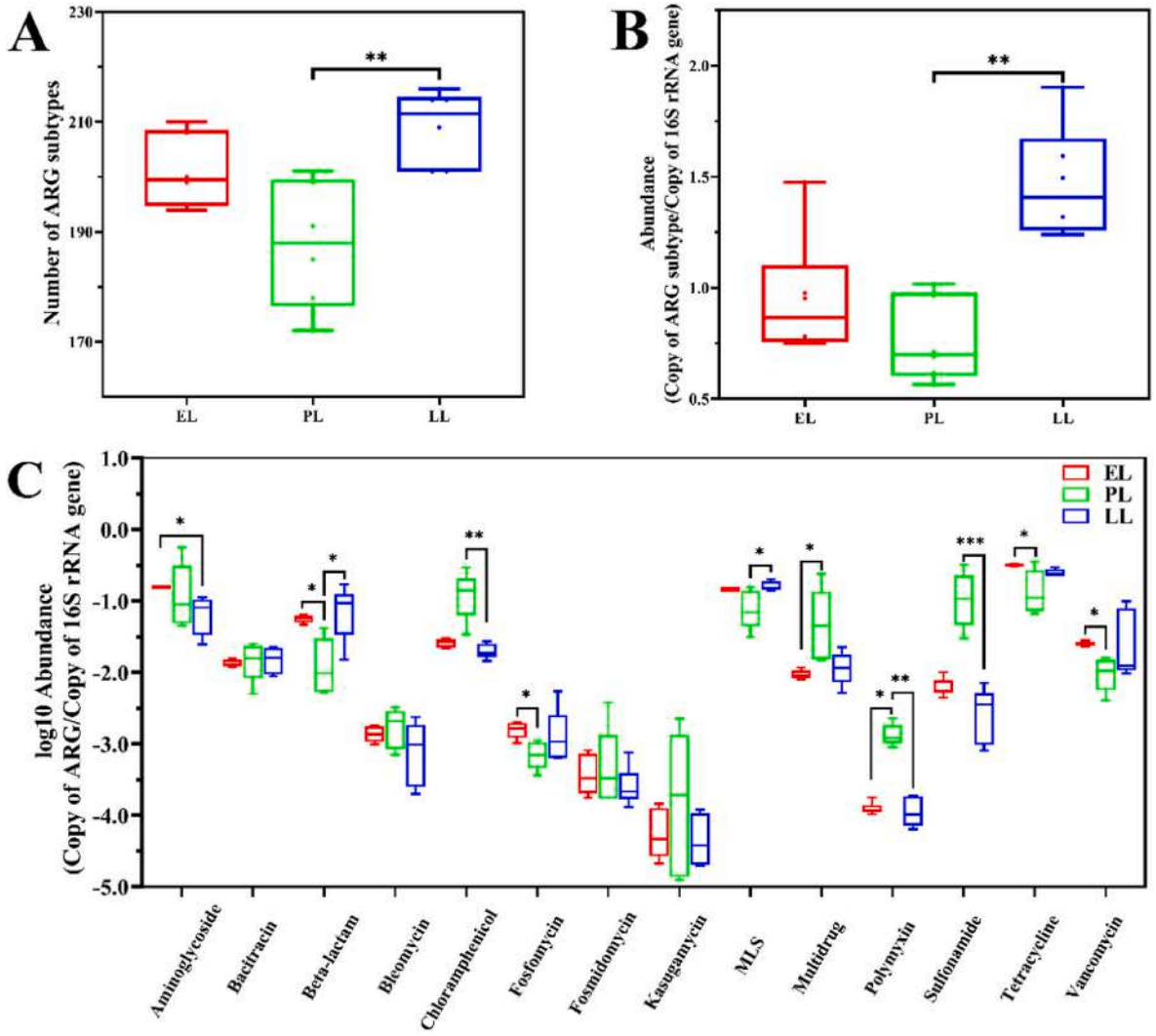

Fig. 4. The number and relative abundances of ARGs detected in different air samples of the three laying periods. (A) Comparison of the total number of ARG subtypes. (B) Comparison of total ARG subtype relative abundance. (C) Comparison of total ARG relative abundance for different antibiotics. Wilcoxon rank sum test: *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$). Abbreviations: EL, early laying period; PL, peak laying period; LL, late laying period. MLS: macrolide-lincosamide-streptogramin.

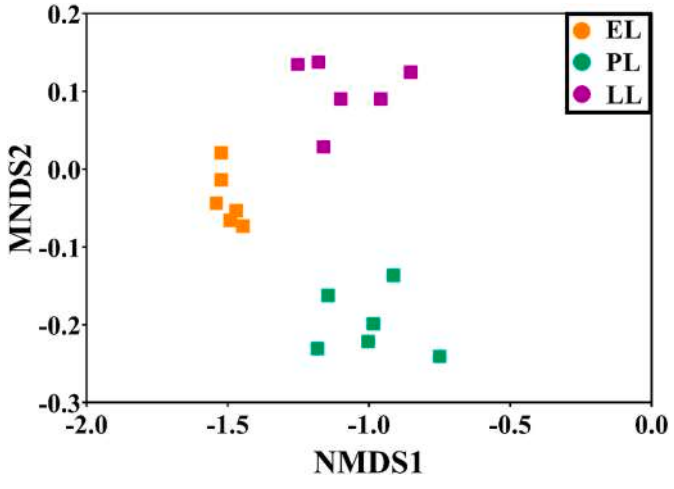

Fig. 5. Nonmetric multidimensional scaling (NMDS) plot of air samples using Bray–Curtis distance matrices based on ARG subtype abundances showing differences in ARG composition. Abbreviations: EL, early laying period; PL, peak laying period; LL, late laying period.

Giesbergeria, and *Helcococcus* were potential hosts for most ARGs, including tetracycline (tet32、tet39、tetH、tetS、tetX、tetX2、tetX3 and tetZ), MLS (erm(TR)、ermX、lnuB、lsa and macB) and aminoglycoside (aac(6′)-I、aac(6′)- II、adeJ、ant(2″)-I、ant(9)-I and aph(3′)-I) (Fig. 6). The genera *Arcobacter*, *Giesbergeria*, *Helcococcus*, *AcetoAnaerobium*, *Acidovorax*, *Anaerobium*, *Peptoniphilus*, *Chryseobacterium*, *Trueperella*, *Arcobacter*, *Neofamilia*, *Paludibacter*, *Simplicispira* and *HalAnaerobium* carried more than 10 ARG types, *Jeotgalibaca*, *Akkermansia*, *Lysinibacillus*, *Lagierella* and *Dysgonomonas* carried 3–9 ARG types, while the genera *Oscillibacter*, *Facklamia*, and *Kocuria* were found to be relevant to only one ARG type (Fig. 6, Table S5). There were obvious co-occurrence patterns within or across ARG types. The network consists of 67 nodes and 323 edges (Fig. S4, Table S6). Among them, beta-lactam (OXA-21), tetracycline (tetX, tetX3, tetH), chloramphenicol (cmlA), vancomycin (vatB), MLS (erm(TR), ereA, macB, lsa), aminoglycoside (aac(6′)-II, aad(9), ant(2″)-I) and multidrug (mexB) resistance genes were associated with more than 10 ARG subtypes. For example, OXA-21 associates with aac(6′)-II, aac(6′)-I, adeJ ant(2″)-I, ant(9)-I, aph(3′)-I, catB, catQ, cmlA, ermX, lnuB, lsa, mexB, OXA-209, tet39, tetG, tetH, tetX3 and tetZ.

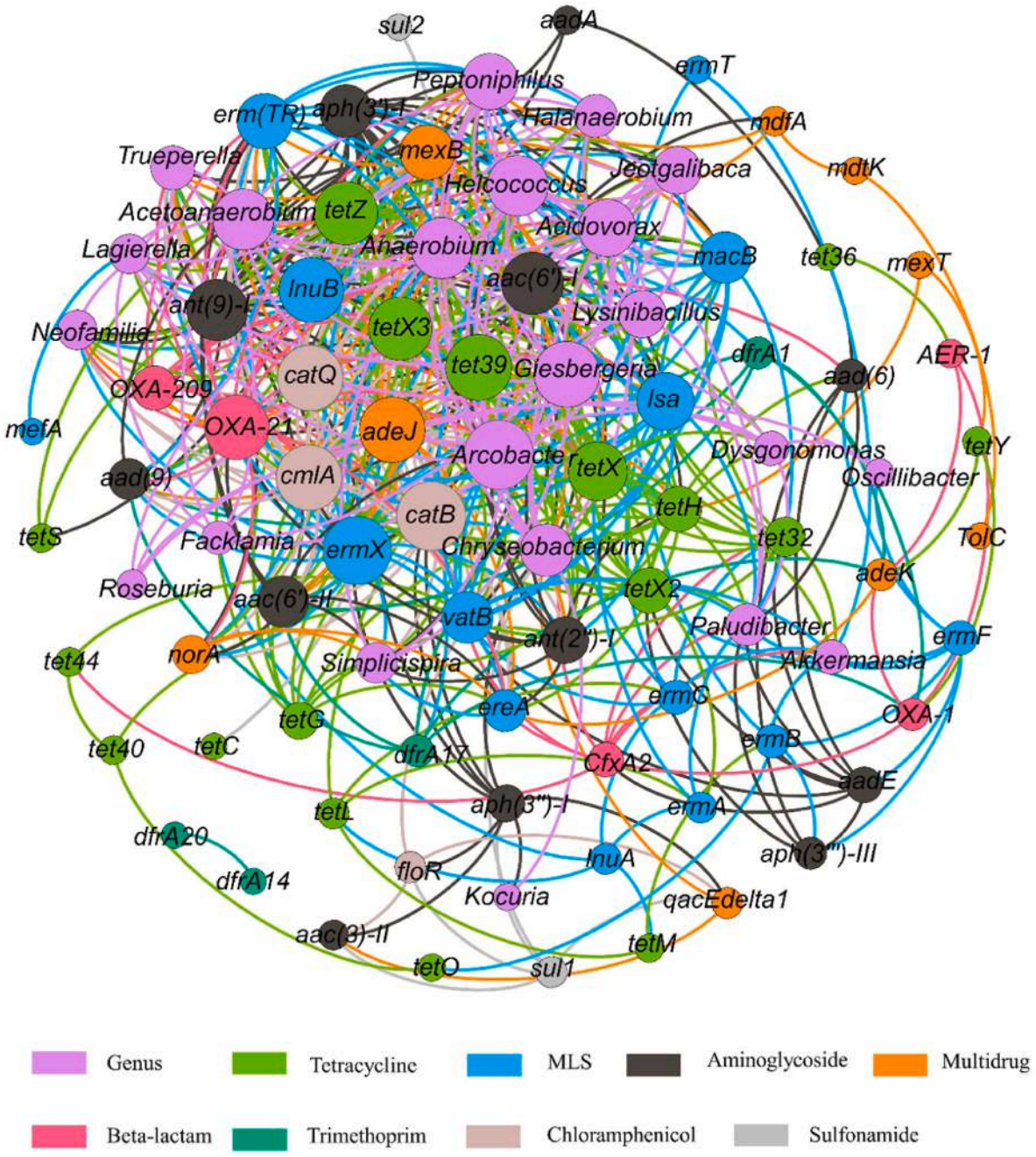

Fig. 6. Network analysis revealing the co-occurrence patterns between ARGs and bacterial genera. A connection represents a strong ($r > 0.9$) and significant ($P < 0.001$) correlation. The nodes were colored according to ARG types and genus. MLS: macrolide-lincosamide-streptogramin.

4. Discussion

In this study, bacterial diversity and resistome profiles were investigated in the air of 18 chicken houses at different laying periods and revealed that they are characterized by unique features. In addition, it was found that the resistome profiles in the air were closely related to the bacteria, and potential pathogenic bacteria and possible hosts of ARG were also identified. This study provides new insights into airborne bacterial microbiota and drug resistance in chicken houses during different laying periods.

4.1. Relationship between airborne bacteria and laying period

In this study, airborne bacteria from the same laying period showed similar characteristics, which might be attributed to the fact that these chicken farms belonged to the same company with the same management (Joat et al., 2021). The difference in bioaerosol samples in different laying periods might be influenced by the laying period. The bacterial diversity in the house air during the PL period was significantly higher than that in the EL period, which may be due to the changes in the physiological and endocrine structure of layer hens after the layer peak supporting the increase in egg production (Dai et al., 2022), leading to the increase in intestinal microbial diversity to improve nutrient utilization and lipid metabolism rate (Videnska et al., 2014). Bacillus subtilis strain DSM 29784 could modulate the cecal microbiome, concentration of short-chain fatty acids, and apparent retention of dietary components in shaver white chickens during grower, developer, and laying periods. The relative abundance of Firmicutes increased from EL to PL, similar to

previous studies, because Firmicutes could provide more nutrients to peak-layer hens through the production of SCFA from complex glycans (Gibiino et al., 2018). In the EL, the high abundance of Proteobacteria in the gut of layer hens was associated with the promotion of immune system development (Oakley et al., 2014). In the PL period, Bacteroidetes replaced Proteobacteria as the second dominant phylum. *Bacteroides* could promote the absorption of carbohydrates in layer hens and provide more energy for the intestinal development of layer hens (Dai et al., 2020; Polansky et al., 2016). This could be explained by the fact that in the PL period, the composition of the intestinal flora was stable, while in the LL period, the relative abundance of Firmicutes decreased and that of Bacteroidetes increased. These changes might reduce the pH of the intestinal chamber, inhibit the colonization of pathogenic bacteria and improve the utilization rate of nutrients to adapt to the new physiological requirements (Polansky et al., 2016). These results were similar to those found when the gut microbiota of layer hens changes during growth (Dai et al., 2022).

This pattern associated with the period of egg production has also been found in bacterial genera associated with potentially airborne pathogens. For example, the relative abundances of *Bacteroides*, *Corynebacterium* and *Fusobacterium* all increased significantly over time. *Bacteroides* could play a dual role according to their position in the host. Usually, *Bacteroides* are beneficial in the intestinal tract, but when they appear in other parts, they have the chance to be pathogenic (Zafar and Saier, 2021). Some *Bacteroides* can induce infectious pathogens in the formation of abscesses (Wexler, 2007). Some species of *Corynebacterium* are opportunistic commensals in the environment and can cause upper respiratory diseases such as laryngitis, nasopharyngitis or tonsillitis (Hoefer et al., 2021a). *Fusobacterium* is an opportunistic pathogen that colonizes the oral mucosa and can cause bacteremia and various rapidly progressive infections (Brennan and Garrett, 2019).

Previous studies have examined changes in the relative abundance of genera associated with potential airborne pathogens in layer hens and broilers (Hong et al., 2012). However, no study has addressed the association of potential airborne pathogens in layer hens with different periods of egg production. This might explain the different respiratory symptoms affecting layer hen and broiler workers (Just et al., 2013). In this study, *Lactobacillus*, *Facklamia*, *Comamonas*, *Jeotgalicoccus*, and *Stenotrophomonas* were the dominant bacterial genera in the bioaerosols of chicken houses and were usually considered relatively harmless (Yang et al., 2018). This study provided valuable information for assessing inhalation exposure and possible human health effects of bioaerosols in layer hens at different periods of egg production.

4.2. Relationship between the air resistance group and laying period

Previous studies on ARGs in chicken houses have shown that chicken manure is one of the major sources of aerosol resistance in chicken houses (Mbareche et al., 2019; Yang et al., 2021). The resistance gene types in the chicken house bioaerosol microbiota in this study were resistant to almost all major antibiotic classes commonly used for clinical and agricultural purposes. According to the previous study, aminoglycosides, tetracycline and MLS resistance genes were the most abundant types of ARGs in all laying periods, and they were also the main ARGs in poultry feces (Wen et al., 2019). The abundance of resistance genes was associated with antibiotic use during feeding (Zhang et al., 2017). Our study showed that the abundances of the ARGs chloramphenicol, multidrug, polymyxin and sulfonamide in the PL period were higher than those in the EL or LL period. This suggests that higher microbial diversity may facilitate horizontal gene transfer in air samples during the PL period. A high abundance of aminoglycoside, fosfomycin, tetracycline and vancomycin genes was observed in the EL period bioaerosols in this study. This might be related to the use of many antibiotics in the growth period of layer hens. Previous studies have reported high levels of aminoglycoside, fosfomycin, tetracycline and vancomycin, which are used to promote growth, in the feces of layer hens in the EL periods (Rivera-Gomis et al., 2021; Zhu et al., 2021). In this study, more than 172 subtypes of ARGs were found, and the abundance of ARGs varied greatly in different laying periods. The order from high to low was LL, EL, and PL. This is consistent with previous studies on ARGs in chicken manure samples at different growth periods and further validates that ARGs in the air may be derived from feces (Zhu et al., 2021). Generally, chicken farms will use many antibiotics before opening and reduce antibiotics during the laying period, resulting in a higher relative abundance in the EL period than in the PL period. In addition, the need to control diseases associated with intensive farming in the LL periods of egg production is also aimed at improving productivity, and large amounts of antibiotics continue to be used (Page and Gautier, 2012; Rivera-Gomis et al., 2021). In addition, the different distribution of ARG subtypes in the air indicated that the laying periods may be the key factor affecting the resistome of bioaerosol in the layer house.

4.3. Relationship between microbiome and resistome profiles

In total, 67 ARG subtypes belonging to 7 ARG types were detected, and 22 bacterial genera were estimated to be potential hosts by network analysis. Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria were the dominant phyla carrying ARGs. Interestingly, they were also the main dominant phyla of airborne bacteria. This suggested that the identified bacterial hosts were under constant antibiotic selection pressure, as the relative abundance of these phyla was found to be different at different laying periods. *Arcobacter*, *Giesbergeria*, *Helcococcus*, *AcetoAnaerobium*, *Acidovorax*, *Anaerobium*, *Chryseobacterium*, *Peptoniphilus*, *Trueperella* and *Neofamilia* carry multiple ARGs, including resistance genes of aminoglycoside, beta-lactam, chloramphenicol, MLS, multidrug and tetracycline. Several possible ARG hosts have been identified in previous studies. For example, *Arcobacter*, known for its pathogenicity, is reported to be an emerging zoonotic foodborne pathogen (Patyal et al., 2011). *Arcobacter* carries several ARG subtypes, such as aminoglycoside, chloramphenicol, MLS, and tetracycline, which is consistent with previous studies (Ferreira et al., 2013; Jia et al., 2017; Lu et al., 2015). These results help to understand the possible host relationship between bacteria and ARGs and provide precise dosing strategies for the treatment of resistant pathogens. However, further study is needed to obtain a more complete picture of antibiotic resistance in complex ecosystems (Gupta et al., 2021; Yu et al., 2020). Significant co-occurrence associations were observed between the internal types of ARGs, such as aminoglycosides, and the external types, such as MLS and chloramphenicol, which was consistent with previously reported results (Gupta et al., 2021; Yang et al., 2021). Overall, the bacterial bioaerosols of chicken houses were found to be an important factor regulating their resistance profiles.

5. Conclusions

In conclusion, this study analyzed the characteristics of airborne bacteria in commercial layer hen houses during different laying periods, providing new insights into the dynamics of bacteria and the resistome, and identified potential ARG host bacteria. The airborne microbiome and resistome varied greatly between different laying periods. Our results indicated that bioaerosols in layer houses might serve as potential reservoirs for bacterial pathogens and ARG hosts. There is a need for further study on the interactions between airborne bacteria and the resistome in different laying periods. Given the evidence of possible resistance to the large number of antibiotics used in poultry production, we recommended reducing the use of antibiotics. In addition, routine surveillance of some potential ARG host bacteria was also recommended to monitor their pathogenicity and ability to transmit antibiotic resistance to the environment.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

All experiments were approved by the relevant regulatory agency of the Animal Ethical Committee of Changchun Veterinary Research Institute (document number: SCXK201906097).

Author contributions

ZG, ZW and JL designed the project. HC, CZ, KZ, JP and YK performed the experiments. Data were analyzed by ZC, YZ, YC, CL, SD and LZ. Manuscript drafted by CZ and HC. ZG and JL revised the manuscript. All authors contributed to the study and approved the final version.

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Declaration of competing interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114173.

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Exposure of Swedish adolescents to elements, persistent organic pollutants (POPs), and rapidly excreted substances – The Riksmaten adolescents 2016-17 national survey

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ABSTRACT

Adolescence is a period of significant physiological changes, and likely a sensitive window to chemical exposure. Few nation-wide population-based studies of chemical body burdens in adolescents have been published. In the national dietary survey Riksmaten Adolescents (RMA) 2016–17, over 13 chemical substance groups, including elements, chlorinated/brominated/fluorinated persistent organic pollutants (POPs) were analysed in blood, and in urine metabolites of phthalates/phthalate alternatives, phosphorous flame retardants, polycyclic aromatic hydrocarbons (PAHs), and pesticides, along with bisphenols and biocide/preservative/antioxidant/UV filter substances (N = 1082, ages 11–21). The aim was to characterize the body burdens in a representative population of adolescents in Sweden, and to compare results with human biomonitoring guidance values (HBM-GVs). Cluster analyses and Spearman's rank order correlations suggested that concentrations of substances with known common exposure sources and similar toxicokinetics formed obvious clusters and showed moderate to very strong correlations ($r \geq 0.4$). No clusters were formed between substances from different matrices. Geometric mean (GM) concentrations of the substances were generally less than 3-fold different from those observed among adolescents in NHANES (USA 2015–16) and GerES V (Germany 2014–17). Notable exceptions were brominated diphenyl ethers (PBDEs) with >20-fold lower GM concentrations, and the biocide triclosan and ultraviolet (UV) filter benzophenone-3 with >15-fold lower mean concentrations in RMA compared to NHANES. Exceedance of the most conservative HBM-GVs were observed for aluminium (Al, 26% of subjects), perfluorooctanesulfonic acid (PFOS, 19%), perfluorooctanoic acid (PFOA, 12%), lead (Pb, 12%), MBP (dibutyl phthalate metabolite, 4.8%), hexachlorobenzene (HCB, 3.1%) and 3-phenoxybenzoic acid (PBA, pyrethroid metabolite, 2.2%). Males showed a higher proportion of exceedances than females for Pb, HCB and PFOS; otherwise no gender-related differences in exceedances were observed. A higher proportion of males than females had a Hazard Index (HI) of substances with liver and kidney toxicity and neurotoxicity >1. Industrialized countries with similarly high standards of living, with some exceptions, show comparable average body burdens of a variety of toxic chemicals among adolescents from the general population. The exceedances of HBM-GVs and HIs strongly suggests that further efforts to limit chemical exposure are warranted.

1. Introduction

Chemicals are used in almost every aspect of society and are found ubiquitously in human exposure media, including food, drinking water, air, dust, and consumer products. There are currently over 26,000 unique substances registered with the European Chemicals Agency, of which approximately 1000 are considered potential endocrine

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Abbreviations

Al	aluminium
BBOEP	bis(2-butoxyethyl) phosphate
BBzP	butylbenzyl phthalate
BHA	3-tert-butyl-4-hydroxyanisole
BMDL1%	benchmark dose of 1% risk
BPS	bisphenol S
BPA	bisphenol A
BP3	benzophenone-3
Cd	cadmium
Co	cobalt
Cr	chromium
br-PFOS	branched perfluorooctanesulfonic acid
CNS	central nervous system
cx-MiDP	mono-carboxy-isononyl phthalate
cx-MiNP	mono-(4-methyl-7-carboxyheptyl) phthalate
cx-MINCH	cyclohexane-1,2-dicarboxylate-mono (7-carboxylate-4-methyl)heptylester
DBP	dibutyl phosphate
p,p'-DDE	1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene
p,p'- DDT	dichlorodiphenyltrichloroethane
DEP	diethyl phthalate
DEHP	diethylhexyl phthalate
DiDP	diiso-decyl phthalate
DiNCH	diisononyl-cyclohexane-1,2-dicarboxylate
DiNP	diiso-nonyl phthalate
DPHP	dipropylheptyl phthalate
EFSA	The European Food Safety Authority
GC-MS/MS	gas chromatography – triple quadrupole mass spectrometry
GM	geometric mean
HBM-GVs	human biomonitoring guidance values
HCB	hexachlorobenzene
HCH	hexachlorocyclohexane
Hg	mercury
HI	Hazard Index
HR	Hazard Ratio
ICP-MS	inductively coupled plasma mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
L-PFOS	linear perfluorooctanesulfonic acid
L-PFHxS	linear perfluorohexanesulfonic acid
L-PFOA	linear perfluorooctanoic acid
MEHP	mono(2-ethylhexyl) phthalate
MEP	monoethyl phthalate
Mn	manganese
MBP	monobutyl phthalate
Ni	nickel
OCPs	organochlorine pesticides/metabolites
OH-MiNP	mono-(4-methyl-7-hydroxyoctyl) phthalate
OH-MINCH	cyclohexane-1,2-dicarboxylate-mono-(7-hydroxy-4-methyl)octyl ester
OH-MPHP	6-hydroxy monopropylheptyl phthalate
oxo-MiNP	mono-(4-methyl-7-oxooctyl) phthalate
PAHs	polycyclic aromatic hydrocarbons
PBDEs	polybrominated diphenyl ethers
PCBs	polychlorinated biphenyls
Pb	lead
PFDA	perfluorodecanoic acid
PFNA	perfluorononanoic acid
PFUnDA	perfluoroundecanoic acid
PFAS	per- and polyfluoroalkyl substances
PFOA	perfluorooctanoic acid
PFOS	perfluorooctanesulfonic acid
POPs	persistent organic pollutants
RMA	Riksmaten Adolescents
Se	selenium
SFA	Swedish Food Agency
TPP	triphenyl phosphate
TBEP	tri(2-butoxyethyl) phosphate
TCS	triclosan
TCP	trichloropyridinol
UBA	German Environment Agency
UPLC	ultra performance liquid chromatograph
US	United States
UV	ultraviolet
1-HP	1-hydroxy-pyrene
2-OH-PH	2-hydroxy-phenanthrene
3-PBA	3-phenoxybenzoic acid
4,4-BPF	bisphenol F
5-cx-MEPP	mono-(2-ethyl-5-carboxypentyl) phthalate
5-OH-MEHP	mono-(2-ethyl-5-hydroxyhexyl) phthalate
5-oxo-MEHP	mono-(2-ethyl-5-oxohexyl) phthalate

disrupting compounds (ECHA, 2022; Street et al., 2018). Furthermore, approximately 1000 new chemicals are registered with the United States Environmental Protection Agency per year (EPA, 2022), often with unknown or poorly understood safety profiles. Many chemicals are known to interfere with the human hormonal system and have previously been associated with a wide range of negative health consequences including neurologic, reproductive and other physiological effects (Braun, 2017; Wan et al., 2021; Yilmaz et al., 2020). Thus, considering both the number and effects of chemicals to which humans are exposed, understanding the variation of chemical mixture exposures within human populations (i.e., the chemical 'exposome') is of great importance.

Most studies examining human exposure to chemicals focus on single compound groups, such as elements, per- and polyfluoroalkyl substances (PFAS) and polychlorinated biphenyls (PCBs) (Marques et al., 2021; Preston et al., 2020; Roth et al., 2021), with few looking at the wider part of the chemical exposome. In reality, human exposure to chemicals is a complex process which involves multiple substances and pathways. Combination toxicity may occur when multiple chemicals occurring at sufficiently high concentrations act on a common endpoint to elicit an effect (Kortenkamp, 2014). In general, such mixtures may elicit a combination effect by either 'concentration addition' or 'independent action', although synergistic or antagonistic effects are also possible, albeit less frequently observed in experimental studies (Martin et al., 2021). 'Concentration addition' occurs when a toxicity threshold is exceeded by the sum concentration of multiple chemicals with the same mechanism/mode of action. In comparison, 'independent action' may occur when chemicals with different modes-of-action reach their individual toxicity thresholds on the same endpoint, thus eliciting a toxic effect (Kortenkamp, 2014). Concentration addition presents a unique challenge to risk assessors because individual toxic compounds may not be found in sufficiently high concentrations in study populations to pass observable health-risk thresholds, but may exceed such thresholds in combination with other chemicals sharing the same mechanism/mode of action. Further layers of complexity arise whereby chemical substances with independent modes of action can still share the same adverse health effects (Christiansen et al., 2020).

Biomonitoring of chemical body burdens in blood and urine provides valuable information about cumulative exposure at the time of sampling. Adolescence is likely a sensitive developmental window to

chemical exposure due to the significant physiological changes that occur. Nevertheless, few nation-wide studies have been published on adolescent exposure to a broad range of chemicals. The few well-known examples include NHANES in the United States (US) and GerES V in Germany (GerES, 2021; NHANES, 2022). Additionally, FLEHS IV in Belgium (Schoeters et al., 2022) represents another similar (albeit regional) study on the chemical exposome of adolescents. These studies, to the best of our knowledge, have not fully investigated the correlations between exposure of adolescents to the measured substances/substance groups. Knowledge of such correlations, which may be due to common exposure sources and/or similarities in toxicokinetics, are crucial when interpreting associations between health outcomes and body burdens of chemical mixtures. The previous studies have compared the observed concentrations of measured chemicals with human biomonitoring guidance values (HBM-GVs), in order to identify the chemicals with concern for adolescent health. However, they have not investigated possible gender and age differences in this context, which may be important for health development during this period of rapid physiological change. Moreover, to the best of our knowledge, no such study has attempted to investigate possible combination toxicity risks with substances with common target organs.

The overall aim of the present study is to characterize the body burdens of over 60 different substances across 13 chemical substance groups (elements (e.g., heavy metals), chlorinated, brominated, and fluorinated persistent organic pollutants (POPs), phthalates, phthalate alternatives, bisphenols, phosphorous flame retardants, polyaromatic hydrocarbons (PAH), pesticides, biocides/preservatives and UV-filters) in Swedish adolescents. To address this goal, we compared measured concentrations in whole blood, serum and urine to those reported in similar studies from the United States, Germany and the Flanders region of Belgium. We also examined correlations among individual substances, in an attempt to identify shared sources of exposure. Finally, we compared measured concentrations to published HBM-GVs and determined Hazard Indices in an effort to assess cumulative health risk (Kortenkamp and Faust, 2010).

2. Method

2.1. Study group and design

The study population was a sub-sample of Riksmaten Adolescents 2016–17 (RMA), a cross-sectional national population-based study of dietary habits and chemical exposure of Swedish adolescents, conducted by the Swedish Food Agency (SFA). Details about the recruitment process, study design and data collection are described elsewhere (Moraeus et al., 2018). Briefly, 619 schools were invited, being representative of the entire country across the 5th (median age, range: 11 years, 10–13), 8th (14 years, 11–15) and 11th (17 years, 16–21) school grades. Of these schools, 259 were invited to participate in the biomonitoring part, with 62 school classes across 57 unique schools opting-in (Moraeus et al., 2018) (Fig. S1, supplement). In total, 1305 students, out of 2377 invited, accepted to be sampled and 1082 had sufficient biomonitoring data to be included in our analysis (Table 1 & Table S1, Supplement). The study design included online-based questionnaires (RiksmatenFlexQ) addressing background factors such as age and gender (Moraeus et al., 2018).

2.2. Ethics

Ethical approval was obtained from the Regional Ethical Review Board in Uppsala (No 2015/190). Participants in grades 8 and 11 gave written informed consent to participate in the study, whilst legal guardians gave consent for 5th graders.

Table 1
Gender differences in substance concentrations among RMA participants.

Substances[a]		Geometric mean (95% CI)			p-value[b]
Abbreviation	Unit/ Matrix	Male (N = 476)	Female (N = 606)	Total (N = 1082)	
Cr	µg/L whole blood	0.58 (0.57–0.60)	0.55 (0.54–0.57)	0.57 (0.56–0.58)	0.092
Mn	µg/L whole blood	10.1 (9.67–10.4)	11.0 (10.7–11.2)	10.6 (10.3–10.8)	<0.001
Co	µg/L whole blood	0.11 (0.10–0.11)	0.14 (0.14–0.15)	0.13 (0.12–0.13)	<0.001
Ni	µg/L whole blood	0.62 (0.61–0.64)	0.62 (0.61–0.64)	0.62 (0.61–0.63)	0.466
Se	µg/L whole blood	94.7 (91.8–97.7)	95.0 (93.9–96.1)	94.8 (93.4–96.3)	0.141
Cd	µg/L whole blood	0.11 (0.11–0.12)	0.13 (0.13–0.14)	0.12 (0.12–0.13)	<0.001
Hg	µg/L whole blood	0.75 (0.69–0.81)	0.60 (0.56–0.64)	0.66 (0.63–0.69)	<0.001
Pb	µg/L whole blood	8.17 (7.77–8.58)	6.72 (6.49–6.97)	7.32 (7.11–7.55)	<0.001
HCB	pg/mL serum	47.3 (45.8–48.9)	38.5 (37.3–39.8)	42.2 (41.2–43.2)	<0.001
p,p'-DDE	pg/mL serum	124 (115–133)	99.7 (93.2–107)	110 (104–115)	<0.001
PCB-118	pg/mL serum	7.01 (6.67–7.36)	6.41 (6.14–6.70)	6.67 (6.45–6.89)	0.006
PCB-138	pg/mL serum	30.3 (28.6–32.2)	23.3 (22.1–24.6)	26.2 (25.2–27.2)	<0.001
PCB-153	pg/mL serum	49.0 (46.1–52.1)	36.9 (34.9–39.0)	41.8 (40.0–43.6)	<0.001
PCB-170	pg/mL serum	13.7 (12.8–14.7)	9.86 (9.24–10.5)	11.4 (10.9–12.0)	<0.001
PCB-180	pg/mL serum	27.7 (25.7–29.8)	19.7 (18.5–21.1)	22.9 (21.8–24.1)	<0.001
PCB-187	pg/mL serum	6.14 (5.71–6.61)	4.49 (4.20–4.80)	5.15 (4.90–5.41)	<0.001
L-PFOA	ng/g serum	1.26 (1.21–1.31)	1.17 (1.13–1.22)	1.21 (1.18–1.25)	0.001
PFNA	ng/g serum	0.39 (0.37–0.41)	0.34 (0.32–0.35)	0.36 (0.35–0.37)	<0.001
PFDA	ng/g serum	0.15 (0.14–0.16)	0.15 (0.14–0.16)	0.15 (0.14–0.16)	0.219
L-PFHxS	ng/g serum	0.55 (0.50–0.61)	0.39 (0.36–0.42)	0.45 (0.43–0.48)	<0.001
L-PFOS	ng/g serum	2.43 (2.29–2.59)	1.92 (1.82–2.03)	2.13 (2.05–2.22)	<0.001
br-PFOS	ng/g serum	1.12 (1.05–1.19)	0.85 (0.80–0.90)	0.96 (0.92–1.00)	<0.001
MEP	ng/mL urine	31.5 (28.8–34.6)	51.0 (46.4–56.2)	41.3 (38.6–44.2)	<0.001
MBP	ng/mL urine	38.9 (36.5–41.3)	43.7 (41.6–45.9)	41.5 (39.9–43.1)	0.001
MBzP	ng/mL urine	6.33 (5.73–6.99)	7.61 (6.98–8.30)	7.02 (6.57–7.49)	0.006
MEHP	ng/mL urine	1.61 (1.52–1.71)	1.82 (1.72–1.93)	1.73 (1.66–1.80)	0.005
5OH-MEHP	ng/mL urine	7.81 (7.35–8.29)	8.19 (7.69–8.73)	8.02 (7.67–8.38)	0.230
5oxo-MEHP	ng/mL urine	5.98 (5.62–6.37)	6.69 (6.31–7.10)	6.37 (6.10–6.65)	0.033
5cx-MEPP	ng/mL urine	6.80 (6.39–7.24)	7.69 (7.25–8.16)	7.29 (6.98–7.61)	0.010
2cx-MEHP	ng/mL urine	2.05 (1.92–2.18)	2.18 (2.06–2.32)	2.12 (2.03–2.22)	0.120
OH-MiNP	ng/mL urine	4.23 (3.89–4.61)	4.93 (4.52–5.38)	4.61 (4.33–4.91)	0.042
oxo-MiNP	ng/mL urine	1.94 (1.79–2.09)	2.34 (2.16–2.54)	2.15 (2.04–2.28)	0.001

(continued on next page)

Table 1 (*continued*)

Substances[a]		Geometric mean (95% CI)			p-value[b]
Abbreviation	Unit/ Matrix	Male (N = 476)	Female (N = 606)	Total (N = 1082)	
cx-MiNP	ng/mL urine	6.34 (5.84–6.88)	7.89 (7.27–8.57)	7.17 (6.76–7.60)	0.001
cx-MiDP	ng/mL urine	0.36 (0.34–0.39)	0.44 (0.42–0.47)	0.41 (0.39–0.43)	<0.001
OH-MPHP	ng/mL urine	1.07 (1.00–1.15)	1.16 (1.08–1.24)	1.12 (1.07–1.18)	0.189
oxo-MiNCH	ng/mL urine	1.20 (1.07–1.33)	1.36 (1.24–1.50)	1.29 (1.20–1.38)	0.032
cx-MINCH	ng/mL urine	0.88 (0.78–0.98)	1.01 (0.92–1.12)	0.95 (0.88–1.03)	0.034
OH-MINCH	ng/mL urine	0.92 (0.82–1.03)	0.99 (0.89–1.10)	0.96 (0.89–1.03)	0.321
DPP	ng/mL urine	1.82 (1.71–1.93)	2.13 (2.01–2.25)	1.99 (1.91–2.07)	0.001
DBP	ng/mL urine	0.13 (0.12–0.14)	0.17 (0.15–0.18)	0.15 (0.14–0.16)	<0.001
BPA	ng/mL urine	0.87 (0.80–0.95)	0.88 (0.81–0.95)	0.88 (0.83–0.93)	0.536
BPS	ng/mL urine	0.12 (0.11–0.13)	0.16 (0.15–0.18)	0.14 (0.14–0.15)	<0.001
4,4BPF	ng/mL urine	0.09 (0.08–0.11)	0.11 (0.10–0.13)	0.10 (0.09–0.11)	0.026
2-OH-PH	ng/mL urine	0.16 (0.15–0.17)	0.17 (0.16–0.19)	0.17 (0.16–0.18)	0.160
TCP	ng/mL urine	1.24 (1.16–1.31)	1.35 (1.28–1.43)	1.30 (1.25–1.35)	0.021
3-PBA	ng/mL urine	0.27 (0.25–0.29)	0.29 (0.28–0.31)	0.28 (0.27–0.30)	0.029
TCS	ng/mL urine	0.25 (0.23–0.29)	0.33 (0.30–0.37)	0.30 (0.27–0.32)	0.002
BHA	ng/mL urine	0.27 (0.21–0.36)	1.33 (1.05–1.69)	0.66 (0.55–0.80)	<0.001
BP3	ng/mL urine	0.64 (0.56–0.73)	1.56 (1.35–1.81)	1.05 (0.95–1.17)	<0.001

[a] Substances with over 50% of samples < LOQ or LOD are not included in this table. For the purpose of calculating geometric means and 95% confidence intervals (CI), determined concentrations < LOQ or < LOD were used and concentrations determined to be 0 were converted to the lowest concentration above 0 divided by sqrt(2) (see section Handling of data below LOQ/LOD). When LOD was available PFAS concentrations < LOD were converted to LOD/sqrt(2), else concentrations < LOQ were converted to LOQ/sqrt(2).

[b] Wilcoxon rank sum test on comparison of concentrations by gender. p-values <0.05 indicate significant difference of concentration between genders.

2.3. Sample collection

One single-spot non-fasting blood and urine sample, along with anthropometric data, were collected from each participant by trained staff during school visits on weekdays throughout 2016 (September)-2017 (May). Blood samples (10 ml) for serum were collected in Vacutainer serum tubes with coagulation activator (Becton Dickinson, article # 367896, Sweden), centrifuged at 1500×*g* for 10 min and then aliquoted to cryotubes. Whole blood samples (4 ml) were collected in lithium-heparin Vacuette tubes (Greiner Bio-one, article# 454056, Germany). Urine samples were collected in acetone-rinsed paper cups by the individual participants during the school visit, and these samples were then aliquoted to polypropylene tubes. All aforementioned samples were promptly frozen at −20 °C at the site of collection and shipped frozen to the SFA where they were stored at −80 °C until being sent to external laboratories for analysis. None of the samples analysed in this study had known disruptions in the cold chain.

2.4. Sample analysis

Full names and abbreviations of the analysed substances, CAS numbers, and their main uses are provided in Table S2 (Supplement). Decisions on inclusion of substances were made by experts from the SFA at the time of study design and were based on toxic properties of the substances and presence in food and/or drinking water. Some essential/suspected essential elements were also included based on suspected interactions with non-essential toxic elements in the body (chromium (Cr), manganese (Mn), selenium (Se), cobalt (Co)) (Table S2, supplement).

2.5. Elements

Samples were analysed for Cr, Mn, Co, nickel (Ni), Se, cadmium (Cd), mercury (Hg), lead (Pb), and aluminium (Al) by the Department of Laboratory Medicine, Lund University, Lund, Sweden (Table S2, Supplement). Concentrations of Pb, Cd and Hg in RMA have been published previously (Almerud et al., 2021). Sample analyses were performed in duplicate. In short, the samples were treated as previously described (Barany et al., 1997). The concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS; iCAP Q, Thermo Fisher Scientific, Bremen, GmbH) equipped with collision cell with kinetic energy discrimination and helium as collision gas. The limit of detection (LOD) varied from 0.05 to 5.0 μg/L. Method precision varied from 2.8 to 15% depending on analyte.

2.6. PCBs, organochlorine pesticides/metabolites (OCPs) and polybrominated diphenyl ethers (PBDEs)

The Department of Health Security, National Institute for Health and Welfare, Kuopio, Finland analysed the chlorinated and brominated POPs in serum (Table S2, Supplement) as described previously (Gasull et al., 2019). Some results from the analyses have been published previously (Zamaratskaia et al., 2022). In brief, concentrations were measured using gas chromatography – triple quadrupole mass spectrometry (GC-MS/MS). The instrument used was an Agilent 7010 GC-MS/MS system (Wilmington, DE, U.S.), GC column DB5MS UI (J&W Scientific, 20m, ID 0.18 mm, 0.18 μm). Limits of quantification (LOQ) ranged from 5 pg/mL for PCB congeners and trans-nonachlor to 40 pg/mL for 1, 1-dichloro-2,2-bis(p-chlorophenyl)ethylene (p,p'-DDE). Two blank samples and two control samples (NIST SRM 1958) were included in each batch of samples. Measured concentrations of chlorinated and brominated POPs in SRM1958 were 80–105% of the certified/reference concentrations. The coefficient of variation (CV%) from SRM 1958 (n = 18) was <3.6% for all compounds.

2.7. PFAS

Serum samples were analysed for PFASs by the Department of Environmental Science, Stockholm University, Sweden (Table S2, Supplement). Details of the extraction and instrumental analysis are described in Nyström et al. (2022). Briefly, samples were fortified with a suite of isotopically-labelled internal standards and then extracted twice with acetonitrile in an ultrasonic bath, followed by clean-up with acidified graphitized carbon. The cleaned-up extract was diluted and fortified with volumetric standards and thereafter stored at −20 °C prior to analysis. Instrumental analysis was carried out on a Acquity ultra performance liquid chromatograph coupled to a Waters Xevo TQS triple quadrupole mass spectrometer operated in negative electrospray ionisation, multiple reaction monitoring mode. Quantification was based on isotope dilution or an internal standard approach (see Nyström et al. (2022) for details). LOQs are summarized in Table S3 (Supplement). In the present study only data on selected perfluoroalkyl acids: linear perfluorooctanoic acid (L-PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), linear perfluorohexanesulfonic acid (L-PFHxS), branched and linear perfluorooctanesulfonic acid (br-PFOS, L-PFOS), were used due to the low detection frequency of other PFASs analysed in RMA (Nyström et al., 2022).

2.8. Substances measured in urine

Analyses were performed at the Department of Laboratory Medicine, Lund University, Sweden, as previously described (Alhamdow et al., 2017; Cequier et al., 2014; Liljedahl et al., 2021) with some modifications. In brief, the samples were analysed on a liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS; QTRAP 5500, AB Sciex, Framingham, MA, USA). Two urinary quality control samples were included in the analysis, one authentic and one spiked. The CV% calculated from QC samples included in all sample batches (N = 34) as a between-run precision, did not exceed 20% for almost all compounds with the exception of mono-carboxy-isononyl phthalate (cx-MiDP, 27% at concentration of 0.6 ng/mL), bisphenol S (BPS, 25% at concentration of 0.8 ng/mL), dibutyl phosphate (DBP, 41% at concentration of 0.1 ng/mL) and 3-tert-butyl-4-hydroxyanisole (BHA, 21% at concentration of 0.8 ng/mL). The analyses of bisphenol A (BPA), 1-hydroxy-pyrene (1-HP), benzophenone-3 (BP3), triclosan (TCS), 3-phenoxybezoic acid (3-PBA), trichloropyridinol (TCP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP), mono-(2-ethyl-5-oxohexyl) phthalate (5-oxo--MEHP), is part of G-EQUAS inter-laboratory control program (University of Erlangen-Nuremberg, Germany). The laboratory also participates in the HBM4EU QA/QC program, and has qualified as HBM4EU laboratory for the analysis of: BPA, BPS, BPF, 1-HP, monobenzyl phthalate (MBzP), mono-(2-ethylhexyl) phthalate (MEHP), 5-OH-MEHP, 5-oxo--MEHP, mono-(2-ethyl-5-carboxypentyl) phthalate (5-cx-MEPP), mono-(4-methyl-7-hydroxyoctyl) phthalate (OH-MiNP), mono-(4-methyl-7-oxooctyl) phthalate (oxo-MiNP), mono-(4-methyl-7-carboxyheptyl) phthalate (cx-MiNP), cyclohexane-1,2-dicarboxylate-mono (7-carboxylate-4-methyl)heptylester (cx-MINCH), cyclohexane-1,2-dicarboxy late-mono-(7-hydroxy-4-methyl)octyl ester (OH-MINCH). Urine concentrations were adjusted for individual differences in urine density in the RMA population as described by (Carnerup et al., 2006). LODs are summarized in Table S4 (Supplement).

2.9. Handling of data below LOQ/LOD

The number of samples with concentrations below LOQ/LOD for each substance are presented in Tables S3 and S4 (Supplement). Except for PFASs, concentrations below LOQ or LOD were handled as previously described (Darnerud et al., 2015; Gyllenhammar et al., 2017; Svensson et al., 2021). Briefly, concentrations above 'background noise' in the analyses were determined to the concentration estimated in the analytical run. When a concentration was estimated not to be above 'background noise' in the chemical analyses, the concentration was set to zero (AMC, 2001). In the case of PFAS, concentrations below LOQ but above LOD were used when available, while concentrations below LOD were set to zero. Concentrations determined to be below LOD or LOQ are more uncertain and should be regarded as semi-quantitative. Nevertheless, the use of these determined concentrations in statistical analyses, as opposed to using substituted or imputed data, is generally considered to result in less statistical bias (AMC, 2001; Bergstrand and Karlsson, 2009). Fig. S2 (Supplement) provides examples of Al, PCB-183 and bis(2-butoxyethyl) phosphate (BBOEP) showing the change in concentration distribution of the substances after replacement of concentrations < LOQ or LOD with determined concentrations instead of fixed concentrations at LOQ or LOD/$\sqrt{2}$. Nevertheless, it is important to be aware of that use of data with a large proportion of determined concentrations below LOQ or LOD will add uncertainty in the interpretation of the results. For GM calculations, concentrations determined to be zero were by necessity further converted to the lowest determined concentrations above zero divided by $\sqrt{2}$. In these cases, PFAS concentrations below LOQ or LOD were set to LOQ or LOD/$\sqrt{2}$.

2.10. Statistical analysis

Analysis was performed using *R* (ver: 4.0.2). In the cluster analysis, the 'varclus' function from the 'hmisc' package (ver: 4.4–0) was used to visualise the clustering of the chemical substances using spearman correlation coefficients with complete linkage. Correlation analyses of relationships between substance concentrations were assessed using Spearman Rank Correlation coefficients. By using results from the cluster analysis in combination with the correlation analysis it is possible to get a more comprehensive description of the relationships between individual substances. It is usually not probable that the cluster analysis identifies clusters including all substances that are similarly correlated to one another. In this case, the correlation analysis provides information about correlations between substances separated in different clusters. In both the cluster and the correlation analysis, substances with more than 70% of concentrations above zero were included in the analyses (Tables S3 and S4, supplement), with the full range of values used for calculations. The strength of the correlations was assessed using definitions proposed by (Schober et al., 2018), where a correlation coefficient of 0.00–0.39 is classified as negligible to weak, 0.40–0.69 is moderate, 0.70–0.89 is strong, and 0.90–1.00 is a very strong correlation. Wilcoxon rank sum and signed rank test ($\alpha = 0.05$) was used to analyse if there were gender-related differences in concentrations, and a Kruskal-Wallis rank sum test ($\alpha = 0.05$) to analyse if there are significant concentration differences in at least one of the school grades compared to the others. The Chi-Squared Test was used to test if there were gender- or school grade-related differences in proportions of exceedances of HBM-GVs and HI > 1 (see section 'Comparisons with HBM-GVs and HI assessment' below).

2.11. Study comparison

The literature was searched for nation-wide population-based studies that analysed the larger chemical exposome in both blood and urine amongst adolescents during the same period as RMA. We found two studies matching this criteria, United States NHANES 2015–17 (NHANES, 2022), German GerEs V 2014–17 (GerES, 2021), and additionally the regional Flemish Belgium FLEHS IV 2016–2020 (FLEHS, 2020).

Concentrations for NHANES were mostly reported as weighted GMs. In the case of chlorinated and brominated POPs, concentrations were instead reported as arithmetic means of pooled samples from separate ethnic groups and genders. We therefore included the reported minimum and maximum ethnical group/gender mean concentrations reported in NHANES 2015-17 within our comparison table (Table 2). Values for GerES V were reported as GMs but sometimes included younger children than those included in RMA in the reporting of certain substances. Values for FLEHS IV were reported as GMs for the adolescent study population, ages 14–15. Comparatively, we reported the RMA results as GMs including all ages (ages 10–21) analysed within our study population.

2.12. Comparisons with HBM-GVs and HI assessment

To assess the potential health concerns associated with chemical exposure in Swedish adolescents, HBM-GVs were obtained for the investigated substances from the peer-reviewed literature. We focussed on published values from the German Environment Agency (UBA) and the HBM4EU project (Apel et al., 2020; UBA, 2015) (Table 4). If HBM-GVs were published by both organizations, the HBM4EU value was used. In certain cases when HBM-GVs from these two sources were lacking we used published biomonitoring equivalents (Hays and Aylward, 2009), or published health-based values from other sources that could be used when comparing with our observed concentrations (Table 4). While the search for HBM-GVs was not exhaustive, data were obtained for many of the substances in the present work.

In order to examine possible combination toxicity of measured substances, the HI assessment approach was used (Borg et al., 2013). This approach assumes concentration addition between measured substances

Table 2
Comparison of geometric mean (GM) of substance concentrations in whole blood and serum observed in RMA with those reported for 12–19 years old adolescents from NHANES, USA, 3–17 years old children/adolescents from GerES V, Germany, and 14–15 years old adolescents from the Flemish regions of Belgium (FLEHS IV)[a].

Substances[b]	RMA (2016–2017) GM (N = 1082)	NHANES (2015–2016) Weighted GM[e] (N = 353–565)	GerES (2014–2017) GM (age) (N = 516–2256)		FLEHS IV (2016–2020) GM (N = 428)
Whole blood (μg/L)					
Mn	11	11			9.4
Se	95	190			
Cd	0.12	0.13	<0.12	(12–17)	0.19
Hg	0.66	0.40			
Pb	7.3	4.7	8.4	(12–17)	7.7
Serum (pg/ml)[c]					
HCB	42	24–37000 (NHBF – AHM)	<70	(14–17)	25
beta-HCH	3.1	<4–60 (NHBF - AHM)	<20	(3–17)	3.7
Oxychlordane	0.13	<4–7900 (NHBF - AHM)			4.0
trans-Nonachlor	2.9	9.3–12000 (NHBF – AHM)			2.5
p,p'-DDT	0.16	<4–16000 (NHBF - AHM)	<20	(14–15)	5.9
p,p'-DDE	110	160. – 690000 (NHBF - AHM)	134	(14–17)	135
PCB-74	3.5	<2–51 (NHWF - NHWM)			
PCB-99	3.9	0.2–3800 (MAF - AHM)			
PCB-118	6.7	4.1–5500 (MAF - AHM)	<20	(3–17)	
PCB-138	26	6.2–10000 (MAF - AHM)	45	(14–17)	
PCB-153	42	8.2–13000 (MAF - AHM)	62	(14–17)	
PCB-156	3.8	<0.8–6.2 (NHBF - NHWM)			
PCB-170	11	2.1–2900 (NHBF - AHM)			
PCB-180	23	4.1–7000 (MAF - AHM)	32	(14–17)	
PCB-183	2.1	<0.8–4.1 (NHBF - AM)			
PCB-187	5.2	2.1–2300 (MAF - AHM)			
∑PCB-138, 153, 180	92		140		71
BDE-47	0.78	27–83 (AF - NHBM)			<1
BDE-99	0.29	7.9–20 (AF - MAM)			<1
BDE-153	0.68	16–54 (AF - NHBM)			<1
Serum (ng/g serum)[d]					
L-PFOA	1.2	1.1	1.1	(12–17)	1
PFNA	0.36	0.47	<0.49	(3–17)	0.3
PFUnDA	0.10	<0.10	<0.24	(3–17)	
PFHxS	0.45	0.89	0.35	(12–17)	0.47
L-PFOS[f]	2.1	2	2.4	(12–17)	2.1
br-PFOS	0.96	0.85			

[a] (GerES, 2021; NHANES, 2022; Schoeters et al., 2022).
[b] For the purpose of calculating geometric means in RMA, determined concentrations < LOQ or < LOD were used and concentrations determined to be 0 were converted to the lowest concentration above 0 divided by sqrt(2) (see section Handling of data below LOQ/LOD). When LOD was available, PFAS concentrations < LOD were converted to LOD/sqrt(2), else concentrations < LOQ were converted to LOQ/sqrt(2).
[c] NHANES/GerES/FLEHS; ng/g lipid of chlorinated and brominated POPs converted to pg/ml serum using average lipid content of serum in RMA of 0.33%.
[d] NHANES/GerES/FLEHS; PFAS ng/mL converted to ng/g using the specific gravity of serum (1.0275) (Sunderman and Boerner, 1949).
[e] Chlorinated POPs; arithmetic mean in pooled samples (N = 2–11, each consisting of 8 individuals) from different ethnical groups: NHBF = non-hispanic black females. AHM = all hispanic males. MHWF = non-hispanic white female. NHWM = non-hispanic white male. MAF = mexican american females. AM = asian male. AF=Asian female. MAM = Mexican american male.
[f] GerES & FLEHS linear + branched isomers.

in the assessment of possible cumulative health concerns. A conservative approach was used by grouping substances with the same target organ of critical effects (Table 4), specifically in this present study kidney toxicity, liver toxicity and neurotoxicity. Each participant's Hazard Ratio (HR) of the substance in question was calculated by dividing the measured concentration of the substance and its most conservative HBM-GV. Each individual's HRs were summed together to a HI according to the shared target organ of critical effects (see formula below). A potential concern to human health exists if the HI is > 1 for each target organ. See Table 5 for selected substance HRs included within each HI.

$$\text{Hazard Index} = \frac{C1}{GV1} + \frac{C2}{GV2} + \frac{C3}{GV3} + \ldots\ldots\ldots\frac{Ci}{GVi}$$

C_i: concentration of substance i in serum/whole blood/urine. GV_i: the HBM-GV for substance i (see Table 4). Hazard Index = the sum of the HRs of each substance in an individual with the same target organ of critical effects (see Table 5)

3. Results and discussion

RMA showed ubiquitous exposure of Swedish adolescents to mixtures of many of the studied elements, chlorinated pesticides, PCBs, and PFASs (Table 1 & Table S3, Supplement). Moreover, >50% of the participants had quantifiable concentrations of urinary metabolites of phthalates/phthalate alternatives, as well as metabolites of phosphorous flame retardants, PAHs, and pesticides, and bisphenols, and biocide/preservative/UV filter chemicals (Table 1 and Table S4, Supplement). This is in line with results from similar studies on adolescents from Germany and the USA (GerES, 2021; NHANES, 2022), and the regional study in Belgium (Schoeters et al., 2022).

Many of the studied elements and POPs have half-lives in the human body ranging from months to years (Nordberg et al., 2014; Verner et al., 2009); consequently, concentrations are representative of medium-to long-term cumulative exposure prior to sampling. In contrast, urine substances have short elimination half-lives (i.e., hours to a few days) and are therefore representative of very recent exposure (Perrier et al., 2016). Nevertheless, the almost ubiquitous quantification of these 'short-lived' substances in adolescent urine suggests that exposure

Table 3
Comparison of geometric means (GM) of substance concentrations in urine observed in RMA, with those reported for 12–19 years old adolescents from NHANES and 14–17 years old adolescents from GerES.

Substances	RMA (2016–2017) GM (N = 1082)	NHANES (2015–2016)[a,b] Weighted GM (N = 405)	GerES (2014–2017)[c] GM (N = 516–2256)	FLEHS IV (2016–2020)[d] GM (N = 416)
Urine (ng/mL)				
MEP	41	35	26	38
MBP	42	12	21	20
MBzP	7	6.1	3.1	3
MEHP	1.7	1.2	1.4	1.1[e]
5OH-MEHP	8	5.8	11	6.7
5oxo-MEHP	6.4	3.8	7.6	4.2
5cx-MEPP	7.3	9.4	12	16
OH-MiNP	4.6		6.9	3.9[e]
oxo-MiNP	2.2	2.6	2.8	
cx-MiNP	7.2		5.9	1.7[e]
cx-MiDP	0.41	2.2	0.9	1.2[e]
OH-MPHP	1.1		0.3	
oxo-MiNCH	1.3		0.93	
cx-MINCH	0.95	0.58	1.1	0.98[e]
OH-MINCH	0.96	0.97	2.3	1.2[e]
DPP	2	1.4		
DBP	0.15	0.21		
BPA	0.88	1.2	1.9	1.1
BPS	0.14	0.37		0.13
4,4BPF	0.10	<0.2		0.17
2-OH-PH	0.17	0.06	0.09	
1-HP	0.07	0.16	0.12	
3-PBA	0.28	0.64		
TCS	0.30	5.2	<1	
BP3	1.1	16	<2	

[a] DPP and DBP from 2013 to 2014 instead. 2-OH-PH from 2011 to 2012 instead. 1-HP and 3-PBA from 2013 to 2014 instead.
[b] (NHANES, 2022) Volumes II & III, updated March 2021.
[c] (Murawski et al., 2020; Schwedler et al., 2020a, 2020b; Tschersich et al., 2021).
[d] (Schoeters et al., 2022).
[e] (Bastiaensen et al., 2021).

occurred on a more-or-less daily basis in Sweden during the RMA study period. However, the use of a single-spot urine sample from each participant makes it difficult to estimate individual long-term exposure due to large intra-individual temporal variation in exposure (Perrier et al., 2016). Nonetheless, the RMA results give estimates of the overall exposure situation among adolescents in Sweden 2016–17 (Aylward et al., 2017).

3.1. Correlations between substance concentrations

This is, to the best of our knowledge, one of the most comprehensive studies of relationships between whole blood, serum and urine concentrations of chemicals in a national population study of adolescents. In the cluster analysis, most elements with diverging dietary exposure sources (Livsmedelsverket, 2017), did not form clear cluster groupings and in most cases negligible to weak correlations were observed (Fig. 1 and Fig. S3, Supplement). Moreover, with a few exceptions, correlations between elements and POPs were negligible to weak (Fig. S3, Supplement), most likely due to differences in toxicokinetics (much shorter half-lives of most elements, many POPs being lipid soluble) (Andersen et al., 2021; Nordberg et al., 2022; Verner et al., 2009) and/or exposure sources (mainly plants-based foods vs foods of animal origin) (Livsmedelsverket, 2017). Within-group clusters were observed among the POPs with similar dietary exposure patterns (Krauskopf et al., 2017; Lignell et al., 2011; Livsmedelsverket, 2017; Nyström et al., 2022) and toxicokinetics (Andersen et al., 2021; Verner et al., 2009) (Fig. 1). However, no overlaps were observed between the chlorinated, brominated and fluorinated POPs (Fig. 1). As suspected from the very short half-lives and diverging exposure sources, the urine substances did not cluster together with any of the substances measured in blood (Fig. 1) and correlations were negligible to weak (results not shown). Among substances measured in urine, metabolites of diethylhexyl phthalate (DEHP), diiso-nonyl phthalate (DiNP), and diisononyl-cyclohexane-1, 2-dicarboxylate (DiNCH) clustered separately from one another and displayed moderate to strong correlations within a given cluster (Fig. 1 & Fig. S4, Supplement). Se and Hg, and Co and Cd, formed separate clusters among the elements (Fig. 1). Hypothetically, Se and Hg relationships could be due interactions between these elements in the body (Bárány et al., 2003; Bates et al., 2006; Glynn and Lind, 1995; Ralston and Raymond, 2010). For Co and Cd similar dietary exposure sources (cereals and sugar/sweets) (Livsmedelsverket, 2017) and toxicokinetics (sharing of iron-transporting mechanisms) (Bárány et al., 2005) may have contributed. Hexa-to hepta-chlorinated PCBs clustered separately from tetra-to penta-chlorinated PCBs as well as from the chlorinated pesticides (Fig. 1). The indoor environment may still be a significant exposure source of the latter PCBs (Johansson et al., 2003; Meyer et al., 2013; Wingfors et al., 2006). For HCB, hexachlorocyclohexane (HCH) and p,p'-DDE, fish consumption has been a less important dietary source than for PCBs in Sweden (Livsmedelsverket, 2017; Törnkvist et al., 2011). BDE-47 and -99 formed a cluster separate from BDE-153 (Fig. 1), whereas BDE-153 was moderately correlated with highly chlorinated PCBs (Fig. S3, Supplement), as also observed among nursing women from Sweden (Lignell et al., 2011). The indoor environment appears to be a more significant source of human BDE-47 and BDE-99 exposure than of BDE-153 exposure (Björklund et al., 2012; Fromme et al., 2016). Among young Swedish women, BDE-153 body burdens were significantly related to consumption of fish (as was the case for certain PCBs), whereas no such relationship was found for BDE-47 and -99 (Lignell et al., 2011). As in a previous RMA study using PCA clustering (Nyström et al., 2022), L-PFHxS, L-PFOS and br-PFOS clustered separately from L-PFOA, PFNA, and PFDA (Fig. 1), which was suggested to be due to exposure to the former PFASs from drinking water (Nyström et al., 2022).

Apart from phthalates/phthalate alternatives with common 'mother

Table 4
Estimated fraction (%) of exceedances of toxic substance concentrations among RMA participants (N = 1082) in relation to non-cancer HBM-GVs, gender and school grade differences in exceedances, and critical effects of the HBM-GVs.

Substance (metabolite)	Type of HBM-GV[a]	Matrix	HBM-GV	Participants exceeding HBM-GV (%)	Male/female participants exceeding HBM-GV (%)	Participants in grade 5/8/11 exceeding HBM-GV (%)	Critical effect	Reference for HBM-GV
Se	Biomonitoring equivalents (BE)	Whole blood	400 µg/L	0			Selenosis	Hays et al. (2014)
Cd	BE	Whole blood	1.4 µg/L	0.8			Kidney toxicity	Hays et al. (2008)
Hg	Human biomonitoring I (HBM I) children/adults	Whole Blood	5 µg/L	0.4			Neurotoxicity	Apel et al. (2017)
	HBM II children/adults	Whole blood	15 µg/L	0			Neurotoxicity	Apel et al. (2017)
Pb	BMDL1% developmental neurotoxicity children	Whole blood	12 µg/L	12	17/8.8*	12/13/11	Neurotoxicity	EFSA (2010)
	BMDL1% systolic blood pressure	Whole blood	15 µg/L	7.1	8.4/6.1	7.3/7.4/6.6	Hypertension	EFSA (2010)
	BMDL10% chronic kidney disease	Whole blood	36 µg/L	0.4			Kidney toxicity	EFSA (2010)
Al	Occupational threshold adverse effects	Serum	6.8 µg/L	26	27/25	27/26/25	Neurotoxicity	Riihimäki et al. (2000)
HCB[b]	BE	Serum	80 pg/mL	3.1	5.9/1.2*	2.7/4.4/2.3	Liver toxicity	Aylward et al. (2010)
p,p'-DDE[b]	Safe level developmental effects	Serum	12.8 ng/mL	0			Reproductive toxicity	WHO (2012)
NDL-PCB[c]	HBM I (138 + 153+180x2)	Serum	3.5 ng/mL	0			Neuro-/immunotoxicity	Apel et al. (2017)
	HBM II	Serum	7 ng/mL	0			Neuro-/immunotoxicity	Apel et al. (2017)
PBDE-99[b]	BE	Serum	1.7 ng/mL	0			Neurotoxicity	Krishnan et al. (2011)
PFAS[d]	PFOA + PFNA + PFHxS + PFOS at TWI young women	Serum	6.9 ng/mL	21		34/15/20*	Immunotoxicity	EFSA (2020)
PFOA[d]	HBM I general population	Serum	2 ng/mL	12	12/13	16/6.9/14*	Mixed toxicity	Hölzer et al. (2021)
	HBM II general population	Serum	10 ng/mL	0			Mixed toxicity	Schümann et al. (2021)
	HBM II females child-bearing age	Serum	5 ng/mL	0.3			Developmental toxicity	Schümann et al. (2021)
PFOS[d]	HBM I general population	Serum	5 ng/mL	19	27/14*	25/19/18*	Mixed toxicity	Hölzer et al. (2021)
	HBM II general population	Serum	20 ng/mL	1.7	1.7/1.8	5.5/0.25/0*	Mixed toxicity	Schümann et al. (2021)
	HBM II females child-bearing age	Serum	10 ng/mL	3.6		9.8/1.8/1.4*	Developmental toxicity	Schümann et al. (2021)
DEP	BE (MEP)	Urine	18000 µg/L	0			Body growth	Aylward et al. (2009)
DBP	HBM guidance value (HBM-GV), children (MBP)	Urine	120 µg/L	4.8	5.5/4.5	5.2/4.0/5.8	Reproductive/developmental toxicity	Lange et al. (2021)
	HBM-GV adults/adolescents	Urine	190 µg/L	1.5	1.7/1.3	1.2/1.7/1.4	Reproductive/developmental toxicity	Lange et al. (2021)
BBzP	HBM-GV children (MBzP)	Urine	2000 µg/L	0			Reproductive/developmental toxicity	Lange et al. (2021)
	HBM-GV adults/adolescents	Urine	3000 µg/L	0			Reproductive/developmental toxicity	Lange et al. (2021)
DEHP	HBM-GV children (5-oxo-MEHP+5-OH-MEHP)	Urine	340 µg/L	0.2			Reproductive/developmental toxicity	Lange et al. (2021)
	HBM-GV adults/adolescents	Urine	500 µg/L	0.09			Reproductive/developmental toxicity	Lange et al. (2021)
	HBM-GV children (5cx-MEPP+5-OH-MEHP)	Urine	380 µg/L	0.2			Reproductive/developmental toxicity	Lange et al. (2021)
	HBM GV adults/adolescents	Urine	570 µg/L	0.09			Reproductive/developmental toxicity	Lange et al. (2021)

(*continued on next page*)

Table 4 (*continued*)

Substance (metabolite)	Type of HBM-GV[a]	Matrix	HBM-GV	Participants exceeding HBM-GV (%)	Male/female participants exceeding HBM-GV (%)	Participants in grade 5/8/11 exceeding HBM-GV (%)	Critical effect	Reference for HBM-GV
DPHP	HBM GV adults (OH-MPHP)	Urine	220 μg/L	0			Thyroid toxicity	Lange et al. (2021)
	Children	Urine	140 μg/L	0			Thyroid toxicity	Lange et al. (2021)
DiNP	BE (OH-MiNP + oxo-MiNP + cx-MiNP)	Urine	14700 μg/L	0			Liver toxicity	Hays et al. (2011)
DiNCH	HBM GV children (OH-MINCH + cx-MINCH)	Urine	3000 μg/L	0			Kidney toxicity	Lange et al. (2021)
	Adults/adolescents	Urine	4500 μg/L	0			Kidney toxicity	Lange et al. (2021)
BPA	HMB GV children	Urine	135 μg/L	0			Kidney toxicity	Ougier et al. (2021)
	HMB GV adults	Urine	230 μg/L	0			Kidney toxicity	Ougier et al. (2021)
3-PBA	BE Tier 1	Urine	1.7 μg/L	2.2	2.3/2.5	3.3/1.2/2.9	Neurotoxicity	Aylward et al. (2018)
	BE Tier 2	Urine	87 μg/L	0			Neurotoxicity	Aylward et al. (2018)
TCS	HBM I children	Urine	2000 μg/L	0			Haemato-/spleen toxicity	Apel et al. (2017)
	HBM I adults	Urine	3000 μg/L	0			Haemato-/spleen toxicity	Apel et al. (2017)

[a] HBM I is the threshold for early warning and concern, whereas HBM II is the threshold for an increased risk for adverse health effects.
[b] Recalculated from concentration in blood lipids to concentration in serum using a mean lipid content in RMA serum of 0.33%. *p ≤ 0.05, Chi-square test.
[c] Non-dioxin like.
[d] PFAS ng/g converted to ng/mL using the specific gravity of serum (1.0275) (Sunderman and Boerner, 1949).

Table 5
Hazard index (HI) in RMA (N = 1082) based on the same target organ critical effect.

Target organ[a]	Composition	Total N with hazard index >1 (%)[b]	Median (range) of HI	Median (range)[c] of HI > 1
Liver	HCB, DiNP	35 (3.2)	0.53 (0.15–93)	1.1 (1.01–93)
Kidney	Cd, Pb, DiNCH, BPA	16 (1.5)	0.31 (0.01–4)	1.64 (1.08–4)
Central nervous system	Hg, Pb, Al, NDL[d]-PCB, PBDE-99, 3-PBA	1018 (94)	1.82 (0.58–15)	1.86 (1–15)

[a] Critical effect target for HBM-GVs as seen in Table 4.
[b] HI calculated per target organ critical effect by dividing participants blood/serum/urine concentrations with corresponding HBM-GV (hazard ratio, HR) from Table 4, then summing HR together. Total numbers of those with HI > 1 shown.
[c] Median and range calculated for participants with HI > 1.
[d] Non-dioxin-like.

substances', the dibutyl phthalate (DBP) metabolite monobutyl phthalate (MBP) and the butylbenzyl phthalate (BBzP) metabolite MBzP formed a cluster suggesting common exposure sources (Fig. 1). MBP and MBzP were also moderately correlated with the DEHP metabolites (Fig. S4, Supplement), as correspondingly observed for MBzP among Swedish and Danish children, and for MBP among Flemish adolescents (Bastiaensen et al., 2021; Langer et al., 2014; Larsson et al., 2014). Common exposure from building materials/consumer products may contribute to the observed relations (KEMI, 2015). As among Swedish and Danish children, and Flemish adolescents (Bastiaensen et al., 2021; Langer et al., 2014; Larsson et al., 2014), in RMA only negligible/weak correlations were observed between the metabolite of diethyl phthalate (DEP), monoethyl phthalate (MEP), and the other phthalate metabolites (Fig. S4, Supplement). DEP is currently the main phthalate used in cosmetic products in the EU (KEMI, 2015). DiNP, diiso-decyl phthlate (DiDP) and dipropylheptyl phthalate (DPHP) have been introduced on the European market more recently than the other phthalates (Fréry et al., 2020), and the metabolites of these three phthalates were moderately correlated with each other (Fig. S4, Supplement). DiNP and DPHP metabolites were also moderately correlated with the DEHP metabolites (Fig. S4, Supplement), also observed for DiNP and DEHP metabolites in Flemish adolescents (Bastiaensen et al., 2021). DiNP is a direct alternative to DEHP as a general plasticizer and are used together with both DiNP and DPHP in PVC production (KEMI, 2015). The two PAH metabolites 2-hydroxy-phenanthrene (2-OH-PH) and 1-HP formed a cluster and were moderately correlated (Fig. 1 & Fig. S4, Supplement), as expected due to the common exposure sources (Murawski et al., 2020)

3.2. Comparisons with other studies

Comparisons of RMA results with other studies of adolescents are somewhat uncertain due to differences in study design/populations and analytical methods. Nevertheless, mean concentrations of elements, POPs and urine substances with a few exceptions varied less than 3-fold between RMA against NHANES (USA), GerES (Germany) and FLEHS (Flanders, Belgium) (Tables 2 and 3). The relatively small differences in average exposure of adolescents between the four studies are most probably due to the similarly high standard of living in these populations, in combination with world-wide distribution of chemicals and products. NHANES showed large ethnic differences in mean serum concentrations of chlorinated and brominated POPs, in some cases up to several orders of magnitude (Table 2) (NHANES, 2022). In RMA, mean concentrations of chlorinated POPs were within these ranges or somewhat lower (Table 2). The concentrations of the three PBDEs were in most cases below the LOQ (<20 pg/ml serum) in RMA (Table S3, supplement). However, the determined concentrations below LOQ in RMA strongly suggested considerably lower mean serum PBDE concentrations than in NHANES, with a minimum of a 23-fold difference (BDE-153) (Table 2) (NHANES, 2022). The higher body burdens of PBDE in the US than in Sweden and other European countries is in line with studies of adults (Fromme et al., 2016). Differences in PBDE legislation between the US and Europe most likely contributes to the exposure differences (Horton et al., 2013). The mean concentration of

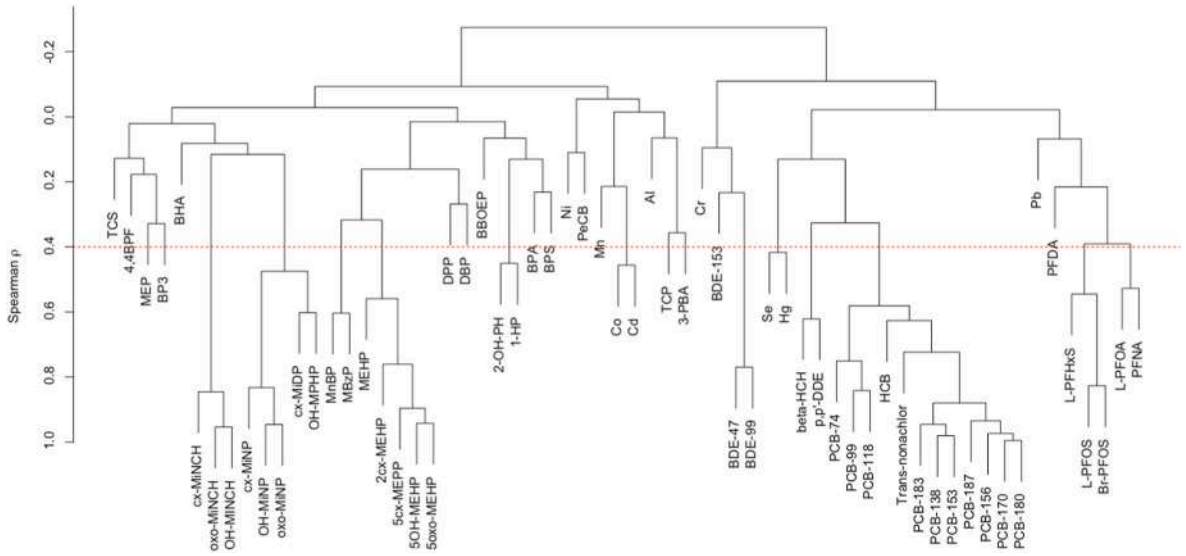

Fig. 1. Cluster analyses of correlations between toxic substance concentrations among adolescents in RMA (N = 1082). Correlation coefficients should be read at where branches separate and not on the names. Figure shows absolute spearman correlation coefficients with both negative and positive correlations. The dotted red line represents the set cut-off for moderate correlation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

dichlorodiphenyltrichloroethane (p,p'- DDT) was reported to be 37-fold higher in FLEHS compared to RMA, but the concentration of the DDT metabolite p,p-DDE did not differ markedly (Table 2). Studies of breast milk suggest similar average p,p'-DDT exposures among women in child-bearing age in Sweden and Belgium around the same time period as the RMA study (Aerts et al., 2019; Kallerman et al., 2021). Further studies are needed to determine the reason behind the large differences in p,p'-DDT concentrations in RMA and FLEHS.

The average concentrations of phthalate metabolites in urine did in many cases not differ more than 3-fold between RMA and the other studies, suggesting similar exposure levels among the adolescents (Table 3). Among the European adolescents this may be due to the common plastics regulation within the EU (CEP et al., 2022; Pereira et al., 2022). Nevertheless, the exceptions to the <3-fold difference were MBP, cx-MiNP, cx-MiDP and 6-hydroxy monopropylheptyl phthalate (OH-MPHP). The mean MBP concentrations in NHANES, GerES and FLEHS were lower than in RMA (Table 3), at most being 3.6-fold lower in NHANES compared with RMA. Similarly, the mean cx-MiNP was also lower in GerES and FLEHS (not reported in NHANES), occurring at a 4.2-fold lower mean concentration in FLEHS. However, the differences in mean concentrations of the other DiNP metabolites were less than 3-fold and RMA did not consistently show the highest concentrations (Table 3). cx-MiDP was lowest in RMA, at most 5.4-fold lower than in NHANES (Table 3), and OH-MPHP was 3.7-fold lower in GerES than in RMA. There was no consistent pattern in the observed differences in mean concentrations of the four phthalate metabolites between RMA and the other three studies, suggesting diverging substance-related exposure sources. The largest differences of concentrations of urine substances were observed for TCS and BP3, being on average about 15-fold higher in NHANES adolescents compared to RMA (Table 3). Although concentrations of these substances were below LOQ in GerES, they were also clearly lower than in NHANES. Differences in the levels of TCS and BP3 in consumer products and also in usage patterns, between Germany and the US were suggested as explanations of divergence in TCS exposure between GerES and NHANES (Tschersich et al., 2021), and is likely also the reason for the large difference between RMA and NHANES.

3.3. Comparisons with HBM-GVs and HI assessment

We surveyed the literature for HBM-GVs and found several from multiple sources (Table 4). There are uncertainties when comparing the different proposed HBM-GV, in part due to variation in the quality of the toxicological database, in procedures used in the development of the different values, safety margins used between point-of-departure of critical effect concentrations and HBM-GVs, and the fact that some of them have not been updated based to the most recent risk assessments.

Moreover, some HBM-GVs listed in Table 4 were specifically developed for distinct sub-populations like children, adolescents, adults or women in childbearing age. UBA, Germany (see Table 4 references) developed HBM I and HBM II values, and in the view of UBA there is no risk of adverse health effects if concentrations of substances are below HBM I. If the concentrations are above HBM I, but below HBM II, efforts should be made to search for potential sources of exposure and to mitigate identified exposure sources. An exceedance of HBM II is by UBA regarded as an increased health risk, and therefore there is a need for acute action to reduce exposure and biomedical advice should also be provided. Biomonitoring Equivalent (BE) values are based on existing health-based guidance values, such as tolerable intakes, and exceedances of BE may be regarded as a health concern (Hays et al., 2007). HBM4EU has developed HBM-GVs that in reality are equivalent to UBA's HBM I values (Apel et al., 2020). The values for Pb, Al, and $PFAS_4$ (Table 4) were based on benchmark concentrations/effect level concentrations in humans without any safety factors (EFSA, 2020; Riihimäki et al., 2000). Moreover, the Al value was based on relationships between occupational exposure and health among aluminium welders (Riihimäki et al., 2000). Despite all these uncertainties, as a conservative measure, exceedances of the HBM-GVs among the RMA participants were regarded as indications of a health concern.

The largest degree of exceedance of HBM I, HBM-GVs for children, and benchmark concentrations/effect levels were observed for Al, $PFAS_4$, PFOS, PFOA and Pb in declining order, all being ≥12% of the adolescent population when looking at the most conservative values (Table 4). Based on the size of the adolescent population in Sweden during 2016 for ages 12, 15 and 18 (N = 324776) (SCB, 2022), the number of adolescents exceeding the HBM-GVs can be estimated to range from 32700 ($PFAS_4$, females only) to 84400 (Al). For HCB, MBP and 3-PBA percent exceedances corresponded to 10000, 16300 and

7100 adolescents, respectively (Table 4). The percentages of exceedances of PFASs are likely overestimated since 5% of the participants were living in areas with a history of PFAS contamination of drinking water (Nyström et al., 2022), which is disproportionally high from a nation-wide population perspective. To the best of our knowledge, the PFAS contamination in some areas of Sweden is a special case not representative for the other substances included in our study. Nevertheless, contribution of unknown local hotspot contamination of other substances to the exceedances of HBM-GVs cannot be excluded. Exceedances were also observed for Cd, Hg, DEP, and DEHP, although to a lesser degree (Table 4). Overall, 54% of the participants exceeded at least one of the HBM-GV, 18% at least two, and 5% at least three. Taken together the results show that a significant fraction of the adolescent population had overall high exposures to several of the toxic substances in relation to the HBM-GVs. When using HBM-GVs for adults and/or adolescents (Pb, DBP, DEHP) or HBM II values (Hg, PFOS) the percentages exceeding the HBM-GVs were less frequent (Table 4). Considering that the concentrations of toxic substances in RMA, GerEs V and FLEHS were comparable (Tables 2 and 3), it is not surprising that GerES and FLEHS also reported exceedances of HBM-GVs of Cd, Pb (FLEHS), Hg (GerES), HCB (FLEHS), PFASs, DBP, DEHP (GerES) and 3-PBA (FLEHS) (Schwedler et al., 2020b; Vogel et al., 2021).

As suggested by the higher GM concentrations of Pb, HCB, and PFOS among males than females in RMA (Table 1), fewer females exceeded the benchmark dose of 1% extra risk (BMDL1%) for neurotoxicity of Pb in children, the BE for HCB, and the HBM I value for PFOS (Table 4). However, observed gender differences in concentrations of the toxic substances (Table 1) did not necessarily result in gender differences in exceedances of HBM-GVs, as observed for PFOA, the DBP metabolite MBP, and 3-PBA (Table 4). Moreover, no gender differences in exceedances of HBM-GVs were observed for Al, and for less conservative HBM-GVs of Pb, PFOS, and MBP. When looking at age-dependent differences in exceedances in relation to observed associations between age and concentrations there were no general trend, except in the case of PFASs that showed a higher degree of exceedances among 5th graders (Table 4), most probably due to a particular subset of the 5th-grade participants (N = 58) having a known high exposure of PFHxS and PFOS from drinking water in Uppsala and Ronneby (Nyström et al., 2022).

Efforts to eliminate environmental pollution of Pb, HCB, PFOA, PFNA, PFHxS and PFOS have resulted in slowly declining temporal trends in different Swedish populations (Gyllenhammar et al., 2021; Lundh et al., 2020; Miaz et al., 2020; Norén et al., 2021; Wennberg et al., 2017). Similar efforts to reduce Cd pollution have not resulted in declining Cd exposures during the last few decades, whilst mixed trends for Hg exposure (no decrease/slow decrease) have been reported (Kippler et al., 2021; Lundh et al., 2020; Wennberg et al., 2017). Taken together, further efforts to reduce exposures to Pb, Hg, Cd, Al, HCB and PFASs in Sweden are clearly needed. A study of young women in Sweden have reported decreasing exposures to DEP, DBP and DEHP over the last few decades, suggesting that the exposure situation also is slowly improving for these rapidly metabolized substances (Gyllenhammar et al., 2017).

In accordance with the RMA findings of exceedances of HBM-GVs of Pb, Hg (methyl-Hg), Al, and PFASs, The European Food Safety Authority (EFSA) concluded that the tolerable intakes of these substances from the diet were exceeded by parts of the EU population at the time of risk assessment (EFSA, 2012a, 2012b, 2010, 2008). Regarding phthalates, EFSA stated that the group tolerable intake of DBP, BBP, DEHP and DiNP (as DEHP equivalents) were not exceeded by EU populations even when considering the worst-case total dietary intake mainly originating from plastic food contact materials (CEP et al., 2019). However, diet is only one of several potential exposure sources of these phthalates, and the contribution of the additional sources most likely contributes to the high concentrations of metabolites observed in a small fraction of the RMA participants (Bastiaensen et al., 2021; Langer et al., 2014; Larsson et al., 2014; Schwedler et al., 2020b). However, considering that RMA only included single-spot urine sample there are still uncertainties about the risks of long-term individual exceedances of the HBM-GVs for these phthalates among adolescents in Sweden.

BPA concentrations in urine did not exceed the published HBM-GV, the GM concentration being 148-fold lower than the HBM-GV for children (Tables 1 and 4). However, in 2021 an EFSA draft opinion on health risks of BPA in foods proposed a considerably lower tolerable intake compared to the pervious assessment in 2015 (EFSA, 2021). It was proposed that populations with both average and high exposures to BPA in all age groups exceed the new tolerable intake. The publication of the final risk assessment will reveal if the exposure of the Swedish adolescent population will be regarded as too high or not from a health risk assessment perspective.

In the cumulative health risk assessment liver toxicity, including HCB and DiNP, showed a median HI (range) of 0.53 (0.15–93), with 3.2% of the participants showing a HI > 1 (Table 5). HCB was the driver of the health concern contributing with 99.9% (median, range: 98.8–100%) to the HI among participants with HI > 1. As with HCB alone, the highest proportion of participants with a health concern HCB was observed among males (5.9%), with a significantly lower proportion among females (1.2%, Chi-square test $p \leq 0.05$). In the case of kidney toxicity, the median HI (range) was estimated to 0.31 (0.006–4.0) and 1.5% of the participants had a HI > 1 (Table 5). Cd contributed 76.3% (3.1–91%) to the HI among participants with HI higher than 1, followed by Pb 23% (7.7–96%). The proportion of males and females with a HI higher than 1 was 1.6% and 1.3%, respectively, with gender difference not being statistically significant. Neurotoxic substances consisting of Hg, Pb, Al, NDL-PCB, PBDE-99 and 3-PBA showed a higher median HI of 1.8 (range: 0.58–15) compared to substances with liver and kidney toxicity, with 94% of the participants showing a health concern (HI > 1). Al contributed the most 39.5% (0–93%) to the HI among participants with HI higher than 1, followed by Pb 33.8% (0–96%). As with liver and kidney toxicity males showed a higher proportion of 96% exceeding HI = 1, whereas the proportion of females was 93% (Chi-square test $p \leq 0.05$). The HI analysis indicates that if the assumption of cumulative additive target organ toxicity effect holds true, significant portions of the adolescent population may be at risk of having too high cumulative exposures to neurotoxic substances. However, granted that the target organ of the central nervous system (CNS) is highly complex and the neurotoxicity of each individual contributing substance may affect different sections or processes of the CNS, the observation should be considered preliminary.

3.4. Strengths and limitations

As previously eluded to, a major strength of this nation-wide population-based study of adolescents is the inclusion of wider groups of toxic substances. There are some minor demographic/life-style differences between the RMA participants and the adolescent population in Sweden, yet participants are still considered as representative of the adolescent population in Sweden with regard to school type/size, and parental education and income (Moraeus et al., 2018). The urine substances have very short half-lives in comparison to the elements and POPs. As a consequence there is intra-individual variation of urine substance concentrations depending on timing of sampling. Other studies have shown that there may be seasonal differences in concentrations for some of the substances in urine (Bastiaensen et al., 2021). Although the possibility of seasonal differences in concentrations were not analysed statistically in the present study, the present results may be regarded as representative for the general adolescent population in Sweden during the sampling period. While aspects of the present work are comparable to prior studies (e.g. NHANES and GerES) some caution is warranted when comparing between studies due to potential differences in study design, analytical methods, and data processing/analyses. Human biomonitoring initiatives of toxic substances has been increasingly moving

towards combination and mixture effects. Yet, most studies still only focus on single substances/substance groups. Inclusion of a wider portion of exposome as in the present study, uncovered patterns of chemical body burdens that are valuable for interpretation of future RMA health studies. In the analyses of urine substances, the precision of cx-MiDP, BPS, DBP and BHA was >20%. This introduced an additional uncertainty in the statistical analyses, and in comparisons with other studies and HBM-GV.

The HBM4EU initiative, as well as UBA, has made steady progress to developing HBM-GVs for the general population (Apel et al., 2020), but nevertheless many toxic substances currently lack established HBM-GVs. We attempted to include the HBM-GVs appropriate for a general adolescent population, but if unavailable we instead used either those developed for children/adults or values proposed for occupational exposure (Al), making comparison of the results uncertain. Moreover, there are uncertainties connected to our limited HI approach of grouping of substances with the same target critical effect organs, and the HI results therefore should be considered as hypothesis generating for future studies of possible combination effects of the included chemicals.

4. Conclusion

Although many biomonitoring studies are available involving a range of individual chemical substances, our study is one of the few attempts at looking at the wider chemical exposome within a single study of adolescents, giving a more holistic and clearer picture of the current state of the chemical body burden in Swedish adolescents. With few exceptions, chemical body burdens in the present work were similar to those observed in adolescents from Germany and the US, showing a similar exposome for adolescents in these economically and industrially developed countries from two different continents. In RMA, the moderate to strong correlations between substances within the same chemical groupings were most likely due to common sources of exposure and/or similar toxicokinetics. However, while the chlorinated, brominated and fluorinated POPs included here share common properties of environmental persistence and bioaccumulation in humans, measured concentrations of the different contaminant classes were rarely correlated. Moreover, no correlations were generally observed between substances measured in different matrices. These observations are important for better interpretation of results in future evaluations of RMA data with regards to possible combination effects. The gender patterns observed, with generally higher average body burdens of neurotoxic Pb, Hg and some POPs in males compared to females, reflected in males having higher proportions of HI $>$ 1 for neurotoxicity also adds important knowledge for future combination effect studies. Our results give support to further efforts to reduce human exposures to toxic chemicals.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114196.

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Impact of diesel exhaust exposure on urinary 1-hydroxypyrene in underground salt and potash workers

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ABSTRACT

Background.

Diesel engine exhaust (DEE) and some of the polycyclic aromatic hydrocarbons (PAH) it contains are carcinogenic to humans (for example benzo(a)pyrene) and can cause lung cancer in workers. The objective of this study was to assess exposures to DEE and its component PAH and the potential associations between these two health hazards in a salt and potash mining population.

Methods.

Between 2017 and 2019, 1003 underground workers (mining n = 801, maintenance n = 202) and 243 aboveground facility workers from two German mines participated. Personal exposure to DEE was assessed in air as elemental carbon for diesel particulate matter (EC-DPM), whereas exposure to PAH was assessed in pre- and post-shift urine samples in terms of 1-hydroxypyrene (1-OHP). Associations between EC-DPM and 1-OHP were studied using linear regression models.

Results.

The highest EC-DPM exposures were measured in mining workers (median 0.06 mg/m^3) followed by workers in the maintenance (0.03 mg/m^3) and facility areas (<0.02 mg/m^3). Exposures above the current German occupational threshold level of 0.05 mg/m^3 were observed in 56%, 17%, and 5% of mining, maintenance and facility workers, respectively. 1-OHP increased statistically significantly across a work shift in underground workers but not in facility workers. Regression analyses revealed an increase of post-shift 1-OHP by almost 80% in mining and 40% in maintenance compared with facility workers. 1-OHP increased with increasing EC-DPM among underground workers. However, internal exposure of 1-OHP mainly remained at levels similar to those of the German general population in more than 90% of the urine samples.

Conclusions.

While exposures to DEE above the current German OEL for EC-DPM are quite common in the studied population of underground salt and potash miners (39.5% overall), urinary concentrations of 1-OHP did not reflect these findings.

1. Introduction

Emissions from diesel engines are complex mixtures of diesel particulate matter (DPM) containing elemental and organic carbon and gases such as carbon and nitrogen oxides, among others (IARC, 2014). Diesel engine exhaust (DEE) also contain polycyclic aromatic hydrocarbons (PAH) and several studies have specifically investigated the PAH content of DEE (Corrêa and Arbilla, 2006; Marr et al., 1999).

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Although PAH profiles in DEE can considerably vary, no significant differences for total PAH concentrations could be detected (Borrás et al., 2009).

DEE is a major contributor to air pollution and global warming (Campbell-Lendrum and Prüss-Ustün, 2019) and has been classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC, 2014). In recent decades, increasingly stringent emission standards have been introduced, especially for road vehicles. Exhaust gas treatment and fuel efficiency have been improved, resulting in lower emissions (Frey, 2018).

DEE poses also an occupational health risk. A review of occupational exposure to DEE found highest exposure concentrations for enclosed underground work sites where heavy equipment is used (e.g. mining and mine maintenance), and lowest when working outdoors or separated from the exposure source (Pronk et al., 2009). A suitable surrogate marker for DEE commonly used for occupational monitoring is elemental carbon in diesel particulate matter (EC-DPM) as it constitutes a large fraction of the particulate mass and can be quantified at low levels (Birch and Cary, 1996). However, external exposure measurements cannot estimate internal workers exposure.

To assess internal exposure to DEE, 1-hydroxypyrene (1-OHP), a metabolite of pyrene, has been commonly used as a surrogate biomarker (Ciarrocca et al., 2014; Hansen et al., 2008; Louro et al., 2022). Reasons include that pyrene has been found in DEE emission. For example, pyrene was the sixth most abundant of the 16 designated high priority PAHs by the US Environmental Protection Agency in a study exploring underground DEE in a Swedish iron ore mine (Gren et al., 2022). In addition, an increase in 1-OHP over the course of a shift was also seen in miners from an Australian goldmine (Du et al., 2019) suggesting that 1-OHP is an appropriate surrogate biomarker to study exposures to DEE and PAH in miners. 1-OHP has also been used in other studies for assessing exposure to PAH thus allowing cross-study evaluation of PAH exposures between different occupational settings rather than settings with DEE exposure alone. For example, the highest 1-OHP concentrations have been previously reported for workers in petrochemical industries (coke-oven workers) and metallurgy workers (Hansen et al., 2008). Less pronounced exposure have been found in firefighters due to their use of high-level personal protective equipment (Hoppe-Jones et al., 2021; Taeger et al., 2023).

Urine concentrations of 1-OHP are also elevated by non-occupational sources of PAH such as tobacco smoking, air pollution, dietary intake, dermal absorption of pharmaceuticals, or contact with contaminated soil (Jongeneelen, 2001). It has been shown that the strongest predictor of urinary 1-OHP excretion in the general population is tobacco smoking (Wilhelm et al., 2008).

To reduce health hazards from DEE in the workplace, an occupational exposure limit (OEL) of 0.05 mg/m^3 for EC-DPM was established in Germany in 2017; a transition period has been granted for underground mining (GESTIS Substance Database). As part of this 5-year transition plan to reduce exposure in salt and potash mining, an epidemiological study was conducted to investigate potential health effects within an 8-h shift. First results on selected cardiovascular, inflammatory and respiratory effects of this study have been published recently (Gamrad-Streubel et al., 2022). The aim of this additional analysis was to determine the exposure to PAH using urinary 1-OHP and to study its relationship with DEE in a salt- and potash-mining population commonly exposed to exhausts from large diesel-powered mining equipment and vehicles that are required for underground mining operations.

2. Material and methods

2.1. Study design and study population

The study population of this analysis comprised 1246 men employed at two salt and potash mining sites in Germany. The rationale, design, and conduct of the underlying cross-sectional study were previously described in detail (Gamrad-Streubel et al., 2022). In brief, all employees working for at least one year in the facility above ground or in the mine underground were eligible. Facility workers who had previously been employed underground or who were regularly occupationally exposed to higher diesel exhaust levels (e.g., by operating small diesel-powered machines) were excluded, as were underground workers who had previously been employed in other mines. According to the workplace risk assessment, neither underground nor surface workers wear respiratory protection in their daily work.

Data were collected on a single 8-h shift between August 2017 and January 2019. Each participant completed a pre-shift survey and was medically examined before and after the shift. Body height and weight were measured before the shift to assess the body mass index (BMI). Smoking status was categorized as current daily smoker, former smoker, and non-smoker (i.e., never and occasional smokers with less than five cigarettes per week).

The study was approved by the Ethics Committee of the Ruhr University Bochum, Germany (Reg. No. 176024). Written informed consent was obtained from all participants.

2.2. Urine sampling and analysis

Urine samples could be collected from 1229 participants before and for 1240 participants after the same shift on any day during the work week. These were stored at −20 °C until analysis. Urinary 1-OHP [µg/L] was evaluated and analysed by high-performance liquid chromatography (HPLC) with fluorescence detection. Urinary creatinine [g/L] was measured to standardize for diuresis. Creatinine-adjusted 1-OHP [µg/g creatinine] was calculated for creatinine levels between 0.3 g/L and 4.0 g/L although only values between 0.3 g/L and 3.0 g/L have been suggested to represent normally hydrated adults (Bader et al., 2020). However, we used an upper value of 4.0 g/L for creatinine adjustment because of the higher muscle mass of the studied miners compared to the general population. In 1224 urine samples before and in 1234 urine samples after the shift, 1-OHP could be determined. Of these, 559 (49%) pre-shift and 473 (38%) post-shift 1-OHP concentrations were below the limit of detection (LOD) of 0.1 µg/L. Due to missing creatinine values or values outside the range of 0.3 and 4.0 g/L for creatinine, 48 pre-shift and 56 post-shift values of creatinine-adjusted 1-OHP could not be determined. Therefore, a total of 1176 pre-shift and 1178 post-shift values of creatinine-related 1-OHP concentrations have been evaluated.

In Germany, the MAK Commission (Permanent Senate Commission of the Deutsche Forschungsgemeinschaft for the Investigation of Health Hazards of Chemical Compounds in the Work Area) established a biological reference value (BAR) of 0.53 µg/L and 0.3 µg 1-OHP/g creatinine (Deutsche Forschungsgemeinschaft, 2022; Klotz, 2021; Wilhelm et al., 2008). The BAR represents the background concentration (95th percentile) of 1-OHP of non-smoking adults in the general population of Germany who are not exposed to PAH at the workplace. Because no BAR exists for current smokers, the 95th percentile of 1-OHP of smoking individuals from the general population was based on the 1998 Environmental Survey in Germany (Becker et al., 2002) and set to 1.03 µg/L and 0.73 µg 1-OHP/g creatinine. We compared these evaluation standards of the non-occupationally exposed general population to the measured concentrations of 1-OHP in salt and potash miners to assess the extent of occupational exposure. Furthermore, Biological Exposure Index (BEI®) levels published by the American Committee of Governmental Industrial Hygienists (ACGIH) exist to evaluate occupational health hazards and risks. In the case of PAH exposures, the BEI® is based on the relationship between urinary 1-OHP and various genotoxic endpoints and should be adjusted to the specific PAH mixture of the workplace. As this has not been determined in salt and potash mining sites, the default value of 2.5 µg/L proposed by the ACGIH has been used for interpreting our results (ACGIH, 2017).

2.3. Ambient measurements

The exposure of DEE was assessed by measuring EC-DPM. Each participant was equipped with personal dust samplers during the entire shift. The respirable dust sampling system PGP FSP2 cyclone with a SG 5100ex battery-driven sampling pump (GSA Messgerätebau, Ratingen, Germany) was used (Gamrad-Streubel et al., 2022). The shift mean values of EC-DPM [mg/m^3] related to an 8-h shift were used as personal exposure index. Sampling rate and measurement duration have a decisive influence on the limit of quantification (LOQ) for EC-DPM, resulting in different values per measurement. At a sampling rate of 2 L/min and an 8-h shift, the LOQ for EC-DPM was 0.025 mg/m^3. EC-DPM could not be determined for 11 subjects and 33% of EC-DPM measurements were below LOQ (n = 406).

2.4. Statistical analysis

Continuous variables to characterize the study population were presented with the arithmetic mean and range. Exposure characteristics were described with median, range, and the number of values below LOD. Boxplots with median, interquartile range (IQR), and whiskers representing the minimum and maximum were used to show the distribution of creatinine-adjusted 1-OHP. Group comparisons were performed by Kruskal-Wallis tests (KWT) or Chi^2 tests, accordingly. Location differences between pre- and post-shift values were tested using signed rank tests (Wilcoxon tests).

We used a maximum likelihood estimation method for multiple imputations of values below LOD. Assuming an equal probability distribution below and above LOD, values below LOD were imputed 100 times at random from a log-normal distribution (Lotz et al., 2013). The results of the imputation analyses were combined using the SAS procedure PROC MIANALYZE.

The monotonic relationships between internal and external exposure were presented by Spearman rank correlation coefficients (r_S) with 95% confidence intervals (95% CI) and p-values. Following a study of miners from a Western Australian gold mine (Du et al., 2019), we captured the association between external exposure and post-shift creatinine-adjusted 1-OHP using linear regression models that included imputed values below LOD. As exposure variable, we included either exposure group (facility, maintenance, mining) or EC-DPM as a continuous variable. In all models, pre-shift creatinine-adjusted 1-OHP was included as an independent variable. In further models, a second adjustment set consisting of age [per 10 years], BMI [kg/m^2], and smoking status (current, non-current) was considered. We used the method of Harel to estimate the coefficient of determination R^2 as a measure of goodness of fit (Harel, 2009).

Statistical analyses were performed using SAS, version 9.4 (SAS Institute Inc., Cary, NC, USA). Figures were prepared using GraphPad Prism, version 9 (GraphPad Software, La Jolla, California, USA).

3. Results

The study population consisted of 243 above-ground facility workers and 1003 exposed underground workers, of whom 202 were in the maintenance group and 801 in the mining group and were stratified by exposure group (Table 1). Mining workers were slightly younger (p_{KWT}<0.001) and duration of employment was slightly shorter (p_{KWT} = 0.005) compared to the other study groups. In addition, current smoking was more prevalent in the mining group.

Facility workers served as reference group due to their low personal exposures to EC-DPM. Accordingly, 82% of the EC-DPM concentrations were below LOD in the facility workers (median < LOD, range < LOD-0.11 mg/m^3), followed by maintenance (49.5% <LOD, median 0.025 mg/m^3, range < LOD-0.17 mg/m^3) and mining (13.7% <LOD, median 0.06 mg/m^3, range < LOD-0.34 mg/m^3) (Table 2). Accordingly, the German OEL was exceeded least frequently in the facility (5%) and more frequently underground in maintenance (17%) and mining (56%) (p^2_{Chi}<0.001). Overall, 488 of the workers studied were above the OEL for EC-DPM (39.5%).

Internal exposure measured by 1-OHP also differed between groups. Volume-adjusted urinary 1-OHP [µg/L] was below LOD in more than 70% of facility workers (reference group), with little difference between pre- and post-shift concentrations. Among underground workers, 1-OHP concentrations below LOD were less frequent, especially after the shift, with median exposures increasing. In example, post-shift 1-OHP was below LOD in 208 miners (26%) only and their median level was 0.3 µg/L. Miners also had the highest creatinine-adjusted 1-OHP concentrations (pre-shift 0.10 µg/g creatinine, post-shift 0.16 µg/g creatinine).

Fig. 1 presents the creatinine-adjusted 1-OHP concentrations before and after the shift per exposure group and stratified by smoking status. Among underground workers, internal exposure increased statistically significantly during the shift. Among facility workers, an increase of creatinine-adjusted 1-OHP was observed only in current smokers who, unlike underground workers, were allowed to smoke during shift breaks. Evaluation standards (BAR levels) for 1-OHP were partially exceeded. For creatinine-adjusted values, exceedances were most common among workers in mining (current smokers, 5.2%; non-smokers, 9.4%), whereas the corresponding frequencies were lower among maintenance workers (3.8% and 1.5%, respectively) and facility workers (3.2% and 1.8%, respectively) (Table S1).

Table 1
Characteristics of the study population of 1246 men.

	Above-ground facility				Underground							
	Reference group (n = 243)				Maintenance (n = 202)				Mining (n = 801)			
	N	Mean	Min	Max	N	Mean	Min	Max	N	Mean	Min	Max
Age [years]	243	41.4	21	65	202	40.4	19	64	801	38.4	19	63
Body mass index [kg/m^2]	243	28.5	18.0	43.0	202	27.8	19.7	44.4	801	28.0	19.6	44.3
Smoking (N, %)												
Non[a]	110 (45.3)				101 (50.0)				342 (42.7)			
Former	64 (26.3)				45 (22.3)				177 (22.1)			
Current	69 (28.4)				55 (27.2)				277 (34.6)			
Missing	0				1 (0.5)				5 (0.6)			
Duration of employment [years]	243	18.0	1.1	46.8	202	16.5	1.6	42.3	801	14.9	0.5	41.6
Hiring date [MM/YYYY]	243	07/2000	03/1971	10/2017	202	11/2001	08/1976	08/2016	801	06/2003	09/1976	02/2018

[a] Non-smokers include never smokers and occasional smokers (<5 cigarettes per week).

Table 2
Exposure characteristics.

	Above-ground facility				Underground							
	Reference group (n = 243)				Maintenance (n = 202)				Mining (n = 801)			
	N	N $_{<LOD}$ (%)	Median	Min-Max	N	N $_{<LOD}$ (%)	Median	Min-Max	N	N $_{<LOD}$ (%)	Median	Min-Max
EC-DPM [mg/m^3]	241	198 (82.2)	<0.02	<0.010–0.11	200	99 (49.5)	0.03	<0.02–0.17	794	109 (13.7)	0.06	<0.02–0.34
Urinary 1-OHP [µg/L]												
Pre-shift	241	170 (70.5)	<0.1	<0.1–3.6	200	102 (51.0)	<0.1	<0.1–1.2	783	327 (41.8)	0.2	<0.1–2.5
Post-shift	242	177 (73.1)	<0.1	<0.1–3.9	201	88 (43.8)	0.1	<0.1–2.1	791	208 (26.3)	0.3	<0.1–3.8
Urinary creatinine [g/L]												
Pre-shift	241	0 (0)	1.59	0.12–4.44	199	0 (0)	1.64	0.19–4.18	786	0 (0)	1.61	0.98–6.74
Post-shift	243	0 (0)	1.35	0.15–3.87	202	0 (0)	1.54	0.14–4.86	794	0 (0)	1.68	0.61–5.54
Urinary 1-OHP [µg/g creatinine][a]												
Pre-shift	231	–	0.05	0.00–2.23	193	–	0.07	0.00–1.85	752	–	0.10	0.00–3.11
Post-shift	230	–	0.05	0.00–2.57	188	–	0.11	0.00–0.92	760	–	0.16	0.00–4.32

1-OHP 1-hydroxypyrene; *EC-DPM* Diesel particulate matter measured as elemental carbon; *LOD* Limit of detection.
[a] Multiple imputed 1-OHP [µg/L] if below LOD and calculation of 1-OHP [µg/g creatinine] only for creatinine concentrations in the range between 0.3 g/L and 4 g/L.

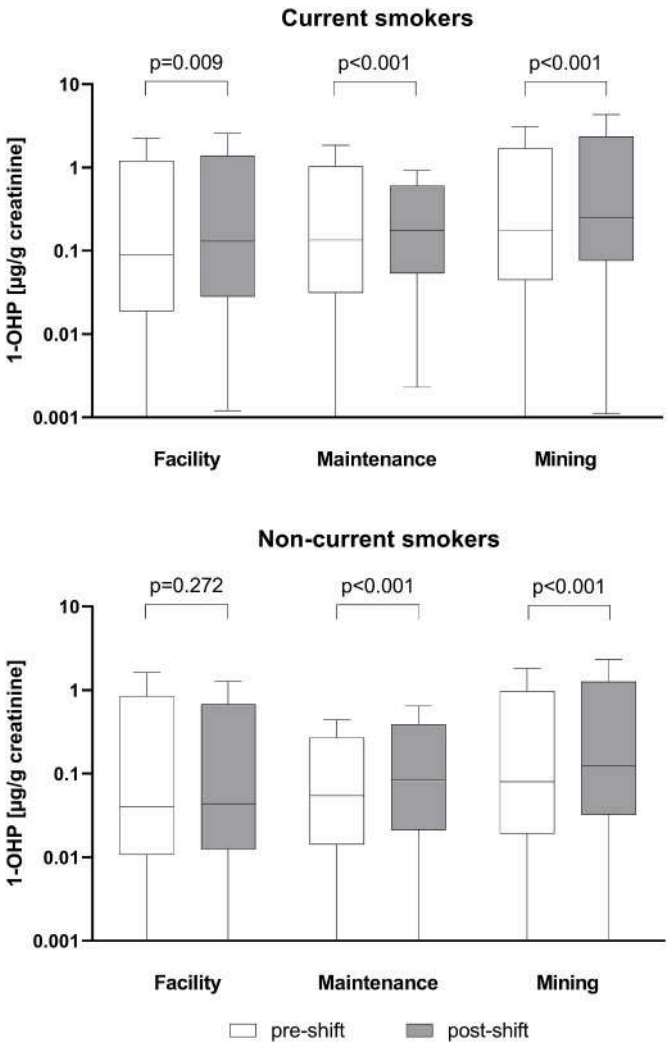

Fig. 1. Boxplots representing median, first and third quartiles, minimum and maximum of imputed creatinine-adjusted 1-hydroxypyrene (1-OHP) stratified by smoking status and exposure group. Location differences between pre-shift (white boxes) and post-shift (gray boxes) concentrations were determined with signed rank tests.

Occupational data for 674 out of the 683 participants of one study site were available and a total of 41 different occupations were reported by those workers. At the facility, one in three participants reported working as a plant operator. The most common occupations among underground workers were large equipment operators (34%), locksmiths (16%), electricians (8%), foremen (7%), and hewers (especially exploration (n = 29) and blasting hewers (n = 8), 7%). Hewers are miners operating cutting and drilling machines underground to loosen minerals (salt and potash) at the road front of the mine. Operators of large diesel engine powered wheel loaders transport the loosened raw material to the next tipping point, where the salt rocks are crushed and carried on conveyor belts to the shaft. Both also operate machines that drill blast holes in the rock, which are then filled with explosives by blasting hewers. Hewers and large equipment operators are higher exposed to DEE compared to other miners since it is difficult to provide a high ventilation rate at the underground road front of the mine (dead end). Large equipment operators and blasting hewers were most likely to be above the OEL (82% and 88%, respectively) as well as above the BAR level for 1-OHP exposure after shifts (16% and 25%, respectively). A total of five participants (all current smokers) were above the BEI® for 1-OHP after the shift. These were four miners (two large equipment operators, one exploration hewer, one miner with no occupational information) and one facility worker without further occupational information.

Spearman correlations of internal (1-OHP) and external (EC-DPM) exposure demonstrated lower correlations of EC-DPM with pre-shift 1-OHP concentrations (creatinine-adjusted 1-OHP: r_S 0.17, 95% CI 0.10–0.23) than with post-shift 1-OHP concentrations (r_S 0.30, 95% CI 0.24–0.36; Table S2). Among mining workers, correlations of EC-DPM and post-shift 1-OHP differed only slightly between current smokers and non-current smokers. However, among maintenance workers, current smokers had slightly higher correlations than their non-current smoking colleagues, indicating a possible effect of smoking in the less exposed underground group compared to mining. As about 80% of EC-DPM were below LOD in the facility, only data of mining and maintenance are presented (Table S2). Nonetheless, there was an association between exceedance of OEL and post-shift BAR in current smokers in the facility and non-current smokers in mining (Table S3).

Positive associations between post-shift creatinine-adjusted 1-OHP and external exposure assessed by either exposure group or EC-DPM were observed after adjusting for pre-shift creatinine-adjusted 1-OHP and persisted when additionally controlling for age, BMI, and smoking status in the statistical models. Compared with facility workers, post-shift creatinine-adjusted 1-OHP was increased by almost 80% in mining workers and 40% in maintenance workers (Table 3). Similarly, there was a statistically significant increase in post-shift creatinine-adjusted 1-OHP with increasing EC-DPM among underground workers (Table 4). Pre-shift creatinine-adjusted 1-OHP concentrations and smoking status had a similarly strong effect on post-shift concentrations. Current smokers had about 50% increased post-shift creatinine-adjusted 1-OHP

Table 3
Linear regression modelling of post-shift creatinine-adjusted 1-hydroxypyrene (1-OHP) concentrations as a function of exposure group.

	Adjustment set 1				Adjustment set 2			
	exp(est)	95%CI		p	exp(est)	95%CI		p
Intercept	0.27	0.22	0.34		0.23	0.14	0.36	
Pre-shift 1-OHP [ln µg/g creatinine]	1.68	1.57	1.80	<0.001	1.57	1.47	1.69	<0.001
Maintenance (ref: Facility)	1.37	1.15	1.65	0.001	1.38	1.16	1.65	<0.001
Mining (ref: Facility)	1.76	1.52	2.03	<0.001	1.76	1.52	2.03	<0.001
Age [per 10 years]					0.99	0.95	1.05	0.843
Body mass index [kg/m^2]					1.00	0.98	1.01	0.533
Current smokers (ref: non-current smokers)					1.58	1.41	1.78	<0.001
R^2	**0.38**	**0.32**	**0.43**		**0.41**	**0.36**	**0.46**	

CI confidence interval.

Table 4
Linear regression models of the association between post-shift creatinine-adjusted 1-hydroxypyrene (1-OHP) and elemental carbon in underground workers.

	Adjustment set 1				Adjustment set 2			
	exp(est)	95%CI		p	exp(est)	95%CI		p
Intercept	0.97	0.73	1.30		0.76	0.45	1.27	
Pre-shift 1-OHP [ln µg/g creatinine]	1.64	1.52	1.77	<0.001	1.54	1.42	1.67	<0.001
EC-DPM [ln mg/m^3 imputed]	1.31	1.20	1.42	<0.001	1.31	1.20	1.42	<0.001
Age [per 10 years]					1.02	0.96	1.07	0.565
Body mass index [kg/m^2]					1.00	0.98	1.01	0.568
Current smokers (ref: non-current smokers)					1.53	1.35	1.74	<0.001
R^2	**0.35**	**0.28**	**0.41**		**0.38**	**0.32**	**0.45**	

CI confidence interval; *EC-DPM* Diesel particulate matter measured as elemental carbon.

concentrations compared with non-current smokers. Linear mixed regression modelling of creatinine-adjusted 1-OHP concentrations at both time points, accounting for the dual measurement per subject, yielded similar risk estimates (maintenance exp(est) = 1.40, 95% CI 1.16–1.70; mining 2.10, 1.81–2.43; EC-DPM 1.37, 1.24–1.50). However, performance was slightly poorer when compared to the presented linear models in Table 3 (R^2 0.12 and 0.41, respectively) and Table 4 (R^2 0.15 and 0.38, respectively).

Due to different EC-DPM exposures at the two study sites (site A mining: median = 0.07 mg/m^3, range < LOD-0.32 mg/m^3; site B mining: 0.04 mg/m^3, <LOD-0.34), we also stratified our analyses. As expected, the linear regression models showed a stronger influence of current smoking on post-shift creatinine-adjusted 1-OHP at the site with lower EC-DPM exposure compared to the site with higher EC-DPM exposure (Table S4).

4. Discussion

The present study aimed to investigate exposure to PAH in terms of 1-OHP in urine and its associations with EC-DPM in workplace air among 801 miners, 202 underground maintenance workers, and 243 workers from above-ground facilities of a German salt and potash mining company. The concentration of 1-OHP increased statistically significantly across an 8-h shift in underground workers but not in surface workers. Although the current German OEL for EC-DPM of 0.05 mg/m^3 was exceeded by almost half of the underground workers, the 1-OHP concentrations were predominantly within the range of the general population that is not occupationally exposed to PAH. This is consistent with the general observation that the workers studied had no clinically relevant indicators of acute cardiovascular, inflammatory, immunologic, or respiratory effects assessed with biomarkers before and after the shift. Most biomarker measurements were within their respective reference ranges, and only a few (thrombocytes, neutrophils, myeloperoxidase, tumor necrosis factor α, immunoglobulin E, fractional exhaled nitric oxide) showed statistically significant post-versus pre-shift differences. However, these differences were independent of exposure group (Gamrad-Streubel et al., 2022).

Consistent with an earlier study (Pronk et al., 2009), miners were exposed to higher concentrations of EC-DPM than underground maintenance workers. The exposure was related to the different amounts of diesel exhaust and the different ventilation conditions in the different areas of the mines. The workplace and specific activity also had an impact on personal exposure. As previously shown in another study at a potash mine in the US (Stanevich et al., 1997), we also observed the most frequent OEL exceedances among large equipment operators with a median EC-DPM exposure of 0.087 mg/m^3. However, this exposure is far less than the average personal exposure to total EC of 0.453 mg/m^3 among ramcar operators that have been observed in a US potash mine and mill (Stanevich et al., 1997). It should be noted that, according to the authors, EC was generated almost exclusively by diesel fuel combustion.

In general, the median EC-DPM exposure of underground workers in our study was quite low (0.048 mg/m^3). A recent study of underground gold miners from Western Australia reported a similar median exposure of 0.056 mg/m^3 (Du et al., 2020). However, earlier studies reported mean exposures in underground mining up to ten times higher (Pronk et al., 2009). The current workplace exposure was up to 53% lower than the exposure observed in the 1990s and early 2000s at the same salt and potash mine. (Dahmann et al., 2007; Lotz et al., 2008). The decrease in exposure within the past 20–30 years could be attributed to increasing efforts to improve diesel engine technology and to switch from standard diesel fuel to renewable fuel in order to reduce DEE in the mining industry. Furthermore, good ventilation is important for reducing underground exposure. However, in the mines studied here, it has been shown that an increase in the ventilation rate is not feasible due to limited cross-sections and, correspondingly, low flow speeds in the shafts (Dahmann et al., 2007).

1-OHP is the most commonly used biomarker in Europe to assess occupational exposure to PAHs (Louro et al., 2022) and has also previously been used as a biomarker to determine exposure to DEE in miners (Du et al., 2019; Scheepers et al., 2002; Seidel et al., 2002). Median results for creatinine-adjusted 1-OHP of facility workers in the present study (pre and post-shift 0.05 µg/g creatinine) were even slightly lower than the median level (0.10 µg/g creatinine) observed in urine samples of the general German population (Becker et al., 2002). Furthermore, the examined non-current smokers from the reference group exceeded

the evaluation standards in less than 2% before and after the shift (Table S1). Similarly, less than 2% of maintenance workers exceeded the BAR levels and thus were also well within the range of the general population. In contrast, post-shift creatinine-adjusted 1-OHP was above the evaluation standard in 46 non-smokers from the mine (9.4%) thus indicating very low exposures to PAH in miners. The median value of all miners was 0.13 μg/g creatinine and therefore comparable to the results of a recent study among Australian gold miners (0.16 μg/g creatinine) (Du et al., 2019). In contrast, much higher concentrations of urinary 1-OHP were found among workers in other industries (Hansen et al., 2008; Louro et al., 2022). For example, studies conducted in Germany (Gündel et al., 2000; Marczynski et al., 2009; Pesch et al., 2011; Strunk et al., 2002) among mastic asphalt workers (0.44 μg/g creatinine), coke oven workers (3.6–19.7 μg/g creatinine depending on the specific workplace), refractory workers (8.4 μg/g creatinine), graphite electrode workers (9.7 μg/g creatinine), converter workers (13.5 μg/g creatinine), and workers producing refractory materials (11.1 μg/g creatinine) were substantially higher. Hence, the concentration of PAH in DEE cannot be considered high.

Urinary 1-OHP is not a specific indicator of internal dose of diesel exhaust as PAH are not only found in DEE but also in tobacco smoke or emissions from other incomplete combustion of organic sources. Thus, internal exposure to PAH measured by 1-OHP is influenced by individual behavioural patterns such as cooking, diet, or smoking in addition to occupational exposure (Hansen et al., 2008). This study also shows statistically significantly higher 1-OHP concentrations for smokers compared to non-smokers, analogous to a previous study (Du et al., 2019). In addition, exceedances of the evaluation standards for creatinine-adjusted 1-OHP were predominantly seen in mining. Not surprisingly, non-current smokers were most affected based on the fact that an important life-style and confounding factor (i.e., smoking) is actually missing in non-smoking workers thus work-related exposures are far easier to track on non-smoking rather than smoking workers.

Due to the presence of nitrogen oxides in diesel and their interaction with PAH, nitrated PAH compounds (nitro-PAH) are also formed during combustion in diesel engines (IARC, 2014). It has been suggested that metabolites of nitro-PAH are more suitable for exposure assessment of DEE, with 1-nitropyrene (1-NP) being a major nitro-PAH and a major constituent of DEE that is partially metabolized to 1-aminopyrene (1-AP) and excreted in urine (Bamford et al., 2003; Riley et al., 2018; Toriba et al., 2007). Some studies among workers exposed to DEE showed statistically significant correlations between 1-NP and urinary 1-AP (Ochirpurev et al., 2022; Wadikar et al., 2021) Furthermore, a study among Australian gold miners also concluded that 1-AP is a more robust and specific biomarker of DEE compared to 1-OHP (Du et al., 2019). However, although 1-AP seems promising for assessing exposures to DEE in future, 1-AP measurements in urine are much less established yet. Most importantly, compared to 1-OHP, simple and robust analytical methods including international round robin programs to guarantee quality-controlled results are missing for 1-AP.

The strengths of the present study are its sample size of more than 1000 underground miners and a high participation rate of 60% among underground workers. In addition, personal exposure to DEE was measured with personal monitors instead of stationary exposure measurements thus allowing a more reliable exposure assessment. However, there are also a few limitations of this study. First, the single-day cross-sectional study design may have caused exposure misclassification if the exposures on the study day were not representative of usual exposure concentrations. Second, due to the study design, the measured 1-OHP concentrations can also not be fully compared with those from other studies where exposure was assessed after the last shift of a working week, as recommended for the BEI®. Third, the interpretation of exposure by job title should take into account the low numbers in some cases, especially among blasting hewers (n = 8).

5. Conclusions

In the two investigated study sites of a salt and potash mining company, the current German OEL for EC-DPM was exceeded, particularly in mining. However, the urine concentrations of 1-OHP do not reflect the expected internal DEE exposure of workers based on the results of the EC-DPM air sampling. Although 1-OHP concentrations increased during a work shift among underground workers, suggesting that this biomarker reflects short-term DEE exposure, the concentrations still remained in the range of the general German population. If at all, individual exceedances occurred predominantly in mining, with non-current smokers being particularly affected. Except for blasting hewers and large equipment operators, workers in salt and potash mining in Germany are unlikely to experience elevated urinary 1-OHP concentrations at current workplace exposure levels of DEE compared to the general population according to our results.

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Declaration of competing interest

Swaantje Casjens, Savo Neumann, Dirk Pallapies, Jürgen Bünger, Heiko U. Käfferlein, Thomas Behrens, Thomas Brüning, and Dirk Taeger, as staff of the Institute for Prevention and Occupational Medicine (IPA), were employed until the end of 2021 at the German Social Accident Insurance Institution for the Raw Materials and Chemical Industry (BG RCI), a public body. IPA is an independent research institute of the Ruhr University Bochum. Jörg Giesen and Volker Neumann, as staff of the Institute for the Research on Hazardous Substances (IGF), Bochum, Germany, are also employed at BG RCI. IGF is an independent research institute. Katrin Rühle, Lisa Gamrad-Streubel, Lisa-Marie Haase, Katharina K. Rudolph, and Thomas Birk are staff of Ramboll, a consultancy commissioned by the BG RCI to conduct the study. All authors are independent of the sponsors in all aspects of this research including study design, collection, analysis and interpretation of data, and the right to publish. The views expressed in this paper are those of the authors and not necessarily those of the sponsors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ijheh.2023.114190.

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Investigation of polychlorinated biphenyls in breast milk from two regions in Bulgaria

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ABSTRACT

Human breast milk is an optimally balanced infant food and a suitable tool for assessing the burden of humans with lipophilic persistent organic pollutants. The aim of this study was to investigate the accumulation profile of polychlorinated biphenyls in breast milk of women living in Bulgaria and to assess the health risk to infants.

Breast milk samples were obtained from 72 healthy primiparae and multiparae mothers, living in two regions in northeastern Bulgaria – Varna region and Dobrich region, in the period October 2019–July 2021. Important information for the study, such as age, body mass, smoking and dietary habits, was collected through a questionnaire. Fifteen congeners of PCBs, including six indicator congeners, were determined by capillary gas chromatography system with mass spectrometry detection.

The lipid content of the tested samples was in the range from 0.5% to 6.7%, with average value 3.25%. The six indicator PCBs in human milk samples formed up to 89% of the total PCBs levels. The most abundant congener was PCB 153, followed by PCB 138 and PCB 180. Five of the 15 PCB congeners (77, 126, 128, 156, 169) were not detected in any of the milk samples. The arithmetic mean PCB levels in milk samples from Varna (32.7 ng/g lw) were found higher than PCB levels in breast milk of mothers from Dobrich (22.5 ng/g lw). The highest PCB levels were found in milk samples from primiparae mothers in 36–40 age group (for both regions). Infant exposure to PCBs present in human milk was estimated using toxic equivalents (TEQ). The health risk to infants was assessed and was compared to the tolerable daily intake (TDI).

Positive correlation was found between the arithmetic mean PCBs levels and two important factors – the age and body mass index of the primiparae group. The mean values of the analyzed PCB congeners in breast milk samples from multiparae were lower than in those from primiparae mothers. The regional differences in PCB concentrations were small, suggesting similar exposures in the studied regions. The levels of PCBs in breast milk were found lower than levels from studies in other European countries. Statistical data does not show any association between PCB levels in milk and dietary habits. The results showed that infants are not at risk of any adverse effects caused by PCBs through breast milk.

1. Introduction

Polychlorinated biphenyls (PCBs) are a class of industrial chemicals that were mass-produced globally and in several Europe countries (Germany, France, Italy, Czech Republic) from the late 1920s to 1985, even after their ban in the end of 1970s (Cerná et al., 2012; Grimm et al., 2015; Komprda et al., 2019).

PCBs are lipophilic, organic compounds from the persistent organic pollutants (POPs) group with the high potential for bioaccumulation and long-distance transfer (WHO Europe, 2003; Alharbi et al., 2018). Due to their environmental and biological persistence, low levels of PCBs are still found in wildlife and humans (Ashraf, M., 2017; Gyllenhammar et al., 2021). Pollutants tend to reach raised concentrations in organisms from higher trophic levels, including humans, due to biomagnification through aquatic and terrestrial food chains (Stancheva et al., 2017; Metcalfe et al., 2022). More than 90% of the total daily human exposure to PCBs is made up of intake from fat-rich food of animal origin (Massart et al., 2008; Sun et al., 2022).

PCBs have low acute toxicity but may pose a health risk in case of chronic human exposure (ATSDR, 2000; ATSDR, 2011). Experimental data indicate that exposure to low levels of PCBs may be associated with chronic non-lethal effects such as endocrine disruption, immune

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dysfunction, neurological disorders, liver injury, diabetes, cardiovascular problems and carcinogenicity (World Health Organization, 2009; Fernández-Cruz et al., 2017; Guo et al., 2019).

Human biomonitoring is a suitable tool for assessing human exposure to PCBs (Zietz et al., 2008). Biomonitoring data directly reflect the total body burden taking into account all routes of exposure, as well as the interindividual variability in exposure levels, metabolism and excretion rates (WHO, 2015). Biomonitoring involves measurements of biomarkers in biological fluids, such as blood, urine, saliva, breast milk and sweat. Human breast milk analysis is a non-invasive method for assessing the actual exposure of the mothers and has an advantage over any other type of human sample due to its high lipid content (Angerer et al., 2007). However, the determination of PCBs in breast milk has its limitations. Samples can be obtained only during lactation and only from lactating women, which excludes other groups of the population. (Brajenović et al., 2018)

The six indicator PCBs (IUPAC № 28, 52, 101, 138, 153 and 180) have been selected by the European Food Safety Authority (EFSA) as the major congeners which are present in various food matrices in high concentrations (EFSA, 2005). Low chlorinated congeners, such as PCB 28, 52 and 101 can metabolize by the human body faster than highly chlorinated PCBs due their half-life of almost 5 years (El-Shahawi et al., 2010; Helou et al., 2019). Highly chlorinated PCB congeners, containing more than five chlorine atoms (PCB 138, 153, 170, 180), tend to bioaccumulate in adipose tissue, human serum lipids and breast milk (Grimm et al., 2015) and can persist for 10–47 years (ATSDR, 2000).

Since 1987, the World Health organization (WHO) and the United Nations Environment Programme (UNEP) have conducted seven global surveys of POPs in human milk (van den Berg et al., 2017). Bulgaria was included in the 3rd Round of WHO coordinated Exposure Study on the POP levels in breast milk. In the third survey (2000–2003) pooled milk samples from three groups of Bulgarian mothers were analyzed for PCBs and organochlorine pesticides. The levels of indicator PCBs measured in milk samples from Bulgaria (2003), reported by van den Berg et al. (2017), were among the lowest in the European countries. There are limited data on PCB levels in breast milk of mothers from Bulgaria in the last twenty years.

The aim of the study was to examine the accumulation profile of polychlorinated biphenyls in breast milk of women, living in two regions in north-eastern Bulgaria, and to assess its relation to individual characteristics.

2. Material and methods

2.1. Study design

The study participants were selected from two regions: Varna (a port city with developed industry) and Dobrich (an agricultural region). Inclusion criteria for the participant mothers: 1) age 25–40 years, 2) breastfeeding period 30–40 days after childbirth; 3) resident in Varna or Dobrich for ≥10 years; 4) informed consent signed. The study protocol was based on the requirements of the WHO coordinated surveys on POPs in human milk (WHO, 2007). The recruitment of participants was done in four medical centers with pregnancy schools. Participants received information about the study and instructions for collecting and storing breast milk samples. Data on age, parity, pre-pregnancy weight, height, smoking and dietary habits were obtained from a validated questionnaire. The interview to fill in the questionnaire for collecting personal characteristics was conducted face-to-face by a qualified person.

The study protocol, the informed consent form and the questionnaire were approved by the Commission for Scientific Research Ethics at Medical University – Varna, Bulgaria (protocol № 85/26.07.2019).

2.2. Sampling and sample preparation

The present study was based on the voluntary participation of donors. Human milk samples were obtained from healthy mothers living in two regions in northeastern Bulgaria – Varna region and Dobrich region, in the period October 2019–July 2021. The recruitment period was extended due to Covid restrictions. In the Varna and Dobrich regions, 47 and 25 individual milk samples were collected, respectively. Participating mothers collected milk samples at home manually or using a manual breast milk pump. Milk samples (≥50 ml) were sampled in sterile containers after and/or during nursing. After sampling, the containers were stored in the refrigerator (+4 °C) for maximum 72 h and then in the laboratory at −20 °C until analysis.

2.3. Chemical analysis

Preparation of milk samples was performed by applying analytical protocol based on EN ISO 1528–1996 the European standardized methods with some modifications. The milk samples were defrosted, then slowly warmed up to 36–37 °C and carefully homogenized. Ten grams of each individual milk sample were weighted in a glass centrifuge tube (50 mL) and the sample was spiked with internal standards (PCB 30 and PCB 204, Dr. Ehrenstorfer Laboratory). Three-step extraction with organic solvents was applied to extract lipids and polychlorinated biphenyls from breast milk. The mix of solvents applied includes hexane/acetone in ratio 1:0 v/v (5 mL), 2:1 v/v (9 mL), 1:1 v/v (8 mL), respectively. After every extraction step, the sample was vortexed for 3 min, then centrifuged by 2500 rpm for 10 min. The hexane layers were collected, and evaporated to near dryness in a rotary vacuum evaporator. The lipid content of the milk samples was determined graphimetrically. The lipid extract was cleaned-up on a multilayer glass column filled with anhydrous sodium sulfate, 2 g of neutral silica (60–230 mesh), 2 g acid silica and 1 g neutral silica from bottom to top. The elution of PCB congeners were performed with 10 mL n-hexane and 20 mL mix n-hexane/dichloromethane in the ratio 9:1 (v/v). The collected eluates were concentrated by a rotary vacuum evaporator to near dryness and resolved in 0.5 mL hexane.

The instrumental determination of the individual PCB congeners was carried out by gas chromatograph GC FOCUS using POLARIS Q Ion Trap mass spectrometer (Thermo Electron Corporation, USA) and equipped with an autosampler AI 3000. The experimental parameters of mass spectrometer were the following: Ion source and Transfer line temperatures 220 °C and 250 °C, respectively; splitless Injector temperature 250 °C; experimental temperature program – 60 °C for 1 min, then 30 °C/min to 180 °C, 2 °C/min to 260 °C, 30 °C/min to 290 °C with a final hold for 2.0 min. Splitless injections of 1 μl were performed using a TR-5ms capillary column coated with cross-linked 5% phenyl methyl siloxane with a length of 30 m, 0.25 mm ID and a film thickness of 0.25 μm. The flow rate of helium as carrier gas was 1 ml/min.

All solvents (acetone, dichloromethane, hexane), reagents and chromatography silica gel (200–300 mesh) used for sample preparation and analysis were HPLC grade from Sigma-Aldrich (St. Louis, MO, USA), USA. Ultra-pure water came from a Milli-Q, IQ Water Purification System. Pure reference standard solution of 15 PCBs (PCB Mix 20 - Dr. Ehrenstorfer Laboratory, Augsburg, Germany) was used for instrument calibration, recovery and quantification of compounds.

Fifteen PCB congeners (IUPAC № 28 + 31, 52, 77, 101, 105, 118, 126, 128, 138, 153, 156, 169, 170 and 180) were measured in the purified extracts from milk samples.

The concentrations of the individual "dioxin-like" PCBs (dl-PCB, UPAC No 77, 105, 118, 156, 126, 169) in milk samples are multiplied by their respective toxic equivalency factors (TEF) according to WHO (2005) and subsequently summed to give total concentration of dioxin-like compounds expressed in TEQs (Van den Berg et al. 2006).

2.4. Quality control

The quality control was performed by analysis of certified reference material: BCR450 (PCBs in milk) – Institute for Reference Materials and

Measurements, European commission. The recovery of PCB congeners in the reference material was in the range of 82.6–98.6% (See Supplementary material). Each series of samples included a procedure blank (Milli-Q water). The limits of detection (LOD) varied for individual PCB congeners from 0.2 to 0.7 ng/g lipid weight (lw).

The limit of detection (LOD) were estimated based on the low concentration of the analytes in matrix samples as 3 times the standard deviation and LOQ is the analyte concentration corresponding to ten times standard deviation. LOD for individual PCBs: 0.2 (PCB 28, 77, 118, 153, 105, 138, 126, 128, 180, 169, 170), 0.4 (PCB 52, 101), 0.7 (PCB 156) ng/g lipid weight. Limit of quantification (LOQ) were calculated from 0.66 to 2.3 ng/g.

2.4.1. Statistical methods

Levene's homogeneity test and the Kolmogorov–Smirnov normality test of the data were applied for analysis of variance. The results showed a normal distribution of the data. The statistical differences between mean values of the data was evaluated by a Student t-test and a significance level of $p < 0.05$ was used. All statistical calculations were made by SPSS V19.0 software package for Windows (SPSS Inc., Chicago, IL, USA). The values under LOD were given as LOD/2 in the calculation of mean concentrations.

3. Results and discussion

3.1. Characteristics and dietary habits of the participants

A total of 72 individual human breast milk samples were collected from two regions in northeastern Bulgaria (47 from Varna region and 25 from Dobrich region). The personal characteristics, residence and dietary habits of women are presented in Table 1. The participants were between 25 and 40 years old, with an average age of 31.7 years (32.5 years for the women from Varna; 30.3 years for the women from Dobrich). These values are higher than those in the representative data for both regions in Bulgaria (Bulgarian National Statistical Institute database, 2021). Authors of similar studies in other European countries (Aerts et al., 2019; Polder et al., 2009; Zietz et al., 2008) reported lower mean mothers' age.

Table 1
Personal characteristics of the participants in the study.

Subject	Characteristics	% of all participants	Varna region	Dobrich region
			n (mean)	n (mean)
Age (years)	25–30	45.8	17 (28.2)	16 (27.1)
	31–35	30.6	18 (33.3)	4 (33.5)
	36–40	23.6	12 (37.4)	5 (38.2)
BMI, kg/m^2	<18.5 (underweight)	15.2	6 (17.3)	5 (18.0)
	18.5–24.9 (normal)	69.4	34 (21.1)	16 (20.8)
	25.0–30.0 (overweight)	9.8	4 (26.5)	3 (25.9)
	>30.0 (obese)	5.6	3 (33.2)	1 (31.1)
			n (%)	**n (%)**
Parity	Primiparae	59.7	25 (53.2)	18 (72.0)
	Multiparae	40.3	22 (46.8)	7 (28.0)
Diet: Fish	None	8.4	2 (4.3)	4 (16.0)
	1 x/month	18.0	8 (17.0)	5 (20.0)
	2 x/month	18.0	10 (21.3)	3 (12.0)
	1 x/week	48.6	24 (51.0)	11 (44.0)
	2 x/week	7.0	3 (6.4)	2 (8.0)
Meat	None	–	–	–
	1 x/week	7.0	1 (2.1)	4 (16.0)
	2 - 4 x/week	59.7	31 (66.0)	12 (48.0)
	>5 x/week	33.3	15 (31.9)	9 (36.0)
Smoking	Yes	29.2	11 (23.4)	10 (40.0)
	No	70.8	36 (76.6)	15 (60.0)

n (%) – number (and %) of participants in the category from the study region.

The results showed a mean pre-pregnancy body mass index (BMI) of the mothers 21.7 kg/m^2 with insignificant difference between the two study regions (21.9 kg/m^2 for Varna and 21.3 kg/m^2 for the women from Dobrich). The mean values of BMI of Bulgarian mothers were lower than the BMI of participants in other European studies on breast milk (Aerts et al., 2019; Polder et al., 2009; Zietz et al., 2008). Almost 70% from all donors were with normal BMI (ranging between 18.6 and 24.4). The underweight women were 15% (mean BMI 17.6 kg/m^2) and 15% were overweight or obese (mean BMI 28.6 kg/m^2).

In our study were included 43 first-time mothers (primiparae) - 60%, and 29 multiparae mothers (second or third child delivery). Mean age of primiparae mothers was 30 years (range 25–40 years) and their body mass index (BMI) was 21.2 kg/m^2. Multiparae mothers' age and BMI were 34 years and 22.4 kg/m^2 respectively. Non-smokers were 77% of all donors from Varna and 60% of all donors from Dobrich. All participants in the present study had a mixed type of diet including fish and meat. Only 8% stated that they do not eat fish and fish products. A small group of mothers (7%) consumed fish according to the recommendations (2 x/week), and 18% consumed fish once a month. Most of the women (59.7%) consumed meat 2 to 4 times a week.

The mean lipid content of the human milk samples was 3.2% (ranging from 0.5 to 6.7%) and no regional differences were observed.

3.2. PCBs levels in human milk samples

The concentrations of fifteen polychlorinated biphenyls (reported as Sum PCBs), including six indicator congeners (I-PCBs) were measured in 72 individual milk samples (Table 2).

The di-ortho congener PCB 153 showed the highest mean concentrations, followed by PCB 138, 180 and 118. A similar distribution pattern of PCB congeners in human milk was reported by several authors in Norway, Slovakia, the Netherlands, Sweden and Croatia (Polder et al., 2009; Čechová et al., 2017; Glynn et al., 2011; Gyllenhammar et al., 2021; Klinčić et al., 2016). The congener PCB 153 was present in all milk samples in both study regions. PCB 138 was found in 97% of the breast milk samples. The most predominant congeners (PCB 153, 138, 118, 180) formed 90% (Dobrich region) and 97% (Varna region) of the total PCB levels in the studied human milk samples.

The mean concentrations of PCB 153, 138, 180 and 118 in the mothers' milk from Varna were found higher than in the milk samples from Dobrich. Five of the analyzed PCB congeners (77, 126, 128, 156 and 169) were detected in concentrations below the limit of detection (LOD) in all breast milk samples from Varna region. In the milk samples from Dobrich region, PCB congeners 28, 52, 77, 105, 126, 128, 156 and 169 were detected below LOD.

The mean PCB levels in milk samples from Varna (32.7 ng/g lw) were found higher than PCB levels in breast milk of mothers from Dobrich (22.5 ng/g lw) and were formed mainly by the sum of the six indicator PCB congeners (28.6 ng/g lw for Varna and 19.7 ng/g lw for Dobrich, respectively) - Fig. 1. These results can be explained by greater potential exposure to PCBs of mothers who live in Varna (region with high industrial and port activities), compared to mothers from Dobrich (rural area). The percentage of detected dl-PCBs in milk samples from Varna and Dobrich was 11.3% (PCB 105, 118) and 8.4% (PCB 118), respectively.

Our results showed that the exposure of mothers is lower than the WHO survey data in the region of Sofia, 2003, demonstrated (sum of the six I-PCBs – 42 ng/g milk fat) (van den Berg et al., 2017).

The mean levels of PCBs found in breast milk samples in our study were in the same range or lower than those found in human milk in other European countries (Čechová et al., 2017; Glynn et al., 2011; Gyllenhammar et al., 2021; Klinčić et al., 2016; Polder et al., 2009; Zietz et al., 2008). Čechová et al., 2017 reported the highest levels of the six indicator PCBs in breast milk from Slovakia (144 ng/g milk fat), followed by Norway (62.02 ng/g milk fat) and the Netherlands (39.09 ng/g milk fat) – Table 3. The sum of the three predominant congeners (PCB 138, 153

Table 2
Polychlorinated biphenyls concentrations (ng/g lipid weight) in the human milk samples collected from Varna and Dobrich regions, Bulgaria.

Compound	Varna region (N = 47)					Dobrich region (N = 25)				
	% of positive samples		ng/g lipid weight			% of positive samples		ng/g lipid weight		
	>LOD	≥LOQ	Mean[a]	95th percentile	Range[b]	>LOD	≥LOQ	Mean[a]	95th percentile	Range[b]
PCB 28* + 31	19	17	0.43	2.16	0.62–4.93	0	0	< LOD	–	–
PCB 52*	4	2	0.22	0.20	0.49–1.00	0	0	< LOD	–	–
PCB 101*	12	10	0.34	1.51	0.66–2.00	40	32	1.87	5.39	2.33–12.79
PCB 77	0	0	< LOD	–	–	0	0	< LOD	–	–
PCB 118	89	89	3.56	7.62	0.69–12.60	64	60	1.87	6.79	1.20–7.13
PCB 153*	100	100	16.41	35.03	3.17–38.98	100	100	10.37	32.38	1.32–34.37
PCB 105	6	4	0.15	0.19	0.31–1.47	0	0	< LOD	–	–
PCB 138*	98	98	7.16	16.48	0.87–20.94	96	96	4.28	9.29	0.54–11.05
PCB 126	0	0	< LOD	–	–	0	0	< LOD	–	–
PCB 128	0	0	< LOD	–	–	0	0	< LOD	–	–
PCB 156	0	0	< LOD	–	–	0	0	< LOD	–	–
PCB 180*	90	90	4.07	8.46	0.77–16.39	80	80	3.17	10.77	1.39–12.36
PCB 169	0	0	< LOD	–	–	0	0	< LOD	–	–
PCB 170	14	10	0.37	2.19	0.21–3.73	20	16	0.53	2.82	1.08–3.91
Sum PCBs			32.74	66.22	4.07–72.99			22.49	59.71	2.76–64.42
WHO-TEQ pg/g lipid weight			1.47	3.10	0.22–5.96			1.02	2.92	0.15–3.34

*Indicator PCB.
[a] Arithmetic mean of values under LOD were given LOD/2 in the calculation of mean concentrations.
[b] Concentration ranges (Min – Max) in positive samples.

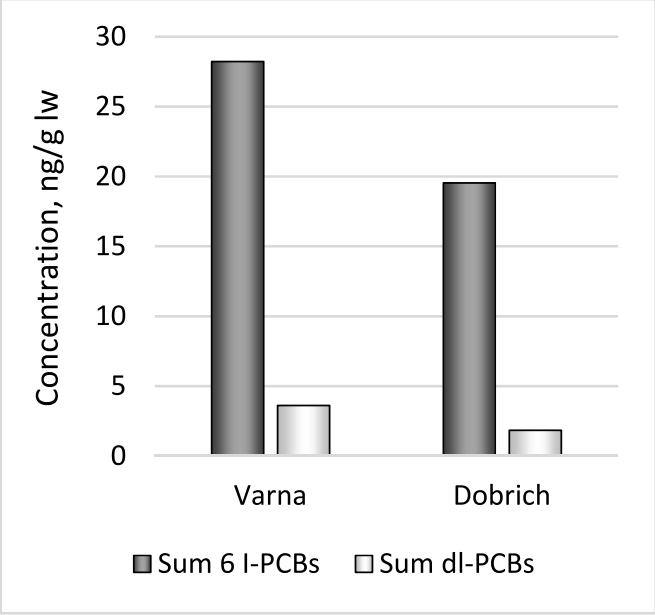

Fig. 1. Sum six Indicator PCBs (I-PCBs) and sum dl-PCBs (ng/g lipid weight) in breast milk from Varna and Dobrich regions.

and 180) multiplied with 1.64 (total PCB) showed a median of 19.2 ng/g lw, which is significantly lower than the data for breast milk samples from two German federal states show – 50.1 ng/g lw (Fromme et al., 2022).

3.3. Impact of maternal age, parity and BMI

There are several individual characteristics that affect PCB levels, such as maternal age, parity, dietary habits, etc. The relation between PCBs and the maternal age of both primiparae and multiparae mothers is given in Fig. 2. Our results showed a rise in PCB concentrations in breast milk with the increase of primiparae mothers' age in both regions studied.

The mothers' age as a determinant of body burden with PCBs were reported in several studies on PCB levels in milk samples from Belgium, Norway, Germany, Tunisia and Poland (Aerts et al., 2019; Polder et al., 2009; Zietz et al., 2008; Ennaceur et al., 2008; Grešner et al., 2021).

The positive correlation between PCB levels in breast milk and the maternal age is clearly expressed in the primiparae group. The mean sum of PCBs in breast milk from primiparae mothers (34.9 ng/g lw) was found higher than that in samples from multiparae mothers (29.1 ng/g lw) for Varna region. The total PCBs in Dobrich milk samples were 24.2 and 15.8 ng/g lw from primiparae and multiparae mothers, respectively. Glynn et al. (2011) showed a positive correlation between PCB levels and mothers age with both younger and older Swedish women and pointed age as the most important determinant of body burdens with PCBs.

Table 3
Comparison of median concentrations (ng/g lipid weight) of PCBs and TEQ (pg/g lipid weight) in human milk from European countries.

Country	Period	N	Sum I-PCBs	WHO TEQ_{2005} dl-PCB	Ref.
Bulgaria	2019–2021	72	18.82	1.09	Present study
Ireland	2016–2018	92[a]	19.25	1.4	Houlihan et al. (2021)
Spain	2016–2019	60	66[b]	0.2	Rovira et al., 2022
Czech	2019–2021	231	80.69	–	Parizek et al. (2023)
Germany	2016–2018	100	51.90	2.45	Fromme et al. (2022)
Slovakia	2010–2012	37	144.10	–	Čechová et al. (2017)
The Netherlands	2011–2014	120	39.08	–	Čechová et al. (2017)
France	2011–2014	96	85.19	4.3	Antignac et al., 2016
Belgium	2009–2010	84	39.7	1.71	Croes et al. (2012)
Norway	2001–2006	388	62.07	–	Čechová et al. (2017)

[a] - Analyzed as 16 pool samples.
[b] - Sum PCB138, 153 and 180.

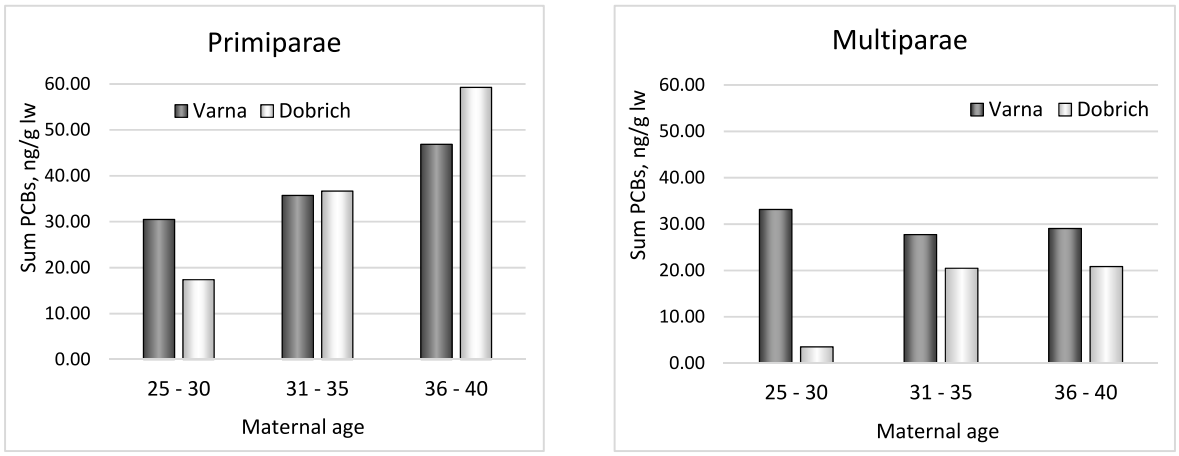

Fig. 2. Total PCB levels in breast milk by parity and age group of participants from Varna and Dobrich regions.

The highest PCB levels were found in milk samples from primiparae mothers in the 36–40 age group (for both regions). The data obtained may be due to the longer exposure to PCBs of the 36–40 year mothers through their diet (as the main route). Lower PCB concentrations (27 ng/g lw) in multiparae mothers' milk in the same age group might be due to previous breastfeeding. Fernández-Cruz et al. (2017) showed breastfeeding as the main route of excretion of lipophilic organic pollutants, so that maternal POPs body burden decreases from 20 to 70% in the course of six months of exclusive breastfeeding.

It is important to note that PCB body load was also associated with the participants' individual BMI. Most mothers (69.4%) had a healthy body mass index (in the range of 18.5–24.9 kg/m^2) before delivery. The results for sum PCBs in milk samples from primiparae mothers increased with the increase of BMI (Fig. 3). The trend was more pronounced in primiparae mothers, while there was no statistically significant correlation in multiparae mothers.

This can be explained by the transfer of human milk lipids, based on a study by Koletzko et al. (2001). The authors found that 70% of the milk lipids in breast milk come from the maternal depots and only 30% from the diet.

3.4. Impact of maternal diet and smoking

The mothers' diet and smoking are among the factors which may affect the residue levels of PCBs in breast milk over the lactation period. All donors in the present study consumed food of animal origin – an important dietary exposure route to persistent pollutants. Seafood consumption has been suggested as a major contributor to human exposure to POPs from a number of authors (Lee et al., 2013; Aerts et al., 2019). In our study group, 93% of the participants consumed fish and seafood much less than the recommendations of the World Health Organization (twice a week) – Table 1. Data from the questionnaires showed that meat was part of the daily food intake of 1/3 of the mothers. About 60% of the mothers consumed meat 2 to 4 times a week. Statistical analysis of the data showed no statistically significant associations between PCB levels in breast milk and the consumption of fish or meat. These results can be explained by the simultaneous influence of different factors. Grešner et al. (2021), found statistically significant relationships between the frequency of fish and dairy consumption and the concentrations of lipophilic pollutants in breast milk.

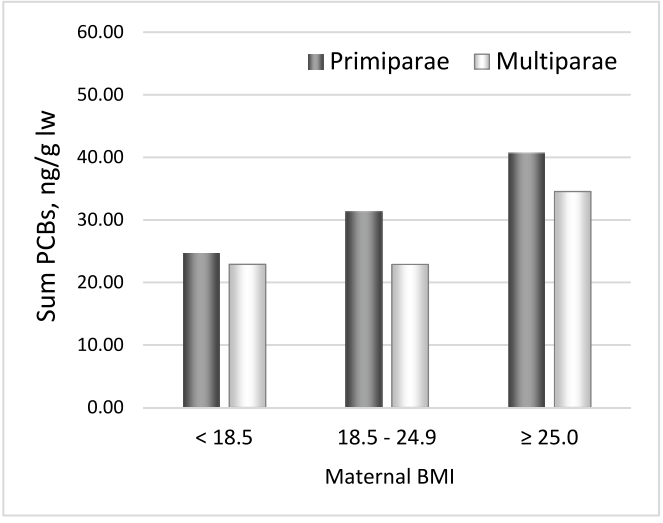

Fig. 3. Total PCB levels in breast milk by BMI group of participants from Varna and Dobrich regions.

The effect of active smoking on persistent pollutant levels in human milk and blood serum has been studied and discussed by different authors (Harris et al., 2001; Glynn et al., 2011; Cerná et al., 2012; Moon et al., 2017). Due to the simultaneous influence of many factors such as age, diet, order of the child, etc. it is difficult to establish a clear relationship between maternal smoking and the content of persistent organic compounds in breast milk. Less than 30% of the participants in the present study defined themselves as smokers before pregnancy – Table 1. Statistical analysis of the data from our study showed that no statistically significant difference was found in the mean PCB levels in the breast milk of smokers and nonsmokers (27.3 and 29.3 ng/g lw, respectively).

3.5. Estimated health risk

Infant exposure to dl-PCBs present in human milk was estimated using toxic equivalents (TEQ), expressed as pg TEQ/g ww (wet weight). TEQ values were calculated by multiplying concentrations of mono-ortho congeners in every sample and toxic equivalency factors (WHO_{2005}-TEF) of dl-PCBs according to Van den Berg et al. (2006). The range of dl-PCB content was in the range 0.0007–0.0179 pg TEQ/g ww (milk samples from Varna) and 0.0004–0.0100 pg TEQ/g ww (milk samples from Dobrich). Commission regulation (EU) No 1259/2011 sets the maximum levels at 0.2 pg TEQ/g ww (Sum of dioxins and dioxin-like PCBs (WHO-PCDD/F-PCB-TEQ)) in infant foods (EC, 2011).

EFSA's CONTAM Panel has set in 2018 a new tolerable weekly intake (TWI) for dioxins and dioxin-like PCBs in food of 2 pg TEQ/kg bw. The main arguments for the reduction of the TWI were the availability of new epidemiological and experimental data on the toxicity of these substances. According recommendation of EFSA the exposure of

breastfed infants should not be compared directly to the TWI (EFSA 2018). The reason is that the new TWI is based on the concentration level in breast milk that would leading to a child serum levels with potentially adverse effects in older children. Instead, it is more appropriate to compare dl-PCBs concentrations to level of 5.9 pg TEQ/g fat, the human milk level likely to result in the NOAEL serum concentration of 7.0 pg WHO_{2005}-TEQ/g fat at 9 years of age (EFSA, 2018; Houlihan et al., 2021).

In Table 2 is present the TEQ values of dl-PCB congeners in the human milk samples from Varna and Dobrich expressed as pg WHO_{2005}-TEQ/g lipid weight in aim to compare the results with European studies. Our results for dl-PCBs in breast milk showed mean levels 1.47 and 1.02 pg WHO_{2005}-TEQ/g lipid weight (for Varna and Dobrich, respectively) and are lower than level of 5.9 pg TEQ/g fat (EFSA 2018). The differences between regions studied are likely due to greater consumption of seafood by the mothers who live in Varna, on the Black Sea coast, compared to mothers from rural region (Dobrich). The percent of participants from Varna, which consume fish and fish products 2 x/month, was more that of mothers from Dobrich. It is well known that seafood is the main contributor to the input of dl-PCBs in humans (Klinčić et al. 2016).

TEQ values for dl-PCB congeners reported in literature are higher (Fromme et al., 2022; Antignac et al., 2016) or comparable (Houlihan et al., 2021; Croes et al., 2012) than those from our study (median values 1.09 pg-TEQ/g lw) – Table 3. The WHO/UNEP global surveys and other international studies of human milk showed a decreasing trend in PCB levels and WHO-TEQs in recent years (van den Berg et al., 2017; Zietz et al., 2008; Čechová et al., 2017; Gyllenhammar et al., 2021). However, even relatively low concentrations of PCBs in human milk, they can cause thyroid hormone disruptions to infants, especially in long-term exposure (Witczak et al., 2022).

Indicator PCBs (PCB 28, 52, 101, 138, 153, 180) are the most frequently analyzed and this group represents about 50% of total non-dioxin like PCB group (ndl-PCBs) measured in food (EFSA, 2018). Commission regulation (EU) No 1259/2011 sets the maximum levels at 1 ng/g ww for sum of I-PCBs in foods for infants. In the current study, the mean values of six I-PCBs in breast milk from Varna and Dobrich were found 0.82 and 0.46 ng/g ww, respectively and did not exceed the maximum levels permitted.

An estimated daily intake (EDI) was calculated to understand the extend of exposure of infants to total PCBs. The calculation was based on assumption that a 5 kg infant ingests 700 g milk per day (Van Oostdam et al., 2005). The EDI (μg/kg body wt./day) was calculated as follows:

$$EDI = \left(c_{milk} \times c_{lipid} \times 700\right) / 5$$

c_{milk} is the mean concentration of total PCBs in the milk samples (μg/kg lw) and c_{lipid} is the lipid content in milk (%).

The guideline standards by Health Canada and WHO proposed tolerable daily intake (TDI) of 1 μg/kg body wt./day (Van Oostdam et al., 2005; Klinčić et al. 2016). Calculated EDI values (0.12 and 0.072 μg/kg body wt./day for Varna and Dobrich, respectively) are one order of magnitude lower than TDI. The results showed that infants are not at risk of any adverse effects caused by PCBs through breast milk. We can conclude that the levels of PCBs in mother's milk are low, and the benefits of breastfeeding far outweigh the possible adverse effects.

4. Conclusions

Positive correlations were found between the mean of the total PCBs levels and two important factors – the age and body mass index of the participants in the primiparae group. The mean values of the analyzed congeners in samples from multiparae were lower than in those from primiparae mothers. The regional differences in PCB concentrations were small, suggesting similar exposures in the studied regions. Women from the more rural area in the northeastern part of Bulgaria (Dobrich) had lower mean levels of indicator PCBs than mothers from Varna. The levels of PCBs in breast milk from the sampled Bulgarian women were found lower than the levels from similar studies in other European countries. The PCB levels were found highest in the breast milk from primiparae mothers in the age group 36–40 years, suggesting bioaccumulation of these pollutants. In conclusion, the presented results show low PCB levels, but it is important to continue the monitoring of organochlorine contamination of both people and other living organisms.

CRediT authorship contribution statement

Stanislava Katelieva Georgieva: Conceptualization, Methodology, Validation, Data curation, Supervision, Project administration, Writing - review & editing. **Temenuga Trifonova**: Methodology, Investigation, Formal analysis, Data curation, Writing - original draft. **Zlatina Peteva**: Methodology, Validation, Investigation, Formal analysis, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114184.

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Modification of low temperature-related hospital admissions for cardiovascular diseases by multiple green space indicators at multiple spatial scales: Evidence from Guangzhou, China

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ABSTRACT

Background: Extreme temperatures have an adverse effect on the occurrence of cardiovascular diseases (CVDs). Previous literatures tend to discuss the modification of CVDs occurrence by green space under high temperature. Relatively less attention is paid to the modification under low temperature. The variation of different attributes and spatial scales of green space in affecting CVDs occurrence are also overlooked.

Methods: This study collected a total of 4364 first-time admission cases due to CVDs in a tertiary hospital in Guangzhou from 2012 to 2018, measured the scale of green space by greening rate (GR) and percentage of landscape (PLAND), the distribution of green space by patch density (PD), mean nearest neighbor distance (ENN_MN) and largest patch index (LPI), and the accessibility of green space by green patch accessibility index (GPAI). Using the time stratified case crossover design method, the modification of low temperature-related CVDs occurrence by the above green space indicators is evaluated in an area with a radius of 100–1000 m which is further divided at an interval of 100 m.

Results: We found high GR, high PLAND, high PD, low ENN_MN, high LPI, and low GPAI corresponds to low risk of CVDs occurrence, the optimal modification scale of each green space indicator, which is radius corresponding to the maximum risk difference between high and low indicator subgroups, is around 800 m (GR), 600 m (PLAND and PD), 500 m (GPAI), and 300 m (LPI and ENN_MN), respectively. As the temperature decreases further, the health benefit from low GPAI at the optimal scale is weakened, whereas the benefits from the others are strengthened.

Conclusions: Low temperature related CVDs occurrence risk can be modified by multiple green space indicators, and these modifications have spatial scale effect. Our findings have important theoretical and practical significance for the formulation and implementation of local green space policies.

1. Introduction

Cardiovascular diseases (CVDs) are a serious threat to global public health (Jagannathan et al., 2019). In China, CVDs are the leading cause of death and premature death (Ma et al., 2020). According to statistics, over 40% of China's population deaths are attributable to CVDs. Existing studies have indicated that abnormal temperature is a key factor in inducing CVDs. Various abnormal temperature factors, such as extreme temperature, cold spells, and heat waves, impact the occurrence of CVDs, among which low temperature has a significant adverse effect on the occurrence of various CVDs subtypes, whereas the adverse effect of high temperature may insignificant in some CVDs subtypes and regions (Phung et al., 2016). Low temperature can induce CVDs through a variety of pathophysiological mechanisms (Freund and Sawka, 1996; Keatinge et al., 1984). Continued attention to the relationship between low temperature and CVDs is crucial (Chen et al., 2019).

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The urban built environment modifies the relationship between air temperature and health, the reason is that various built environment factors affect temperature distribution within the city, this disparity results in different health risks to the public in different parts of the city (Schinasi et al., 2018). Urban "green space" is any vegetation in the urban landscape—a place that provides leisure and recreation for residents and a habitat for nature (Bolund and Hunhammar, 1999; Kabisch and Haase, 2013). Modifying temperature is an important way of green space influencing the human health. It is well known that under high temperature weather, green space can produce cooling effects through shading and evapotranspiration (Davtalab et al., 2020; Yu et al., 2018). Several studies have focused on the modification of high temperature related disease by green space, especially in the context of global warming, for example, Burkart et al. (2016) selected Lisbon, Portugal as the study area to explore the impact of green spaces on heat-related mortality; Bao et al. (2021) studied modification of heat-related stroke by green space in Shenzhen, China. The results of the above studies all show that the high temperature related disease risk decreases with the increase of green space rate. However, as an important climate modification factor, the impact of green space on air temperature is not limited to high temperatures. In low temperatures, green space can protect against cold wind and release sensible heat (Hong et al., 2012; Song and Wang, 2015; Vogel, 1989), this may have a protective effect on human health. To date, little is known about the modification of low temperature-related diseases, including CVDs, by green space. A study conducted in Wuhan, China (a subtropical city) has found that the low temperature related hospital admission risk of ischemic stroke decrease with the increase of green space rate (Li et al., 2021). The modification of low temperature related CVDs by green space is a question worthy of further consideration (Meili et al., 2021; Zhang et al., 2018).

Most of previous studies involving modification of temperature-related CVDs by green space only construct the vegetation scale indicator (Bao et al., 2021; Li et al., 2021), and rarely discuss the spatial distribution pattern of green space. The vegetation density and its arrangement can affect urban microclimate (Hami et al., 2019; Pérez and Perini, 2018), and may affect the relationship between temperature and the occurrence of CVDs. Shen and Lung (2016) selected the Taipei metropolitan area as their study area, and chose the annual mean temperature as the mediating variable. The researchers found that the temperature is positively related to the green patch distance and the green patch fragmentation, and the increase in temperature would increase the mortality of CVDs. However, in this study, the abnormal temperature interval is not set, so the modification by the green space distribution pattern on the low temperature is not analyzed. In addition, the accessibility of green space has been used to study CVDs (Coutts et al., 2010; Ngom et al., 2016). However, there is still a lack of attention to accessibility when discussing the modification of temperature-related CVDs by green space. In terms of spatial scale, previous studies have involved large scales to explore the modification by green space, for example, Shen and Lung (2016) et al. used blocks as research units, and the unit area exceeded 4.3 km^2; Li et al. (2021) took the block divided by the main road network as the research unit, and the unit area could reach 3 km^2. For the elderly, who are most prone to CVDs, the scope may exceed the daily activity space of this group (World Health Organization, 2016). In addition, Previous studies focused on a single spatial scale of green space (Bao et al., 2021; Burkart et al., 2016; Li et al., 2021), the modification by green space may change if the scale is altered. In conclusion, exploring the modification of temperature-related CVDs by green space with different spatial attributes and scales is necessary.

Based on the previous literature (Coutts et al., 2010; Lee et al., 2021; Liu et al., 2022; Ngom et al., 2016; Rahnama and Shaddel, 2019), this study constructed six green space indicators from three aspects: green space scale, distribution pattern, and accessibility. The time-stratified case-crossover design (TSCC) is used to explore the modification of the occurrence of low temperature-related CVDs by each indicator in circular buffers with radii ranging between 100 and 1000 m with the patient's home address as the center, at intervals of 100 m (Liu et al., 2022; World Health Organization, 2016; Yeager et al., 2020). We chose Guangzhou, China as the study area. Guangzhou is a subtropical city with a developed economy in China. Previous studies have shown that low temperature has adverse effects on CVDs in Guangzhou (Yang et al., 2015). The purpose of this study is to solve three important questions: 1) Whether green space attributes affect the occurrence of CVDs under low temperature conditions? 2) What are the characteristics of this effect with the change of spatial scales, and which scale has the strongest modification effect? and, 3) How does the modification change as the temperature further decreases? In the context of rapid urbanization, the answers to the above questions are significant to urban planning and related policy formulation.

2. Data and methods

2.1. The study area and population

Guangzhou is located in southern China and is the capital of Guangdong Province. Guangzhou is a first-tier city in China with a Gross Domestic Product (GDP) of 2.5 trillion yuan in 2020, ranking fourth in China. Guangzhou located at longitude 112°57′ to 114°3′ east and latitude 22°26′ to 23°56′ north, covering an area of 7434.4 square kilometers (2870.4 square miles). Guangzhou belongs to the subtropical monsoon climate, with high temperatures and, rainy summer, mild and dry winter, it has an annual mean temperature of 21.5–22.2 °C (70.7–72.0 °F). According to the results of the seventh census, the population of permanent residents is 18.68 million (National Bureau of Statistics of China, 2020). The study area selected for this study is the central urban area of Guangzhou (Fig. 1), including Liwan District, Haizhu District, Yuexiu District, and Tianhe District. The total area of these districts is 279.63 square kilometers, accounting for 3.8% of the city's total area, with a permanent population of 6.239 million, accounting for 33.2% of the city's population, with a GDP of 1.2 trillion-yuan, accounting for 47.4% of city's GDP.

2.2. Cases and meteorological data

We collected the raw data from a well-known third-class hospital in Guangzhou, located in Yuexiu District. The data comprised information on patients admitted to the hospital for the first time due to CVDs; the disease type is I00–I99 under the ICD-10 coding standard. Only including cases from the central urban area of Guangzhou greatly ensures that the patient's residential address is consistent with the registered address for a period of time before admission. The registration information includes personal information such as age, sex, and place of residence, as well as treatment information such as admission time, admission cost, and number of operations. Baidu map and AMAP are two well-known mapping software in China, providing rich application program interfaces services (APIs), among which geocoding service can convert physical addresses into geographic coordinates, which has been applied in multiple previous studies (Hua, 2018; Li et al., 2019). To ensure the accuracy of the patients' address coordinates, we used both Baidu map and AMAP geocoding services to convert the patients' address into coordinates points and excluded the points in the two sets of results where the projected coordinates representing the same address differ by more than 100 m. The coordinate points with ambiguous addresses were also manually excluded. Finally, we conducted follow-up study based on the remaining Baidu coordinate points.

China Meteorological Data Network (http://data.cma.cn/) provides daily meteorological data for this study. We use data from Guangzhou Station (No. 59287), including information on mean temperatures (unit: 0.1 °C), mean wind speed (unit: 0.1 m/s), atmospheric pressure (unit: 0.1 hPa), and relative humidity (unit: 1%). The temperature indicator, converted to the unit of °C, is taken as the explanatory variable, whereas

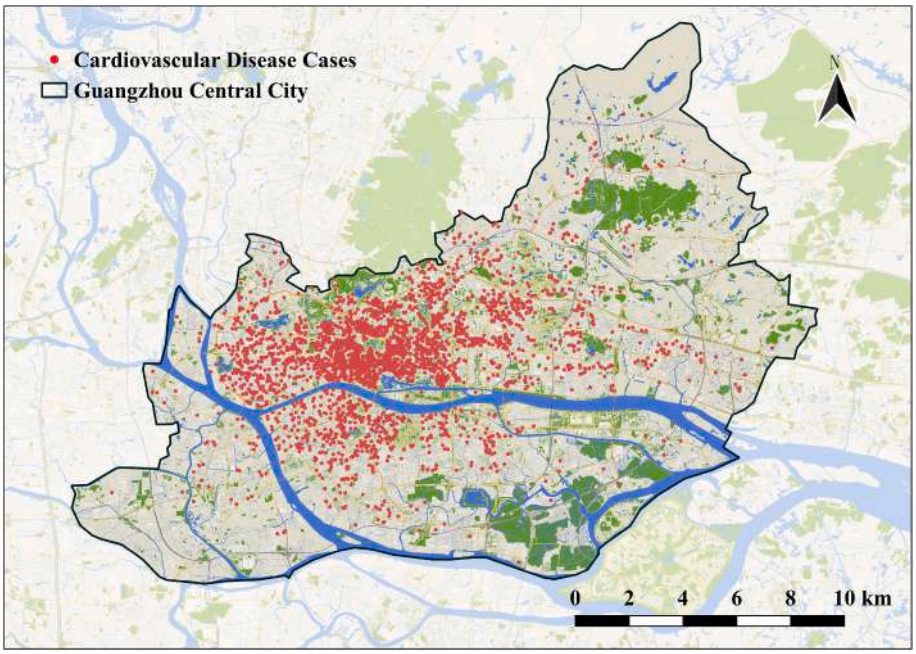

Fig. 1. Overview of the study area and the distribution of cases.

the remaining meteorological indicators are covariates.

2.3. Construction methods of green space indicators

This study reflects the scale, distribution pattern, and accessibility of green space by constructing six different indicators. Indicators reflecting the scale of green space include greening rate (GR) and percentage of landscape (PLAND). Specifically, this study uses the normalized vegetation index (NDVI) to stand for the greening rate. NDVI is widely used to explore the relationship between green space and health due to its simple calculation and good indication. The NDVI data comes from the Landsat spectral indices products over China (Peng et al., 2020) (http://databank.casearth.cn), with a spatial resolution of 30 m and a temporal resolution of 16 days. This study collected all NDVI data from 2012 to 2018. A total of 11 clear sky image products were selected, covering all research years. The pixel values of all images are averaged, and the average image is used as the basis for calculating the GR (Burkart et al., 2016). This study uses the 10-m resolution global land cover in 2017 to calculate the PLAND (Gong et al., 2019; Xu et al., 2017). Referring to previous studies, we select forest, grassland, shrub, wetland (Code: 20, 30, 40, 50) as green space (Bauwelinck et al., 2021), and then combine the four types of green space to calculate the ratio of total green space to buffer area. When constructing the green space distribution pattern and accessibility indicators, the same data as the PLAND is used. The indicators reflecting the layout of green space include: 1) Patch density (PD), the calculation formula is: N/A, where N is the total number of green patches in the buffer, and A is the buffer area. Since we calculate the risk of admission in a specific buffer area, the number of green spaces N is used instead of the density of green spaces. 2) The mean nearest neighbor distance (ENN_MN), the calculation formula is $\sum_{i=1}^{N} D_i/N$, where i represents the green patches in the buffer, D_i is the distance between a green patch and the nearest neighbor green patch, and N is the total number of green patches in the buffer. This indicator expresses the concentration of green space distribution. The larger the value, the more discrete the green space distribution and the lower the adjacency. 3) Largest patch index (LPI) is the ratio of the largest green space patch area in the buffer to the buffer area. The calculation formula of green patch accessibility index (GPAI) is: $\sum_{i=1}^{N} D_{ij}(A - A_i)/A(N-1)$, where j represents the patient's address, D_{ij} is the distance between a patch in the buffer and the patient's address, A_i is the area of a patch in the buffer, and N is the total number of green patches in the buffer. This formula expresses the average distance from the patient's address to each green patch (weighted by the patch area). The value increases with the increase of the distance between the home address and the green patch, and decreases with the increase of the patch area. The larger the value, the less accessible the public is to the surrounding green space.

All the above indicators are counted in the 100 m–1000 m circular buffer centered on the home address. The above-mentioned green space indicators construction and numerical calculation were implemented by ArcGIS10.5 software, QGIS3.16 software, and python language.

2.4. Statistical analysis

In this study, the time stratified case crossover design (TSCC) is used to construct an experimental group and a control group. The TSCC design adopts a self-matching method, taking several time points before and after the onset of the patient as a control, reducing the data volume requirements and eliminating potential confounding factors that do not change with time, such as individual's age, sex, basic health status, socio-economic situation, etc. This approach is widely used when studying the relationship between short-term environmental exposure factors and diseases (Mostofsky et al., 2018). For this study, all identical days of the week within the 30 days prior to admission were selected as controls (Liu et al., 2021). According to the protocol, each case had 3 or 4 controls. Conditional logistic regression is used to fit the TSCC, and we apply a cubic spline function to fit the mean temperature with 4 degrees of freedom (df) and a cubic spline function with 3 df to fit all covariates (Chen et al., 2021; Guo et al., 2017). The formula is as follows:

$$ln(h(t,\ X)) = ln(h_0(t)) + ns(TEM, df = 4)\beta_1 + ns(RHU, df = 3)\beta_2 + ns(WIN, df = 3)\beta_3 + ns(PRS, df = 3)\beta_4$$

Where t is the date of CVDs hospital admission; X represents independent variable, includes explanatory variables and covariates; $ln(h(t,X))$ represents the risk function of exposure to X on the day t; $ln(h_0(t))$ is the baseline risk function; $\beta_1 - \beta_4$ represents the variable coefficients. In our study, the OR (Odds Ratio) is expressed as the risk of a certain temperature value relative to the reference temperature at which admission is not affected, here we set the 50th percentile as the reference tem-

perature. The significance level is set as 0.05, and the 95% confidence interval is calculated. The 5th percentile is used as low temperature calculation point, and the 2.5th percentile is used as the extremely low temperature calculation point to explore the modification by green space when the temperature further decrease. We take 30 days as the preset maximum lag days (Huang et al., 2022; Zhao et al., 2022). To explore cumulative effect of temperature, we use the moving-average lag structure, which calculates the average multi-day temperature within a certain lag period (Zhang et al., 2021). We calculate the cumulative low temperature risk of each lag day, and select the best lag period based on the highest OR and significance of the OR for subsequent research on the modification by green space (Wichmann et al., 2012). In this study, the median of each green space indicator under each buffer radius is used to stratify all cases into two subgroups, by comparing the difference of low temperature-related CVDs admission risk between high and low indicator subgroups, to explore the modification strength by the indicator. For any specific green space indicator, the corresponding radius with the largest OR difference and smallest confidence interval overlap is selected as the optimal modifying scale.

In the sensitivity analysis, we observe the OR and its significance of each cumulative lag period under different dfs (3–7) of the temperature variable, which further help us choose the best lag period for the following study on the modification by green space. This study used the R language (version 4.2.1) Survival package (version 3.3–1) and the DLNM package (version 2.4.7) to achieve the above statistical analysis. The copyright of the R language belongs to the entire open-source community, and anyone is free to use, modify and distribute the code of the R language.

3. Result

3.1. Descriptive statistics

After the above screening process, a total of 4364 cases were included in this study, with a time span of 2012/9/3–2018/9/3. Male cases accounted for 56% of the total number of cases, and elderly cases aged 65 and above accounted for 52% of the total number of cases. There were slight annual variations in admission counts despite the absence of a clear-cut downward or upward trend over the study period (Fig. 2a). The mean temperature indicated an obvious V-shaped fluctuation trend with seasonal changes (Fig. 2b), the temperature ranged from 3.4 °C to 31.1 °C throughout the study time. In exploring the relationship between low temperature and admission to CVDs, the reference temperature is set to 23.4 °C (50th percentiles), the low temperature is set to 10.9 °C (5th percentiles), and the extremely low temperature is set to 9.3 °C (2.5th percentiles).

The GR and the PLAND, the GR and the LPI, and the PLAND and the LPI all have a high positive correlation, especially the correlation coefficients between the PLAND and the LPI in all radius buffers are above 0.86, indicating that the buffer with a larger percentage of landscape is more likely to have a larger single green space patch. The correlation coefficients of PD and ENN_MN in all radii are below −0.66, indicating that the higher the number of green patches, the better the adjacency of the green patches (Table S1). The stratification standards of each green space indicator under different radius buffers are shown in Table 1.

3.2. The hospital admission risk of low temperature-related CVDs in each cumulative lag period

With the increase in the cumulative lag period, the admission risk of low temperature-related CVDs goes through a decline process from the cumulative lag of 0 days to the cumulative lag of 16 days (Fig. 3). The peak OR is reached in the cumulative lag of 2 days (OR: 1.3 [95%CI: 1.03, 1.65]), and there is a OR extreme value which is greater than the ORs on the left and right lags (OR: 1.28 [95%CI: 0.99, 1.64]) in the cumulative lag of 7 days. After the cumulative lag of 16 days, the risk curve becomes stable, and the confidence interval widens. When changing the dfs (3–7) of the explanatory variables, the cumulative lag 2-day and 7-day hospital admission ORs also reach extreme values, and the lag 2-day ORs are significant in all dfs, and the lag 7-day ORs are significant in all dfs except 4 (Table S2). Based on the above results, the modification by green space on low temperature-related CVDs is expressed in terms of cumulative lag of 2 days, and the result of lag of 7 days is used as auxiliary analysis.

3.3. The modification by green space indicators on admission risk of low temperature-related CVDs

3.3.1. The risk modification trend by each green space indicator with the change of radius

In terms of green space scale, results with a cumulative lag of 2 days indicate that low GR and PLAND corresponds to higher admission risk in all radius buffers. For the low GR (Fig. 4a), In the radius of 100 m–600 m, the risk changes to a “V" shape, and 400 m is the turning point of the risk from falling to rising. In the radius of 600 m–800 m, the risk also changes to a “V" shape, and 700 m is the turning point. After 800 m, the modification by GR tends to be stable. In the radius of 800 m, the risk reaches its maximum (OR: 1.52 [95%CI: 1.09, 2.11]), which increases by 42% relative to high GR. For the low PLAND (Fig. 4b), the risk curve on both sides of the 600 m radius changes to a “U" shape, but the change

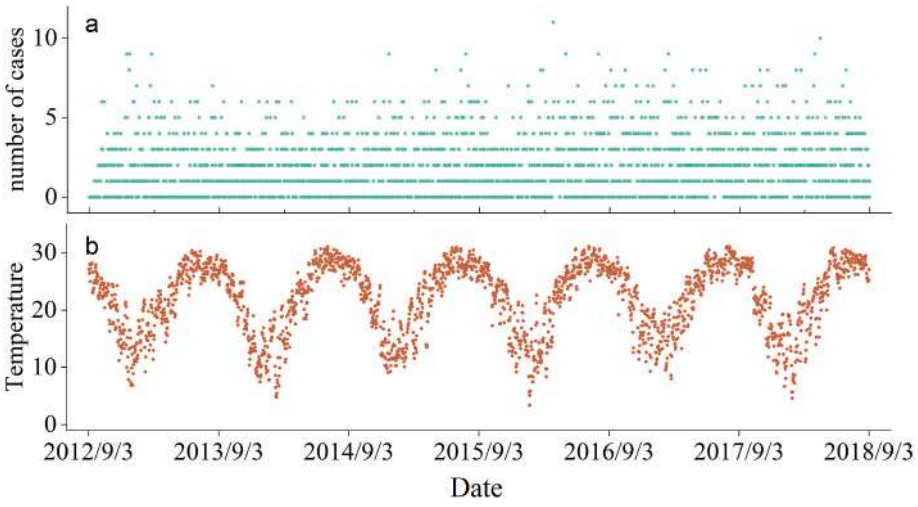

Fig. 2. The daily CVDs cases and daily mean temperature changes.

Table 1
Stratifications for high and low indicator subgroups of each green space indicator under different radius buffers. Abbreviations: Greening rate (GR), Percentage of landscape (PLAND), Patch density (PD), Largest patch index (LPI), Mean nearest neighbor distance (ENN_MN), Green patch accessibility index (GPAI).

Radius(m)	Low GR	High GR	Low PLAND (%)	High PLAND (%)	Low PD	High PD
100	0.11–0.27	0.27–0.6	0–7.26	7.26–78.97	0–6	6–19
200	0.14–0.28	0.28–0.62	0–9.23	9.23–86.14	0–21	21–47
300	0.16–0.28	0.28–0.63	0.19–9.6	9.6–87.6	1–45	45–90
400	0.17–0.28	0.28–0.63	0.88–11.16	11.16–87.01	6–78	78–132
500	0.18–0.28	0.28–0.62	1.47–11.23	11.23–86.05	11–119	119–185
600	0.19–0.29	0.29–0.61	1.65–12.39	12.39–82.29	21–172	172–245
700	0.2–0.29	0.29–0.58	1.88–13.13	13.13–75.35	40–234	234–312
800	0.2–0.29	0.29–0.57	2.09–12.93	12.93–67.12	77–301	301–385
900	0.21–0.29	0.29–0.57	2.44–14.22	14.22–66.33	99–377	377–468
1000	0.21–0.29	0.29–0.57	2.33–13.36	13.36–66.14	115–456	456–571
Radius (m)	Low LPI (m^2)	High LPI (m^2)	Low ENN_MN (m)	High ENN_MN (m)	Low GPAI (m)	High GPAI (m)
100	0–1008	1008–24755	13.56–47.2	47.2–200	0–65.73	65.73–99.93
200	0–3206	3206–107957	19.93–43.6	43.6–226	0–130.47	130.47–198.5
300	184–6291	6291–248295	23.82–42.67	42.67–130	118.79–196.39	196.39–276.45
400	916–9617	9617–436235	30.2–42.29	42.29–120.93	156.03–261.39	261.39–336.88
500	1465–14654	14654–668867	31.14–42.08	42.08–77.29	235.08–326.28	326.28–402.46
600	1557–21533	21533–914535	32.53–41.73	41.73–78.96	298.37–393.16	393.16–470.73
700	1557–32493	32493–1137489	33.22–41.46	41.46–79.1	342.83–459.06	459.06–544.56
800	2656–46066	46066–1307173	34.08–41.33	41.33–66.05	415.24–525	525–642.81
900	2931–61707	61707–1556269	34.34–41.26	41.26–64.97	487.03–591.23	591.23–718.76
1000	3114–84253	84253–1882368	33.9–41.28	41.28–65.59	538.05–657.65	657.65–778.44

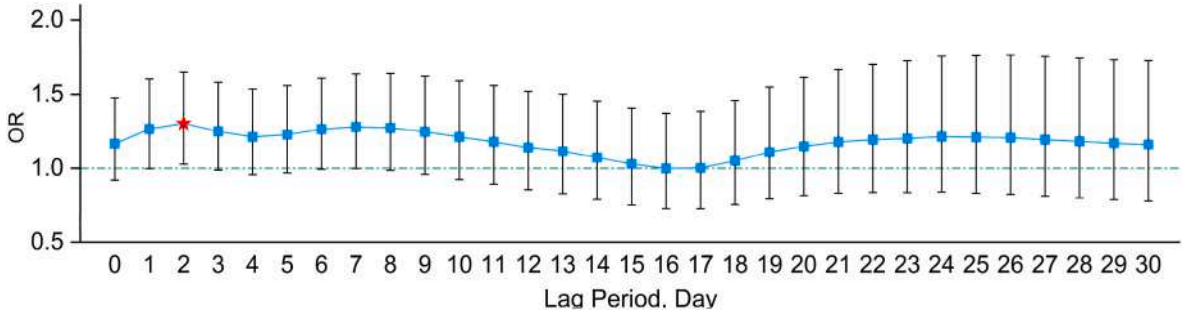

Fig. 3. The lag risk curve of low temperature-related CVDs admission (5th percentiles of temperature distribution). The red star indicates the highest and significant OR with lag 2 days. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

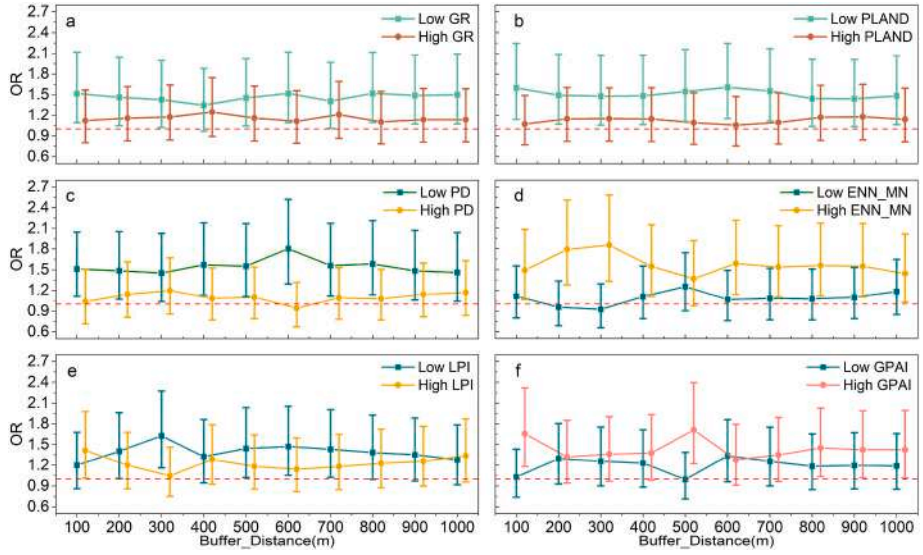

Fig. 4. The admission risk of low temperature-related CVDs corresponding to the subgroups of six green space indicators in all radii (cumulative lag of 2 days). Abbreviations: Greening rate (GR), Percentage of landscape (PLAND), Patch density (PD), Largest patch index (LPI), Mean nearest neighbor distance (ENN_MN), Green patch accessibility index (GPAI). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

process is more gradual than GR. Here, 300 m and 800 m are the turning points of the risk change. In the radius of 600 m, the risk reaches its maximum (OR: 1.61 [95%CI: 1.15, 2.24]), which increases by 56% relative to the high PLAND. The cumulative 7-day lag results also show that the larger green space scale is associated with a lower admission risk (Table S3). As the radius increases, the risk trend is similar to the cumulative lag of 2 days. The modification by GR is the strongest at 900 m, but the degree of modification is not obviously different from that at 800 m. The PLAND also has the strongest modification at 600 m.

In terms of spatial distribution pattern of green space, results with a cumulative lag of 2 days indicate that low PD, high ENN_MN and low LPI correspond to higher risk in vast majority radius buffers. 1) For the low PD (Fig. 4c), The risk in the radius of 100 m–600 m shows an overall upward trend. After 600 m, the admission risk gradually decreases. In the radius of 600 m, the risk reaches its highest (OR: 1.8 [95%CI: 1.29, 2.52]), which is increases by 86% compared to the high PD. 2) For the high ENN_MN (Fig. 4d), the risk is on the rise in the radius of 100 m–300 m. In radius of 300 m–600 m, the risk changes to a "V" shape. The risk gradually weakens after 600 m. In the radius of 300 m, the risk reaches its maximum (OR: 1.85 [95%CI: 1.33, 2.58]), which increases by 93% relative to the low ENN_MN. 3) Except for the radius of 100 m, the low LPI in all radius buffers correspond to a higher admission risk (Fig. 4e). For low LPI, As the radius changes from 100 m to 300 m, the risk gradually increases. In the radius of 300 m–400 m, the risk decrease. After 400 m, the risk shows a slow upward-downward trend. In the radius of 1000 m, the modification by LPI basically disappears. In the radius of 300 m, the risk reaches its maximum (OR: 1.63 [95%CI 1.16, 2.27]), which increases by 58% relative to the high LPI. The results of the cumulative lag of 7 days (Tables S4 and S5) are similar to the results of the cumulative lag of 2 days. The modification by PD is the strongest in 600 m, the ENN_MN has the strongest modification in 300 m, and the LPI has the strongest modification in 300 m.

The results with a cumulative lag of 2 days shows that high GPAI corresponds to a higher admission risk of low temperature-related CVDs in all radii except 600 m (Fig. 4f). For high GPAI, In the radius of 100 m–200 m, the admission risk decrease, and the GPAI has a weak modification in the radius of 200 m–400 m. The risk increases in the radius of 400 m–500 m and decrease in the radius of 500 m–600 m. After 600 m, the risk increases slowly. After 800 m, the risk tends to stabilize. In 500 m radius buffer, the modification reaches its peak (OR: 1.71 [95% CI: 1.22, 2.39]), which increases by 72% relative to low GPAI. The results of the cumulative lag of 7 days (Table S5) show that with the increase of radius, the OR value change trend is similar to that of the cumulative lag of 2 days. The modification by GPAI is the strongest at 900 m, but the degree of modification is not obviously different from that at 500 m.

3.3.2. *Analysis of the modification by green space indicators with the temperature further decreases (from the 5th percentile to the 2.5th percentiles)*

Based on the results of cumulative lag of 2 days and 7 days, the optimal scales are selected, and the modification by each green space indicator is analyzed when the temperature further decreases (Fig. 5). Specifically, a radius of 800 m is selected for the GR, a radius of 600 m for the PLAND and the PD, a radius of 300 m for the LPI and the ENN_MN, and a radius of 500 m for the GPAI.

Regarding the results with a cumulative lag of 2 days, for the low GR, the OR value of the 2.5th percentiles is 1.69 [1.17, 2.43], which increases the risk by 17% compared to the 5th percentiles; for the high GR, the OR value of the 2.5th percentiles is 1.13 [0.77, 1.66], which increases the risk by 3% compared to the 5th percentiles (Fig. 5a). For the low PLAND, the OR value of the 2.5th percentiles is 1.77 [1.22, 2.56], which increases the risk by 16% compared to the 5th percentiles; for the high PLAND, the OR value of the 2.5th percentiles is 1.10 [0.76,1.60], which increases the risk by 5% compared to the 5th percentiles (Fig. 5b). For low PD, the OR value of the 2.5th percentiles is 2.00 [1.38, 2.90], which increases the risk by 20% compared to the 5th percentiles; for the high PD, the OR value of the 2.5th percentiles is 0.97 [0.67, 1.42], which is a 3% increase in risk compared to the 5th percentiles (Fig. 5c). For the low ENN_MN, the OR value of the 2.5th percentiles is 0.99 [0.67, 1.44],

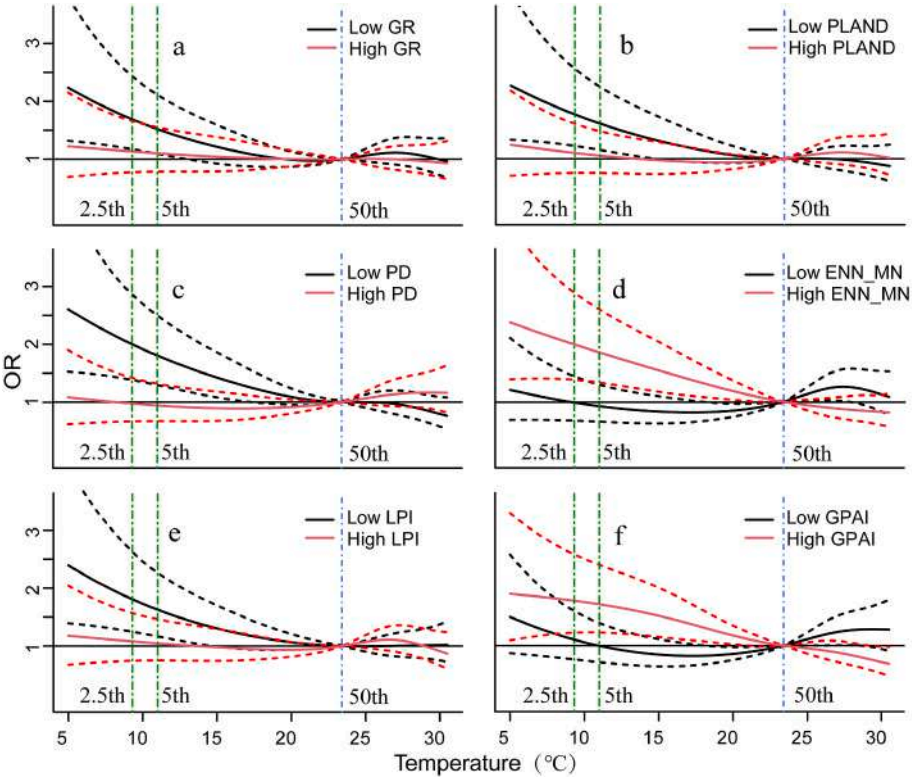

Fig. 5. The admission risk curve of temperature-related CVDs corresponding to the subgroups of each green space indicator at the optimal modification scale (cumulative lag of 2 days). The solid curves represent estimated ORs, and the dashed curves represent upper or lower bounds of 95%CI. The 50th, 5th, and 2.5th respectively represent reference temperature, low temperature, and extremely low temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

which increases the risk by 7% compared to the 5th percentiles; for the high ENN_MN, the OR value of the 2.5th percentiles is 1.99 [1.38, 2.88], which increases the risk by 14% compared to the 5th percentiles (Fig. 5d). For the low LPI, the OR value of the 2.5th percentiles is 1.81 [1.25, 2.62], which increases the risk by 18% compared to the 5th percentiles; for the high LPI, the OR value of the 2.5th percentiles is 1.08 [0.74, 1.57], which increases the risk by 3% compared to the 5th percentiles (Fig. 5e). For the low GPAI, the OR value of the 2.5th percentiles is 1.10 [0.76, 1.60], which increases the risk by 11% compared to the 5th percentiles; for the high GPAI, the OR value of the 2.5th percentiles is 1.77 [1.22, 2.58], which increases the risk by 6% compared to the 5th percentiles (Fig. 5f).

From the above results, it can be concluded that with a further decrease in temperature, the high GR, the high PLAND, the high PD, the high LPI, and the low ENN_MN will produce a smaller incremental risk of hospital admissions, indicating that their degree of risk-modifying increases as temperatures continue to drop. As for the GPAI, although the risk of high GPAI is still higher than that of the low GPAI at extremely low temperatures, the growth rate of the overall risk curve slows down, while the risk curve corresponding to the low GPAI still maintains a certain growth rate, indicating that the health benefit from the low GPAI weakens as the temperature continues to decrease. The changing trend of each risk curve corresponding to the cumulative lag of 7 days and the cumulative lag of 2 days is consistent (Fig. S1).

4. Discussion

4.1. The modification mechanism of the occurrence of low temperature-related CVDs by green space

This study explores the modification by various green space indicators on the occurrence of low temperature-related CVDs, compares the modification strengths at different spatial scales, and then explores the risk-modifying effects when the temperature is further lowered.

This study finds that high GR has a protective effect on low temperature-related CVDs. Li et al. (2021) found that high GR corresponds to a lower admission risk of low temperature-related stroke. The study was carried out in Wuhan, which shares the subtropical monsoon climate with Guangzhou, corroborating our findings. The PLAND, GR, and LPI used in this study can reflect the scale and growth of trees. In addition, the PD and the ENN_MN can reflect the compactness of tree arrangement. Our results express the benefit of dense and compact green space on somatosensory temperature at low temperatures, which is reflected in the reduction in the admission risk of low temperature-related CVDs. Zhang et al. (2018) constructed a Wuhan residential community model to explore the effect of trees on body temperature under low temperature conditions. The researchers found that closely arranged evergreen vegetation is more conducive to creating a good body temperature than deciduous vegetation (the zonal vegetation in Guangzhou is South subtropical monsoon evergreen broad-leaved forest). Meili et al. (2021) researched four cities in different climate zones as study areas and comprehensively evaluated the effects of vegetation and solar radiation interaction, evapotranspiration, and changes in surface roughness on surface temperature. Among them, the warming effect of vegetation can be summarized into two aspects. First, the canopy can absorb more short-wave radiation and release a large amount of sensible heat to the surface. Second, vegetation can reduce the surface roughness of urban neighborhoods, hinder the energy interaction of turbulent flow, and increase the temperature during the day. This warming effect increases with the increase of vegetation density and coverage area. The temperate oceanic climate type cities have an obvious warming effect in winter. The mean temperature in Guangzhou is also above 0 °C in winter, and it is expected that this warming effect will also be obvious in the Guangzhou area. No research has been found on the impact of accessibility on the admission risk of low temperature-related CVDs. The possible explanation is that the better the access to green space, the more direct the public is affected by green space, and thus better able to obtain the benefits of green space (Tamosiunas et al., 2014). When analyzing the modification of each green space with the further decrease of temperature, we find that at the optimal modification scale, the benefits of the high GR, the high PLAND, the high PD, the low ENN_MN, and the high LPI are enhanced. These conclusions further demonstrate the benefits of large-scale, high-density vegetation. However, the GPAI results show that with further reductions in temperature, low GPAI corresponds to a larger increase in risk than high GPAI. That is, the health benefit from the low GPAI gradually decrease with the decrease in temperature. A possible explanation is that at low temperatures, the public with high access to vegetation is more susceptible to the adverse effects of vegetation, as well as its benefits, because vegetation blocks direct sunlight, making the already low temperature even low (Xing et al., 2019), which is not conducive to cardiovascular and cerebrovascular health. The modification of green space on the low temperature-related CVDs may also be related to the time, frequency, and way of public participation in green space. Different accessibility indicators need to be tried in the future.

4.2. The scale effect of green space modification

This study discovers that each green space indicator has a scale effect on the modification of low temperature-related CVDs, and there is a spatial scale with the strongest modification. Our study shows that the optimal modification radii of GR and PLAND are around 800 m and 600 m. Similar to the results of previous studies, for example, Su et al. (2019) selected Barcelona, Spain, as their research area, randomly choosing survey subjects in each block, and studying whether the GR around the participants' home addresses impacted their health. The study includes circular buffers with radii of 50 m, 100 m, 250 m, 500 m, and found that the green space within a radius of 500 m has the greatest health benefits. Browning and Lee (2017) reviewed the impact of green space on the public health at all ages, and found that 500 m–1000 m radius buffer GR centered on home addresses were better predictors of physical health. Liu et al. (2022) reviewed the effect of green space on the occurrence of CVDs, and found that the GR in the 500 m radius buffer has a stronger protective effect on cardiovascular and cerebrovascular health than at 300 m and 1000 m. The above studies all show a scale effect on the impact of GR on physical health, and the optimal modification scale may occur between 500 m and 1000 m, which supports our results.

The "Green Space and Health" document published by the World Health Organization lists several criteria for the selection of green space radii (World Health Organization, 2016), all of which are based on walking elements. Among them, the "Green Infrastructure Planning Standard" in England recommends that there is at least one green space not less than 2 hectares within 300 m around the residence. The European Union suggests the elderly walk a distance of 15 min as the standard, which corresponds to a walking distance of about 500 m and a straight-line distance of about 300 m. The online tool of the US Environmental Protection Agency provides calculation services for green space-related indicators within walking distance of 500 m. Yeager et al. (2020) reviewed the relationship between green space and cardiovascular and cerebrovascular health and found that previous studies mostly calculated green space indicators in a 200 m–500 m radius buffer. The above common radius selection methods are based on two reasons. First, the radius range is more in line with the public's green vision range, and second, the green space within this radius is more likely to be frequently used by the public. Our study constructed the GPAI to express the use opportunity of green space. The results with a cumulative lag of 2 days showed that GPAI modification is strongest within a 500 m radius. The optimal modification scale for results with a cumulative lag of 7 days is larger, and these distances are all larger than the standard value of the World Health Organization. First, the benefits of physical and mental pleasure, emotion regulation, and increased exercise time obtained by the public participating in green space are not within the scope of our

study. Green space may have a larger spatial scope for the modification of low temperature-related CVDs admissions. Second, the 300 m straight-line distance standard is relatively conservative. Millward et al. (2013) chose Halifax, Canada, as the research area, collected a large number of individual behavioral trajectory data, analyzed the public walking mode, and found that most of the public are willing to participate in urban space within a range of 600 m. Finally, farther green spaces with greater recreational opportunities and unique significance can motivate the public to break through distance barriers to participate (Ekkel and de Vries, 2017).

The optimal modification radius of each green space distribution pattern indicator is within the optimal modification radius of green space scale. Specifically, the optimal modification radius of high PD is around 600 m, and the optimal modification radius of high LPI and high ENN_MN is around 300 m. It shows that when urban green space planning is carried out, sufficient vegetation scale and density should be maintained at a larger spatial scale, while at a relatively small scale, more attention should be paid to the compact layout of green space, and a single larger area should be provided within 300 m as much as possible. At the same time, reasonably adjusting the area and the total number of green space patches at the intermediate scale to provide greater accessibility to green space. These measures will be beneficial in protecting the public's cardiovascular and cerebrovascular health under low temperature conditions.

4.3. Limitations

In this study, relative humidity, wind speed, and air pressure are controlled in the process of constructing the relationship between temperature and CVDs admission, but there may still be some confounding factors that are not controlled. In addition, due to the limitation of data acquisition, the number of cases involved in this study is relatively small, and it is necessary to be expanded further to improve the statistical results of the risk model. Our conclusion should be used with caution in an area which the climate, population, economy and other characteristics are very different from us. More empirical research is needed to draw conclusions applicable to different areas. When looking into the future, firstly, in the field of research on the relationship between green space and physical health, more attention should be paid to the influence of green space attributes such as green space distribution patterns and vegetation types, not limited to the scale of green space, especially the relationship between air temperature and CVDs. Secondly, when constructing a model of the relationship between green space and health, attention should not only be paid to spatial scale effects, but also to geographic issues such as variable unit area and uncertainty of spatial background. Finally, in terms of mechanism exploration, it is necessary to clarify the specific path of vegetation in modifying low temperature-related cardiovascular and cerebrovascular health and carry out corresponding empirical research.

5. Conclusion

In this study, the time-stratified case-crossover design method is used to explore the modification of admissions risk of low temperature-related CVDs by green space scale, green space distribution pattern, and accessibility, six specific green space indicators were constructed. It is found that high GR, high PLAND, high PD, low ENN_MN, high LPI, and low GPAI correspond to low risk of CVDs occurrence, the optimal scales are around 800 m (GR), 600 m (PLAND and PD), 500 m (GPAI), and 300 m (LPI and ENN_MN), respectively, with the further decrease in temperature, the health benefit from the low GPAI is weakened, while the benefits from the others are strengthened. For countries and regions where the morbidity or mortality of CVDs is significantly affected by low temperature, the conclusions of this study have important theoretical and practical significance when forming and implementing local green space policies.

Ethical approval

Medical Ethics Committee for Clinical Research and Animal Trials of the First Affiliated Hospital of Sun Yat-sen University.

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Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114193.

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Monitoring ambient air pollution and pulmonary function in asthmatic children by mobile applications in COVID-19 pandemic

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ABSTRACT

Background: Several public health measures were implemented during the COVID-19 pandemic. However, little is known about the real-time assessment of environmental exposure on the pulmonary function of asthmatic children. Therefore, we developed a mobile phone application for capturing real-time day-to-day dynamic changes in ambient air pollution during the pandemic. We aim to explore the change in ambient air pollutants between pre-lockdown, lockdowns, and lockdowns and analyze the association between pollutants and PEF mediated by mite sensitization and seasonal change.

Method: A prospective cohort study was conducted among 511 asthmatic children from January 2016 to February 2022. Smartphone-app used to record daily ambient air pollution, particulate matter (PM2.5, PM10) Ozon (O_3), nitrogen dioxide (NO_2), Carbon Monoxide (CO), sulfur dioxide (SO_2), average temperature, and relative humidity, which measured and connected from 77 nearby air monitoring stations by linking to Global Positioning System (GPS)-based software. The outcome of pollutants' effect on peak expiratory flow meter (PEF) and asthma is measured by a smart peak flow meter from each patient or caregiver's phone for real-time assessment.

Results: The lockdown (May 19th, 2021, to July 27th, 2021) was associated with decreased levels of all ambient air pollutants aside from SO_2 after adjusting for 2021. NO_2 and SO_2 were constantly associated with decreased levels of PEF across lag 0 (same day when the PEF was measured), lag 1 (one day before PEF was measured), and lag 2 (two days prior when the PEF was measured. Concentrations of CO were associated with PEF only in children who were sensitized to mites in lag 0, lag 1, and lag 2 in the stratification analysis for a single air pollutant model. Based on the season, spring has a higher association with the decrease of PEF in all pollutant exposure than other seasons.

Conclusion: Using our developed smartphone apps, we identified that NO_2, CO, and PM10 were higher at the pre- and post-COVID-19 lockdowns than during the lockdown. Our smartphone apps may help collect personal air pollution data and lung function, especially for asthmatic patients, and may guide protection against asthma attacks. It provides a new model for individualized care in the COVID era and beyond.

1. Introduction

Approximately three million people worldwide have asthma, and the burden of this disease is substantial as asthmatic individuals may experience frequent night-waking, loss of work productivity, increased healthcare expenses, and increased time spent on treatment (Dierick et al., 2020). Although treatment for asthma control is currently available, poor asthma control leads to emergency visits and hospitalization

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(Fergeson et al., 2017). Pulmonary function tests such as peak expiratory flow (PEF), forced vitality capacity (FVC), and forced expiratory volume in the first second (FEV1) may be used to monitor respiratory function in asthmatic patients (Enright et al., 1994). Asthma is a multifactorial disease attributable to genetic susceptibility factors, host factors, and environmental exposures. Therefore, avoiding environmental pollutants/irritants besides asthma treatment is crucial to prevent asthma exacerbations (Dharmage et al., 2019).

Previous studies have demonstrated that exposure to hazardous air pollutants may decline pulmonary function (Altman et al., 2023). Various studies have provided evidence that sulfate (SO_4^{2-}), ammonium (NH_4^+), nitrogen dioxide (NO_2), sulfur dioxide (SO_2), particle matter (PM10 and PM2.5), and ozone (O_3) exposure in asthmatic patients were associated with decreased PEF levels, (Edginton et al., 2021; Kim et al., 2021a; Gehring et al., 2020). Additionally, the existing biological hazards in air pollutants such as mites have previously been reported to reduce PEF levels because of their allergy sensitization (Okasha et al., 2021). However, the vivid mechanism remains unclear and needs to be addressed.

Concerning the literature on air pollution and asthma surveillance, previous studies did not amass air pollutants based on geo-located locations. Instead, data were measured in fixed settings (Gehring et al., 2020). These data collection methods may be subject to measurement error because participants may move across areas, and the association observed may have been biased.

To overcome this issue, collecting air pollution data via smartphone applications (apps) may be optimal because smartphones have built-in Global Positioning System (GPS) functions and can personally collect air pollutant exposure based on one's location. Therefore, the collected data may be more precise compared with previous methods. In addition, smartphone apps can assess the dynamic change of air pollutants without burdening the participants and study investigators (Johnston et al., 2018; Loh et al., 2017). In pandemics such as COVID-19, this has been a crucial advantage of smartphone apps, as lockdowns may occur and impact the progress of data collection. Smartphone apps also provide an opportunity to understand the dynamic air quality change between pre-post and during lockdowns. These gadgets are also beneficial in collecting data more efficiently from longitudinal cohorts across multiple years and provide valuable information about asthma and its symptoms (Ambrosini et al., 2017).

Few studies used smartphone applications to amass environmental data and asthma outcomes. A study in Australia collected pollen concentration and respiratory symptoms using smartphone apps (Jones et al., 2021). However, the environmental data collected were not specifically on air pollutants. Another study in the United States developed an app to monitor air pollutants for asthmatic children. Nevertheless, the app still needs to be evaluated in a real-world setting (Kim et al., 2021b). Currently, studies on collecting air pollutants using smartphone apps and assessing their association with asthma outcomes in the pediatric population still need to be made available. Therefore, we developed a smartphone app to collect real-time personal pollutants to explore the change in the ambient air pollutants between pre-lockdown, lockdowns, and lockdowns; and to analyze the association between pollutants and PEF among asthmatic children and observe if the association is modified by mite sensitization and season.

2. Method and material

2.1. Study design, setting, and population

This was a prospective cohort study employing repeated measurements of the outcome and exposure. This study was conducted between January 2016 and February 2022. A total of 511 children were recruited with eligibility criteria of 18 years of age, diagnosed with asthma, and who visited the Taipei Hospital. During the follow-up period, a lockdown (May 19th, 2021, to July 27th, 2021) was imposed due to the COVID-19 pandemic. The hospital's Institutional Review Board approved the study (TH-IRB-0016-0038), and parents provided written consent before their child was enrolled.

2.2. Smartphone app and exposure collection

We collected real-time personal exposure of particulate matter $\leq$10 m and $\leq$2.5 m in aerodynamic diameter (PM10 and PM2.5), sulfur dioxide (SO_2), nitrogen dioxide (NO_2), ozone (O_3), and carbon monoxide (CO) from air monitoring station by the Environmental Protection Administration. There were a total of 77 stations located throughout Taiwan. PM 2.5 ($\mu g/m^3$) and PM10 ($\mu g/m^3$) were detected using beta ray, O_3 (ppb) was measured using ultraviolet absorbance, CO (ppm) was measured using infrared absorbance, SO_2 (ppb) was measured using an ultraviolet fluorescence spectrometer, and NO_2 (ppb) was measured by gas-phase chemiluminescence detection. The relative humidity and temperature were also collected. Participants or their caregivers were required to download a smartphone app that can automatically record the personal air pollution parameters from the closest air pollution stations in real-time using the in-built Global Positioning System (GPS) function. This smartphone app can also send in-time reminders of avoidance of unhealthy air quality to the participants or caregivers when it detects poor air quality. The sensitization to mite allergens was defined as levels of specific IgE $\geq$0.7 U/mL.

2.3. Outcome measurement

Forced vital capacity (FVC), forced expiratory volume in 1 s (FEV1), and FEV1/FVC ratio were measured by spirometry in the baseline assessment. A smart peak expiratory flow meter from each patient or caregiver's phone was used for real-time assessment. PEF is a measure used by asthma patients to record their asthma control conditions. The instrument is constructed with a unique air tube that was 3D printed and a small number of electronic parts that communicate with a smartphone using a unique app. The headphone jack is used to transmit a pressure-dependent frequency signal created when the user blows into the tube to a smartphone app. The specifically designed app reads the signal, transforms the frequency to a flow rate, and displays this information for the patients to see, as well as logging it for condition monitoring.

2.3.1. Quality assurance and control

We adapted the QA and quality control (QA/QC) procedures for PFT that were in place in our laboratory for oscillometry in addition to the suggestions from the ERS guidelines and the Tremoflo user manual (Wu et al., 2020). The ERS guidelines and the Tremoflo user manual suggest that measurements should have a minimum duration of 6 s, valid data points $\geq$70%, and a minimum of 3 valid measurements with a coefficient of variation $\leq$15%. However, these suggested guidelines were not automatically excluded and must be assessed by the operator. In our QA/QC procedures, we gave subjects 3 min of rest before the first oscillometry measurement to stabilize lung volumes because it is a 60-m walk from the waiting area to the laboratory. A minimum of 3 tidal breaths must be observed before each oscillometry recording to ensure subjects are breathing at resting functional residual capacity level to avoid mechanical drifts. A 30 s rest is also given to subjects between each measurement to avoid short-term variability. We increased the minimum rest time from 3 to 10 min to stabilize the lung volumes if forced respiratory maneuvers were performed before the oscillometry test. Biological calibration was also added to ensure the two oscillometry devices function correctly.

2.4. Statistical analysis

We tested the P for the trend of air pollution levels in different periods of the follow-up: before the COVID-19 lockdown (2016 Jan 4th −2021 May 14th), during lockdown (2021 May 15th-July 27th), and

after lockdown (2021 July 28th - 2022 Feb 25th). Interrupted time series analysis assessed the association between air pollutants and the COVID-19 lockdown. Associations between repeated measures of air pollutants at lag 0 lag 1- and lag 2- and PEF were assessed using generalized estimate equations (GEE) (lag 0: on the same day when the PEF was measured: lag1: 1 day before PEF outcome measurements; lag 2: 2 days before PEF outcomes measurement).

Age, gender, body mass index, environmental tobacco smoke, family income, temperature, and relative humidity were adjusted in the regression models. Effect modification was explored by mite sensitization, and stratified associations were presented. For these analyses, two-pollutant models were also examined. Associations between repeated measures of air pollutants and emergency room visits were assessed using GEE with a Poisson distribution, and adjusted results were presented. Because mite is a vital aeroallergen, which may confound the effects of air pollutants, the stratification results by mite sensitization were also presented. All analysis was performed on SPSS software version 22.0 (IBM, Armonk, NY, USA) and R program version 3.5.3 (R Fountin for Statistical Computing, Vienna, Austria).

3. Results

3.1. Baseline characteristics of the study subjects

At baseline, the mean age was 8.1 years, and 213 (41%) participants were male (Table 1). The mean FVC was 3263 mL, and the mean FEV1 was 2680 mL. The mean PEF was 177.2 L/minute, and the mean ACT score was 18.8.

3.2. The change in ambient air pollution between pre-lockdown, lockdowns, and lockdowns

3.2.1. Ambient air pollutant distribution during the follow-up period

The daily mean concentration of air pollutants at lag 0, lag 1, and lag2 during the follow-up period are presented in (Table 2). The mean of each air pollutant did not vary mainly across the three lags. There was evidence of correlations among the air pollutants (Table A.1).

3.2.2. Ambient air pollution levels and COVID-19 lockdowns

There was a trend that the concentrations of NO_2, CO, O_3, and PM10 were lower during the COVID-19 lockdown compared to pre-and post-lockdown periods (Fig. 1). COVID-19 lockdowns (May 19th, 2021, to July 27th, 2021) were associated with decreased levels of all air pollution aside from SO_2 after adjusting for the year 2021 (Table A.2).

3.3. Associations between ambient air pollutants and PEF among asthmatic children

3.3.1. Associations between air pollutants and PEF by single pollutant model

NO_2 and SO_2 were constantly associated with decreased levels of PEF across lag 0 (same day when the PEF was measured), lag 1 (one day before PEF was measured), and lag 2 (two days prior when the PEF was measured) (NO_2: lag 0: 1.01, 95%CI, −1.72 to −0.27, p = 0.03; lag1: 1.22, 95%CI: −1.96 to −0.48, p = 0.005; lag2: 0.89, 95% CI: −1.59 to −0.19, p = 0.031; SO_2: lag 0: 5.23, 95%CI, −9.99 to −0.43, p = 0.017; lag1: 4.83, 95%CI: −9.04 to −0.61, p = 0.025; lag2: 6.10, 95% CI: −11.07 to −1.13, p = 0.005). While PM 2.5 was only associated with lag 2 (−0.79, 95%CI: −1.45 to −0.12, p = 0.031), PM10 was associated with lower levels of PEF at lag 0 (−0.44, 95%CI: −0.77 to −0.10, p = 0.030) and lag 2 (−0.39, 95%CI: −0.76 to −0.02, p = 0.023). CO was associated with decreased levels of PEF only at day 0 (−49.0, 95%CI: −77.97 to −18.33, p = 0.008) and at day 1 (−43.1, 95%CI: −71.81to −14.34, p = 0.01). O_3 was not associated with PEF on either of the days (Table 3).

Table 1
Study characteristics.

	Total (N = 511)
BMI, mean (SD)	18.8 (4.7)
Age, mean (SD)	8.1 (5.2)
Sex (male)	213
Mite (>2vs. ≤ 2) (N = 202)	156 vs.48
FVC(mL)	3263 (817)
FEV1 (mL)	2680 (663)
FEV1/FVC(%)	83.2 (10.6)
FeNO	29.4 (15.6)
PEF	177.2 (98.9)
ACT score	18.8 (6.5)

Table 2
Daily concentrations of ambient air pollutants (N = 511).

	Min	Max	IQR	SD	Mean
Lag 0					
$PM_{2.5}$ (μg/m^3)	2	42	10	14.48	7.43
PM_{10} (μg/m^3)	6	85	19	31.02	13.85
NO_2(ppb)	5	43	4.4	17.30	6.41
CO (ppm)	0	1	0.19	0.46	0.17
O_3 (ppb)	9	68	13.8	30.89	10.82
SO_2 (ppb)	0	8	1.5	2.82	1.33
Lag 1					
$PM_{2.5}$ (μg/m^3)	1	42	10	14.24	7.57
PM_{10} (μg/m^3)	5	77	20	30.37	15.01
NO_2(ppb)	1	40	7.86	15.74	6.58
CO (ppm)	0	1	0.10	0.43	0.17
O_3 (ppb)	8	61	7.83	30.56	11.12
SO_2 (ppb)	0	9	1.11	2.69	1.26
Lag 2					
$PM_{2.5}$ (μg/m^3)	1	45	9	14.19	7.56
PM_{10} (μg/m^3)	7	112	17	29.93	15.80
NO_2(ppb)	4	40	7.78	15.57	6.48
CO (ppm)	0	1	0.20	0.43	0.16
O_3 (ppb)	7	74	14.2	30.02	10.76
SO_2 (ppb)	1	9	1.10	2.74	1.29

Lag 0 : Same day; Lag 1 : 1 day before; Lag 2 : 2 day before.

3.3.2. Associations between air pollutants and PEF by two pollutant model

When including O_3 as a co-pollutant, the association for all pollutants was similar to the results observed in the single pollutant models. However, when having NO_2 or CO as a co-pollutant, the association was slightly different from the effects observed in the single air pollutant models. Only the association between SO_2 and PEF at lag 2 remained significant in these co-pollutant models. When including PM2.5 or PM10 as co-pollutants in the models, only the association between CO, NO_2, and PEF at lag 0 and the association between SO_2 and PEF at lag 2 remained significant (Table A3).

3.3.3. Effect modifications by mite sensitization for PEF outcomes

There was evidence that mite sensitization may modify the effect of specific air pollutants on PEF. In the single air pollutant models, concentrations of CO was associated with PEF only in children who were sensitized to mite in lag 0, lag 1, and lag 2 in the stratification analysis (lag 0: −48.15, 95%CI: 77.97, −18.33, P = 0.002; lag 1: −43.07, 95% CI: −71.81, −14.34, P = 0.003, lag 2: −33.85, 95%CI: −61.75, −5.94, P = 0.017). Additionally, PM10 and PM 2.5 were only associated with PEF in children sensitized to mites in lag 0 and lag2. No effect modification was observed for O_3 (Table 3).

3.3.4. Effect modifications by seasons for PEF outcomes

There was evidence that seasons may modify the effect of specific air pollutants on PEF. In the single air pollutant models, concentrations of CO was associated with PEF only in children who were sensitized to mite in lag 0, lag 1, and lag 2 in the stratification analysis (lag 0: −48.15, 95%

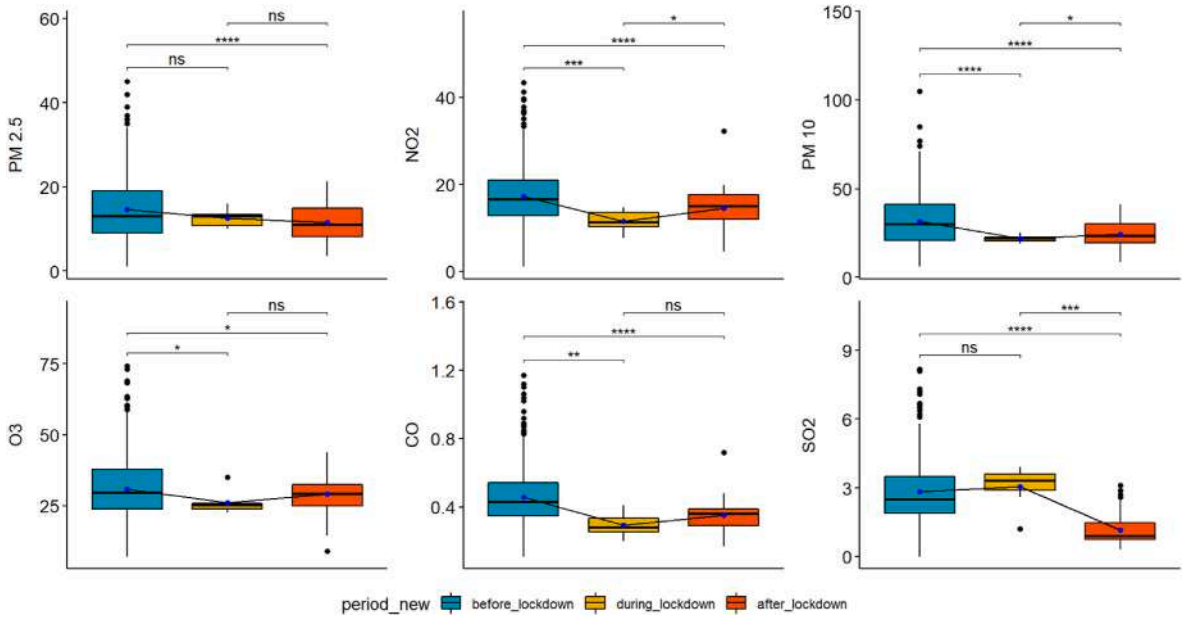

Fig. 1. Air pollution levels during pre-post lockdowns and lockdowns.

Table 3
Single-pollutant models of associations between ambient air pollution exposure and PEF stratified by mite sensitization.

PEF	Total (N = 511)			Mite sensitization (−)			Mite sensitization (+)		
	B	95%CI	P-value	B	95%CI	P-value	B	95%CI	P-value
Lag 0									
$PM_{2.5}$ (μg/m^3)	−0.67	−1.25, −0.09	0.081	0.60	−4.61, 5.82	0.820	**−0.67**	**−1.25, -0.09**	**0.025***
PM_{10} (μg/m^3)	**−0.44**	**−0.77, -0.10**	**0.030***	−0.54	−4.52, 3.43	0.788	**−0.44**	**−0.77, -0.10**	**0.011***
NO_2(ppb)	**−1.01**	**−1.72, -0.27**	**0.031***	0.37	−3.48, 8.22	0.427	**−1.00**	**−1.72, -0.27**	**0.007***
CO (ppm)	**−49.0**	**−77.97, -18.33**	**0.008***	59.5	−149, 268	0.576	**−48.15**	**−77.97, -18.33**	**0.002***
O_3 (ppb)	−0.06	−0.50, 0.39	0.823	−0.32	−3.89, 3.25	0.860	−0.06	−0.50, 0.39	0.802
SO_2 (ppb)	**−5.23**	**−9.99, -0.43**	**0.017***	−6.87	−28.4, 14.6	0.531	**−5.21**	**−9.99, -0.43**	**0.032***
Lag 1									
$PM_{2.5}$ (μg/m^3)	−0.54	−1.18, 0.09	0.128	−1.28	−5.89, 3.34	0.587	−0.54	−1.18, 0.09	0.094
PM_{10} (μg/m^3)	−0.34	−0.74, 0.06	0.081	−0.32	−3.11, 2.47	0.823	−0.34	−0.74, 0.06	0.097
NO_2(ppb)	**−1.22**	**−1.96, -0.48**	**0.005***	−1.76	−9.80, 7.08	0.752	**−1.22**	**−1.96, -0.48**	**0.001***
CO (ppm)	**−43.1**	**−71.81, -14.34**	**0.010***	−32.0	−294, 230	0.811	**−43.07**	**−71.81, -14.34**	**0.003***
O_3 (ppb)	0.36	−0.06, 0.78	0.153	1.41	−1.24, 4.06	0.298	0.36	−0.06, 0.78	0.090
SO_2 (ppb)	**−4.83**	**−9.04, -0.61**	**0.025***	−6.18	−27.9, 15.6	0.578	−4.83	−10.49, 0.84	0.095
Lag 2									
$PM_{2.5}$ (μg/m^3)	**−0.79**	**−1.45, -0.12**	**0.031***	−2.93	−7.67, 1.82	0.227	**−0.79**	**−1.45, -0.12**	**0.021***
PM_{10} (μg/m^3)	**−0.39**	**−0.76, -0.02**	**0.023***	−0.67	−2.20, 0.87	0.393	**−0.39**	**−0.76, -0.02**	**0.040***
NO_2(ppb)	**−0.89**	**−1.59, -0.19**	**0.031***	**−6.47**	**−12.5, -0.40**	**0.037***	**−0.89**	**−1.59, -0.19**	**0.013***
CO (ppm)	−33.8	−61.75, −5.94	0.052	−168.2	−412, 76.1	0.177	**−33.85**	**−61.75, -5.94**	**0.017***
O_3 (ppb)	0.21	−0.27, 0.68	0.422	1.76	−1.31, 4.83	0.262	0.21	−0.27, 0.68	0.398
SO_2 (ppb)	**−6.10**	**−11.07, -1.13**	**0.005***	**−19.4**	**−37.0, -1.82**	**0.030***	**−6.10**	**−11.07, -1.13**	**0.016***

Lag 0 : Same day; Lag 1 : 1 day before; Lag 2 : 2 day beforeModels were adjusted for age, gender, body mass index, environmental tobacco smoke, family income, temperature, and relative humidity.*$p < 0.05$.[a]

[a] Bold font indicates statistical significance

CI: 77.97, −18.33, P = 0.002; lag 1: −43.07, 95% CI: −71.81, −14.34, P = 0.003, lag 2: −33.85, 95%CI: −61.75, −5.94, P = 0.017). Additionally, PM10 and PM 2.5 were only associated with PEF in children sensitized to mites in lag 0 and lag2. No effect modification was observed for O_3 (Table 4).

4. Discussion

In the present study, lower levels of NO_2, CO, O_3, and PM10 were observed during the COVID-19 lockdown compared to pre-and post-lockdown periods in Taiwan, but this trend was not observed in SO_2 and PM2.5. Our findings that real-time monitored NO_2, CO, O_3, and PM10 were lower during COVID-19 lockdown compared with pre/post lockdowns are primarily consistent with previous studies in different settings (Adam et al., 2021), which can be explained by the reduced transportation emission during lockdowns. The trend of SO_2 levels during lockdown is interesting, and the evidence is contraindicated from the literature (Adam et al., 2021; Kenawy et al., 2021). Our findings may be explained by the fact that SO_2 levels are generally higher in summer or spring throughout the year (Lee et al., 2018). Our observation may be possible due to seasonal factors rather than lockdown interventions, as the lockdown occurred in the summer seasons (May–July). Future studies examining the effect of lockdowns and air pollutants should consider seasonal factors.

Table 4
Single-pollutant models of associations between ambient air pollution exposure and stratified by seasons.

PEF score												
Spring				Summer			Autumn			Winter		
	B	95%CI	P-value	B	95%CI	P-value	B	95%CI	P-value	B	95%CI	P-value
Lag 0												
PM2.5	−1.89	−3.68, −0.09	0.039*	−0.38	−1.62, 0.85	0.545	−1.48	−2.72, −0.24	0.019*	1.54	0.43, 2.65	0.006*
PM10	−0.62	−1.72, 0.47	0.263	−0.14	−0.87, 0.59	0.702	−0.76	−1.35, −0.16	0.013*	0.34	−0.19, 0.88	0.209
NO2	−3.20	−5.01, −1.39	0.001*	−1.42	−3.04, 0.19	0.085	−0.86	−2.60, 0.88	0.332	0.70	−0.43, 1.84	0.224
CO	−166.5	−232.2, −100.9	0.000*	−61.00	−130.4, 8.44	0.085	0.72	−64.1, 65.5	0.983	51.9	3.64, 100.1	0.035*
O3	0.23	−0.82, 1.27	0.672	0.21	−0.91, 1.32	0.716	−0.76	−1.58, 0.05	0.067	0.20	−0.50, 0.90	0.568
SO2	−2.19	−14.21, 9.83	0.721	−6.34	−16.41, 3.74	0.218	−4.00	−13.45, 5.46	0.407	0.33	−6.37, 7.04	0.923
Lag 1												
PM2.5	−1.108	−2.476, 0.260	0.112	−0.268	−1.568, 1.032	0.686	−1.212	−2.337, −0.088	0.035*	1.042	0.062, 2.021	0.037*
PM10	−0.286	−1.240, 0.669	0.557	−0.032	−0.876, 0.812	0.940	−0.734	−1.465, −0.004	0.049*	0.406	−0.098, 0.909	0.114
NO2	−2.679	−4.907, −0.487	0.017*	−0.864	−2.650, 0.922	0.343	−1.062	−2.421, 0.297	0.125	0.184	−1.098, 1.466	0.778
CO	−120.047	−193.382, −46.711	0.001*	−48.593	−114.642, 17.457	0.149	−20.622	−78.979, 37.735	0.489	20.353	−26.950, 67.656	0.399
O3	0.456	−0.700, 1.162	0.439	0.694	−0.296, 1.683	0.169	0.101	−0.736, 0.938	0.813	0.385	−0.416, 1.187	0.346
SO2	−3.583	−19.883, 12.716	0.667	−2.904	−10.576, 4.768	0.458	−0.974	−6.120, 8.068	0.788	−0.958	−6.644, 4.728	0.741
Lag 2												
PM2.5	−1.394	−2.801, 0.014	0.052	−0.911	−2.340, 0.517	0.211	−0.730	−2.376, 0.916	0.385	−0.66	−1.39 , 0.08	0.079
PM10	−0.355	−0.974, 0.265	0.262	−1.144	−3.018, 0.731	0.232	−0.361	−1.411, 0.688	0.500	−0.137	−0.68, 0.407	0.622
NO2	−2.349	−4.277, −0.420	0.017*	−1.144	−3.018, 0.731	0.232	−1.038	−2.375, 0.300	0.129	0.625	−0.548, 1.797	0.296
CO	−99.308	−183.504, −15.112	0.021*	−59.880	−137.624, 17.864	0.131	−26.504	−74.586, 21.578	0.280	29.620	−15.303, 74.543	0.196
O3	0.994	−0.064, 2.052	0.066	0.087	−0.808, 0.982	0.849	0.261	−0.813, 1.344	0.634	−0.092	−0.852, 0.669	0.813
SO2	−0.148	−12.202, 11.906	0.981	−3.151	−11.788, 5.486	0.475	−2.405	−9.104, 4.293	0.482	−6.088	−12.162, −0.015	0.049*

Lag 0 : Same day; Lag 1 : 1 day before; Lag 2 : 2 day beforeModels were adjusted for age, gender, body mass index, environmental tobacco smoke, family income, temperature, and relative humidity.*$p < 0.05$.

While PM10, NO_2, CO, and SO_2 were associated with lower levels of PEF in the lag 0 model, PM2.5 was only associated with lower levels of PEF in the lag 2 model in the follow-up period. We found that personal exposure to air pollutants reduced lung function in single- and two-way air pollutant models. The association between air pollutants and respiratory function has been explored by several studies (Kim et al., 2021a; Gehring et al., 2020). Although previous studies also explored other respiratory function parameters such as FEV_1 and FVC, and we only explored PEF, our results were akin to most previous findings (Kim et al., 2021a), (Gehring et al., 2020), (Ye et al., 2021). Such findings may be explained by the oxidative stress produced after exposure to pollutants, which may lead to airway hyper-reactivity and lung injury (Ghio et al., 2012). Alternatively, a possible explanation is that these air pollutants may promote Th2 inflammation (Bleck et al., 2006).

Interestingly, a research group in the UK used a small portable sensor to capture personal air pollutant levels and investigated the association between personal air pollution and respiratory parameters (PEF, FENO, FEV_1, and FVC), but they did not find an association. Although this study also used a personal approach to collect exposure data, the results contradict ours. This may be because of the different methods used for collecting individual measurements. We used a smartphone app linked to the closest air pollution station to obtain the air pollutant levels. However, this study used a sensor to directly capture the sample total oxidants in the ambient air once per minute (Chambers et al., 2018). Future research may explore the different sensitivity and specificity of air pollutants measurements between other methods.

We found evidence that mite sensitization modified the association between air pollutants and PEF levels, with stronger associations in those sensitized to mites during the follow-up period. Our analysis found that mite sensitization modified the associations between air pollutants and respiratory function. This finding further proves the hypothesis of our previous report that exposure to PM2.5 and mite allergens had a synergistic effect on the development of asthma (Wang et al., 2016; Wang, 2013)]. In another study in Puerto Rico, the authors observed a non-significant increase trend of asthma in those who lived closest to the major roads and were exposed to mite sensitization (Stevens et al., 2020). This may be because air pollutants and mite sensitization are related to Th2 inflammation, inducing airway inflammation and airway modeling (Bouazza et al., 2018; Hsu et al., 2020).

Regarding season and PEF, we found that seasons may modify the effect of specific air pollutants on PEF. In the single air pollutant models, concentrations of CO were associated with PEF only in children who were sensitized to the mite. In Taiwan, spring is one of the seasons which reflect high pollution concentration. It is linear with findings, and the natural phenomenon of frequent sand and dust storms transfer from East Asia during the Spring season [28]. It resulted in the additional accumulation of air pollutants and exaggerated PEF in children.

There are some limitations to this study. Firstly, we did not adjust some potential confounding factors, such as seasonality and personal behavior, in our regression model, which may have biased our results. However, we have put average temperature and relative humidity in our model. We have a large sample size. Moreover, repeated measures of air pollutants and emergency room visits were assessed using a generalized estimating equations model with a Poisson distribution to increase power and decrease in this longitudinal study.

The strength of this study is that mobile phone apps are able to collect real-time and precise air pollutant levels on a population scale which allows for capturing the day-to-day dynamics change of these particles. In addition, it easily records the real-time asthma condition by peak flow measurement from smartphone apps. This immediate collection of particles using a smartphone app and its effects on asthmatic patients is also a cost-effective method for collecting data. It overcomes the potential data collection barrier during a pandemic such as COVID-19. By smartphone app internet of Thing platform, data from sensor devices can be shared. This can help physicians to deliver notifications to patients and families about their health condition and to produce health statistics and predictive analysis.

5. Conclusion

In conclusion, smartphone apps may help to collect personal ambient air pollution data. Using our developed smartphone apps, we found that NO_2, CO, and PM10 were higher during the pre-and post-COVID-19 lockdowns than during the lockdown. Exposure to air pollution increased PEF variability in asthma subjects during the monitoring period. Lastly, the association was stronger in children who were sensitized to mites. It is essential to inform asthmatic children about the risk of exposure to high air pollution levels. Ongoing monitoring of air pollution levels is essential for children's health, and sending in time reminders of avoidance of unhealthy air quality may be performed by smartphones to protect children's lung health and acute asthma attacks. The digitally controlled air monitoring and management system and peak flow monitoring may help fight against asthma during and even beyond the pandemic. This smart healthcare model can be the new normal for asthma hybrid care.

Author contributions

I-J.W. designed the studies; **P-Y. L**. performed the analysis; **B-F H** analyzed the data; **P-Y. L. and W.-I. J.** wrote the article. **J-Y W and RP** reviewed and edited, **I.-J. W**. supervision, funding acquisition, and project administration. All authors have read and agreed to the published version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114186.

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"Residential greenness, gestational diabetes mellitus (GDM) and microbiome diversity during pregnancy"

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ABSTRACT

Background: Gestational diabetes mellitus (GDM) is associated with reduced gut microbiota richness that was also reported to differ significantly between those living in rural compared to urban environments. Therefore, our aim was to examine the associations between greenness and maternal blood glucose levels and GDM, with microbiome diversity as a possible mediator in these associations.

Methods: Pregnant women were recruited between January 2016 and October 2017. Residential greenness was evaluated as mean Normalized Difference Vegetation Index (NDVI) within 100, 300 and 500 m buffers surrounding each maternal residential address. Maternal glucose levels were measured at 24–28 weeks of gestation and GDM was diagnosed. We estimated the associations between greenness and glucose levels and GDM using generalized linear models, adjusting for socioeconomic status and season at last menstrual period. Using causal mediation analysis, the mediation effects of four different indices of microbiome alpha diversity in first trimester stool and saliva samples were assessed.

Results: Of 269 pregnant women, 27 participants (10.04%) were diagnosed with GDM. Although not statistically significant, adjusted exposure to medium tertile levels of mean NDVI at 300 m buffer had lower odds of GDM (OR = 0.45, 95% CI: 0.16, 1.26, p = 0.13) and decreased change in mean glucose levels ($\beta = -6.28$, 95% CI: 14.91, 2.24, p = 0.15) compared to the lowest tertile levels of mean NDVI. Mixed results were observed at 100 and 500 m buffers, and when comparing highest tertile levels to lowest. No mediation effect of first trimester microbiome on the association between residential greenness and GDM was observed, and a small, possibly incidental, mediation effect on glucose levels was observed.

Conclusion: Our study suggests possible associations between residential greenness and glucose intolerance and risk of GDM, though without sufficient evidence. Microbiome in the first trimester, while involved in GDM etiology, is not a mediator in these associations. Future studies in larger populations should further examine these associations.

Abbreviations: Directed Acyclic Graph, (DAG); Gestational Diabetes Mellitus, (GDM); Glucose Challenge Test, (GCT); Last Menstrual Period, (LMP); Normalized Difference Vegetation Index, (NDVI); Socioeconomic Status, (SES).

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1. Introduction

As urbanization advances, people live in less green environments. Modern urban life is linked to chronic stress, lack of physical activity and exposure to environmental risk factors. In 2016, the World Health Organization published a summary of dozens of epidemiological studies supporting the findings that living near urban green spaces, such as parks, playgrounds and natural vegetation, promotes mental and physical health and reduces morbidity and mortality among city dwellers (World Health Organization, 2016).

Multiple pathways may mediate the associations between green spaces and health, and several have been suggested. The relatively new biodiversity hypothesis links the reduction in green spaces to reduced host microbial biodiversity, which in turn results in adverse effects on health (Haahtela et al., 2013; Hough, 2014). A recent double blinded placebo-controlled study amongst 26 urban-living children, demonstrated that continuous exposure to microbially rich environments can shift host microbiome composition and regulatory immune responses, supporting the biodiversity hypothesis (Roslund et al., 2022).

Gestational diabetes mellitus (GDM) is defined as any glucose intolerance with the first onset or diagnosis during pregnancy. Although previous studies demonstrated associations between greenness and decreased incidence of type 2 diabetes mellitus, as reported and summarized in a recent meta-analysis (Twohig-Bennett and Jones, 2018), we could only identify a handful of studies regarding GDM incidence and greenness. These studies demonstrated reduced risk of GDM incidence with exposure to higher Normalized Difference Vegetation Index (NDVI) values, a measure of vegetation density and greenness (Choe et al., 2018; Liao et al., 2019; Qu et al., 2020; Yu et al., 2023).

GDM prevalence is affected by a variety of risk factors, such as overweight or obesity, ethnicity, socioeconomic status (SES), diet, genetic polymorphisms, advanced maternal age, and family or personal history of insulin resistance diseases (Zhu and Zhang, 2016). Another factor suggested to affect GDM is the gut microbiome, with reduced richness in GDM pregnancies and changes in relative abundance of certain species compared to healthy pregnant women (Calatayud et al., 2019; Pinto et al., 2023; Turjeman et al., 2021). Gut microbiota also differs significantly in abundance and richness between hosts living in rural and urban environments (Bowyer et al., 2022). Thus, it is possible that GDM occurrence is affected by greenness and biodiversity, through their effect on the gut microbiota richness.

Our hypothesis is that residential greenness is inversely associated with GDM incidence in pregnant women, possibly by affecting gut microbiome richness. Our study aimed to evaluate the association between greenness and GDM risk. The results of this research could have implications for public health by identifying beneficial associations that can be the first step towards developing specific recommendations for greenness exposure standards during pregnancy.

2. Methods

2.1. Study population

Data on the gut microbiome and GDM status was obtained from an Israeli pregnancy cohort, as described by Pinto et al. (2023). Briefly, pregnant women (N = 400) were recruited at three Israeli health care clinics between January 2016 and October 2017 and were followed throughout pregnancy and delivery. Inclusion criteria included healthy pregnant women, aged 18–40, at 11–13 weeks gestational age. Exclusion criteria included non-spontaneous pregnancies, diagnosis of type 1 or type 2 diabetes mellitus before pregnancy, antibiotics usage 3 months prior to recruitment and multiple gestation pregnancies. For our main analysis, we focused on women living in urban areas as defined by the Israeli Ministry of Interior, who met the inclusion criteria and had available data on greenness exposure using the high resolution RapidEye satellite system (N = 269) (Supplemental Fig. 1).

2.2. Residential greenness

Participants' residential addresses (N = 269) during first trimester (provided at recruitment) were geocoded using the Google geocoding server, at the home level when possible (N = 264, 98.14%) or at the settlement level (N = 5, 1.86%), as previously described (Agay-Shay et al., 2013a).

To determine the exposure to residential greenness, we used NDVI derived from RapidEye satellite data, at 5 m × 5 m resolution (Planet, 2014). NDVI is a greenness indicator based on land surface reflectance of visible (red) and near infra-red parts of the spectrum (Weier and Herring, 2000), ranging between −1 and 1, with higher values indicating more greenness and negative values indicating non-biomass (water, cloud, snow). Residential greenness was calculated as the mean NDVI at 100, 300 and 500 m buffers around each maternal residential address (N = 269). NDVI values were generated from RapidEye data by using images obtained on October 24th 2014, after the dry season, that were available for our study region. During the dry season only the evergreen canopies of woodland trees, shrubs and irrigated vegetation contribute to NDVI (Helman, 2018). We selected this month (driest, after the summer) to maximize NDVI contrast and to evaluate effects from exposures to evergreen vegetation including irrigated vegetation. Negative values were converted to zero as previously recommended (Martín-Sotoca et al., 2018; Weier and Herring, 2000). Mean NDVI values were categorized into tertiles of exposure defined as low, medium and high levels of NDVI for each buffer.

2.3. Glucose levels and gestational diabetes mellitus

The primary outcome, GDM, was generally assessed for every participant at 24–28 weeks gestational age using a 50 g Glucose Challenge Test (GCT), regularly performed as a screening test in Israel. Blood glucose levels were measured 1 h after glucose administration, and GDM was defined when glucose levels were above 200 mg/dL (Yogev et al., 2009). Participants demonstrating glucose levels between 140 and 200 mg/dL were required to perform a 100 g oral glucose tolerance test (Committee on Practice Bulletins—Obstetrics, 2018). Participants who presented risk factors for GDM such as large for gestational age (infants larger than 10th percentile for gestational age and sex), GDM in previous pregnancies or polyhydramnios, performed the oral glucose tolerance test instead of GCT (N = 28). During the oral glucose tolerance test, blood glucose levels were measured just before glucose administration whilst fasting, and 1 h, 2 h and 3 h after glucose administration. GDM was defined if any of the following conditions existed: 1) fasting glucose level of 95 mg/dL or above; 2) 1 h blood glucose level of 180 mg/dL or above; 3) 2 h blood glucose level of 155 mg/dL or above; and 4) 3 h blood glucose level of 140 mg/dL or above (Committee on Practice Bulletins—Obstetrics, 2018). Diagnosis using one abnormal value instead of two abnormal values is based on Yogev et al. (2009), showing adverse pregnancy outcomes in pregnant women with one abnormal value, and according to the American College of Obstetricians and Gynecologists' recommendations (Committee on Practice Bulletins—Obstetrics, 2018). Glucose levels measured during GCT were used as a continuous outcome (mg/dL) for 242 participants with available data.

2.4. Microbiome diversity

Microbiome diversity indices were considered as possible mediators in the associations between residential greenness and GDM and glucose levels at GCT. First trimester microbiome diversity was assessed using stool and saliva samples that were collected during recruitment as described previously (Pinto et al., 2023). Briefly, sample collection and processing were in accordance with the National Institutes of Health (NIH) Human Microbiome Project standards (Human Microbiome Project Consortium, 2012). Samples were analyzed using 16S rRNA gene

sequencing to identify and quantify bacterial species composition of the gut microbiome. Bacterial DNA was extracted, amplified and sequenced using Illumina adapter sequences which targeted the highly conserved V4 region of the 16S gene (Goodrich et al., 2014). Alpha (within sample) diversity was calculated using four different indices, and values were considered as mediators: 1) Observed species which measures number of the species (amplicon sequence variants in this case) and represents richness (Wagner et al., 2018); 2) Shannon's diversity index which measures richness and evenness (overall diversity) (Wagner et al., 2018); 3) Faith's phylogenetic diversity which measures richness considering phylogenetic distance (Chao et al., 2016); and 4) Pielou's evenness index which measures equity in species abundance (Finotello et al., 2018).

2.5. Statistical analysis

Linearity of the associations between greenness (exposure) and outcomes was assessed using generalized additive models. Because nonlinear associations were observed (Figs. 1–2), we analyzed exposure variables in categories defined by tertiles of exposure, which allow interpretation relative to 'low', 'medium' and 'high' values.

Directed Acyclic Graphs (DAGs) were drawn (Supplemental Figs. 2 and 3), and were analyzed using DAGitty (Shrier and Platt, 2008; Textor et al., 2016). In the DAGs, we included known covariates that could potentially confound the associations between exposure-outcome, mediator-outcome and exposure-mediator.

The covariates evaluated were: maternal age, gravidity, parity, body mass index, family medical history, comorbidities, chronic medication treatment, smoking, GDM in previous pregnancies, education and ethnicity. Additional factors such as sleeping habits, stress levels, physical activity, diet type and eating habits (calorie and carbohydrate consumption) were also evaluated. Data were collected using self-report questionnaires (Pinto et al., 2023), validated stress questionnaires (Cohen et al., 1983), food diaries, and dietitian interviews. Fasting plasma glucose levels, insulin levels, leptin levels and inflammatory markers levels such as several interleukins, granulocyte-macrophage colony-stimulating factor, interferon gamma and tumor necrosis factor alpha were measured using blood samples collected during recruitment and were additionally considered as confounders or mediators. SES, a possible confounder in the association between residential greenness and GDM (Qu et al., 2020), was evaluated based on the residential address provided at recruitment and the Israel Central Bureau of Statistics' 2017th database (Israel Central Bureau of Statistics, 2017). In addition, season during early pregnancy, which is an important possible confounder (Preston et al., 2020), was defined based on the last menstrual period (LMP) date reported by the participants at recruitment, as hot season (LMP dates between March 31st through September 22nd) and cold season (LMP dates between September 23rd through March 30th) (Agay-Shay et al., 2013b; Alpert et al., 2004).

Based on the constructed DAGs, we selected one minimal set of variables needed to block all biasing paths for all models, and we adjusted the models for the following covariates: SES and seasonality at LMP (Supplemental Figs. 2 and 3).

Mean and standard deviation were calculated for continuous variables and percentages of every category were calculated for categorical variables. Descriptive analyses were performed between the covariates and GDM categories and NDVI tertiles. According to the distribution type, Student's T-tests and Mann-Whitney tests were performed for continuous variables for GDM categories, and analysis of variance tests and Kruskal-Wallis tests were performed for the three NDVI tertiles. Chi-squared tests were performed for categorical variables. Univariate analysis was performed in order to examine the association between exposure-outcome (NDVI-GDM, and NDVI-glucose levels).

Generalized linear models with normal distribution and identity link were used to evaluate associations between greenness and changes in mean glucose levels (beta coefficients with 95% confidence intervals). Generalized linear models with binary family log link function were used to evaluate the changes in odds ratios and 95% confidence intervals of GDM. Crude and adjusted models were evaluated.

Mediation analysis was conducted using four alpha diversity indices of first trimester microbiome to evaluate the percentage of the association between residential greenness at 300 m buffer and GDM and glucose levels at GCT, explained by each of the mediators (and 95% CI), estimated via the non-parametric bootstrap method with 500 simulations (Lapointe-Shaw et al., 2018). Causal mediation analysis was based on the following assumptions: 1) no unmeasured confounding of the exposure-outcome effect; 2) no unmeasured confounding of the mediator-outcome effect; 3) no unmeasured confounding of the

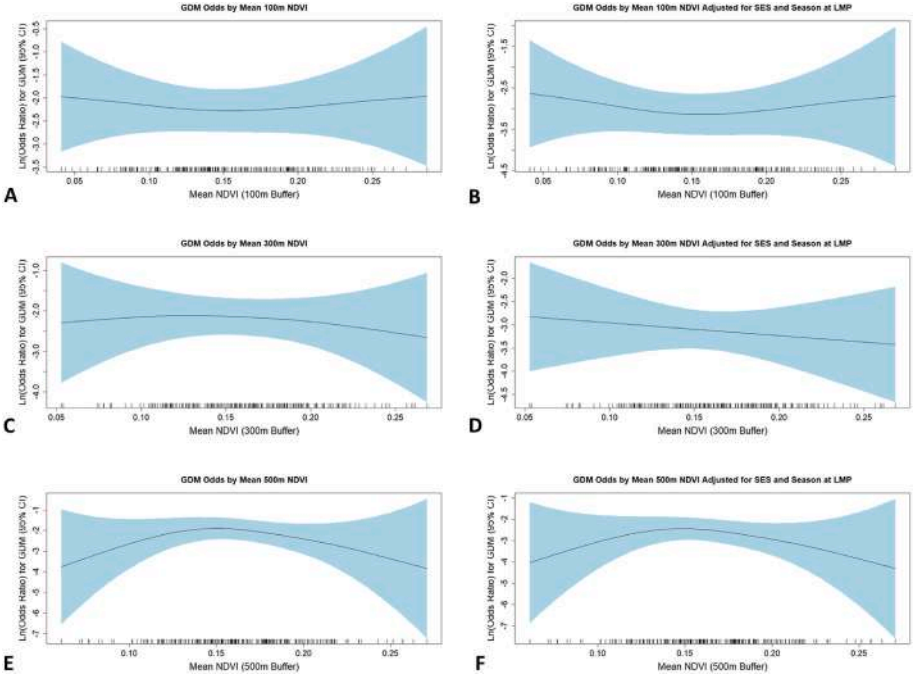

Fig. 1. Unadjusted and adjusted associations between GDM odds and mean NDVI at 100, 300 and 500 m buffers derived from RapidEye data for the urban population (N = 269). A and B present the 100 m NDVI buffer, C and D the 300 m buffer, and E and F the 500 m buffer. Crude models for the different buffers are presented in A, C and E, and adjusted models are in B, D and F. Generalized additive models were used. Models were adjusted for SES and season at LMP. Component smooths are shown with 95% confidence intervals that include the uncertainty about the overall mean (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

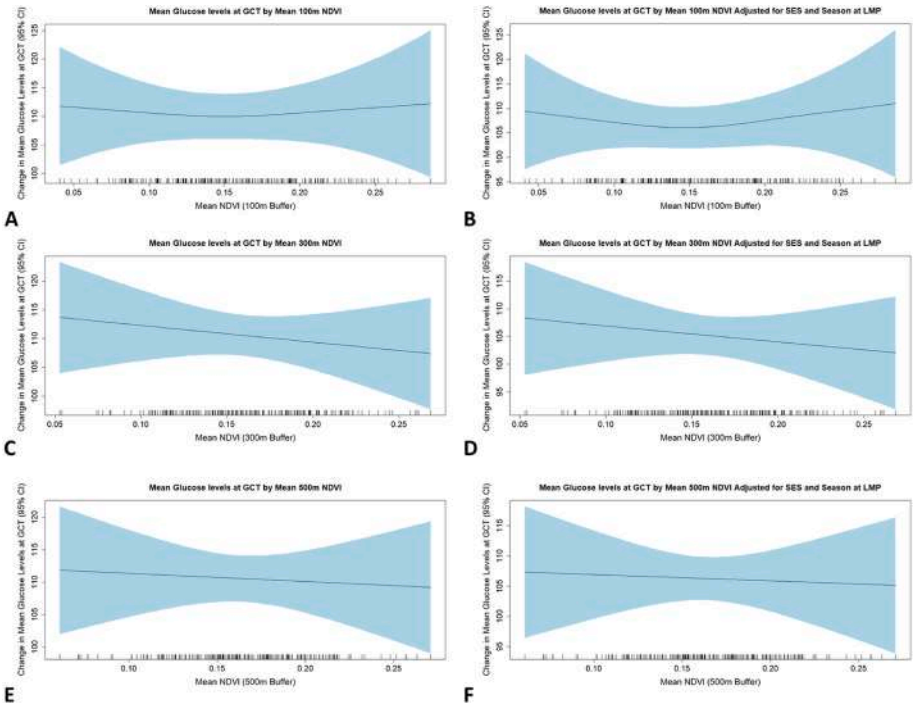

Fig. 2. Unadjusted and adjusted associations between changes in mean glucose levels at GCT and mean NDVI at 100, 300 and 500 m buffers derived from RapidEye data for urban population (N = 242). A and B present the 100 m NDVI buffer, C and D the 300 m buffer, and E and F the 500 m buffer. Crude models for the different buffers are presented are in A, C and E, and adjusted models in B, D and F. Generalized additive models were used. Models were adjusted for SES and season at LMP. Component smooths are shown with 95% confidence intervals that include the uncertainty about the overall mean (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

exposure-mediator effect; and 4) no confounder of the mediator-outcome effect that are affected by the exposure (Rijnhart et al., 2021).

When assumptions of the mediation analysis are met, the proportion mediated describes the proportion of the effect of the exposure (NDVI tertile) on the outcome (GDM/glucose levels at GCT) that goes through the mediator (first trimester microbiome diversity) (Lee et al., 2021). It is calculated by dividing the average causal mediation effect by the total effect, multiplied by 100 to generate percentages. The proportion mediated could be negative if the direct and indirect effects have opposite signs. It is calculated as the sum of the proportion mediated for each mediator at a time. Proportion mediated higher than 1 (larger than 100%) indicates one of the following: 1) there are other mediators with a negative proportion mediated; 2) the mediators affect one another; 3) there are interactions between the effects of the mediators on the outcome (VanderWeele and Vansteelandt, 2014).

A priori statistical significance was defined as $p < 0.05$. R (version 4.1.3; R Development Core Team), RStudio (version 1.3.1073; RStudio Team) and SPSS 25 (version 25.0; IBM SPSS Statistics for Windows) statistical softwares were used for all analyses described above.

2.6. Further analyses

Several sensitivity analyses were performed using Sentinel-2 satellite data and the entire population including non-urban living participants (N = 354). Methods are presented in supplements (Supplemental Methods 1).

2.7. Ethical approval

The study protocol was approved by the Helsinki committee of Rabin Medical Center, Beilinson Campus (No. 0263-15-RMC). Individual written informed consents were provided from all participants at recruitment, in accordance with Clalit's institutional review board approval (No. 0135-15-COM).

3. Results

3.1. Descriptive statistics

Selected maternal sociodemographic and lifestyle characteristics, as well as exposure variables, are presented in Table 1. Out of the study population, 27 participants (10.04%) were diagnosed with GDM. Participants in the GDM group were more likely to be multiparous, had a pre-pregnancy body mass index categorized as overweight or obese, had more GDM-related family history and had more GDM diagnoses in past pregnancies. Moreover, participants in the GDM group presented higher fasting glucose levels during early pregnancy (6–8 weeks of gestation) and were more likely to be smokers (all p values < 0.05). However, assessment of the GDM group compared to the non-GDM group revealed no statistically significant differences in other characteristics examined, including mean NDVI values, seasonality at LMP, SES, medication treatment (although medication types were significantly different), education or ethnicity. These results suggest that, excluding a few variables that were previously known as risk factors for GDM (such as multiparity, weight, and past GDM diagnosis), the study groups are very similar and thus comparable.

Supplemental Table 1 presents the same sociodemographic and lifestyle characteristics, characterized by mean NDVI exposure tertiles at 300 m buffer. There is a statistically significant difference between SES in the different exposure tertiles, with higher mean NDVI values corresponding to higher SES.

3.1.1. GDM diagnosis and mediators

Supplemental Table 2 presents first trimester covariates that were considered as possible mediators in urban study population (N = 269), characterized by GDM incidence. GDM diagnosis-related variables are also presented. As expected, significant differences were observed in first trimester body mass index, GCT and oral glucose tolerance test values, with higher values in the GDM group. We did not observe significant differences in stress levels, sleeping habits, calories and carbohydrates consumption, insulin and leptin levels, alpha diversity indices in saliva samples and most of the indices in stool samples. There was only a statistically significant difference in stool's observed species

Table 1
Study population (urban) characteristics of GDM diagnosed participants (n = 27) compared to healthy non-GDM participants (n = 242). Data reported as mean (SD) or as count (%).

	GDM group (N = 27)	Non-GDM group (N = 242)	Total population (N = 269)	*p* values[a]
Age (Years), Mean (SD)	32.82 (3.93)	31.23 (4.30)	31.39 (4.28)	0.06[b]
Age Category (Years)				0.19[c]
under 25	0 (0.0%)	15 (6.2%)	15 (5.6%)	
25-34	17 (63.0%)	168 (69.4%)	185 (68.8%)	
35+	10 (37.0%)	59 (24.4%)	69 (25.7%)	
Maternal Gravidity, Mean (SD)	2.59 (1.22)	2.33 (1.36)	2.36 (1.35)	0.15[d]
Maternal Gravidity Category				0.05[c]
1	8 (29.6%)	78 (32.2%)	86 (32.0%)	
2	2 (7.4%)	70 (28.9%)	72 (26.8%)	
3	11 (40.7%)	58 (24.0%)	69 (25.7%)	
4+	6 (22.2%)	36 (14.9%)	42 (15.6%)	
Maternal Parity, Mean (SD)	1.15 (1.03)	0.90 (0.91)	0.92 (0.92)	0.20[d]
Parity Category				**0.03**[c]
Nulliparous	10 (37.0%)	95 (39.3%)	105 (39.0%)	
Primiparous	5 (18.5%)	92 (38.0%)	97 (36.1%)	
Multiparous +	12 (44.4%)	55 (22.7%)	67 (24.9%)	
Season At LMP [e]				0.38[c]
Hot Season	9 (33.3%)	102 (42.1%)	111 (41.3%)	
Cold Season	18 (66.7%)	140 (57.9%)	158 (58.7%)	
Height (m), Mean (SD)	1.63 (0.07)	1.64 (0.06)	1.64 (0.06)	0.66[b]
Missing	0	20	20	
Pre-Pregnancy BMI (kg/m^2), Mean (SD)	28.03 (7.44)	22.30 (3.79)	22.92 (4.67)	< **0.001**[d]
Pre-Pregnancy BMI Category [f]				< **0.001**[c]
Missing	0	20 (8.3%)	20 (7.4%)	
Underweight	2 (7.4%)	32 (13.2%)	34 (12.6%)	
Normal	8 (29.6%)	147 (60.7%)	155 (57.6%)	
Overweight	8 (29.6%)	32 (13.2%)	40 (14.9%)	
Obesity	9 (33.3%)	11 (4.5%)	20 (7.4%)	
GDM Related Family History				**0.046**[c]
Missing	4 (14.8%)	81 (33.5%)	85 (31.6%)	
GDM	0 (0.0%)	1 (0.4%)	1 (0.4%)	
Diabetes	7 (25.9%)	20 (8.3%)	27 (10.0%)	
Hypertension	0 (0.0%)	15 (6.2%)	15 (5.6%)	
Diabetes and Hypertension	2 (7.4%)	4 (1.7%)	6 (2.2%)	
No	14 (51.9%)	121 (50.0%)	135 (50.2%)	
Previous GDM Category				**0.01**[c]
Yes	2 (7.4%)	2 (0.8%)	4 (1.5%)	
No	25 (92.6%)	240 (99.2%)	265 (98.5%)	
GDM Related Comorbidities [g]				< **0.001**[c]
Missing	0 (0.0%)	6 (2.5%)	6 (2.2%)	
Yes	9 (33.3%)	14 (5.8%)	23 (8.6%)	
Multiple GDM Related Comorbidities	0 (0.0%)	1 (0.4%)	1 (0.4%)	
Other Comorbidities	5 (18.5%)	74 (30.6%)	79 (29.4%)	
No Comorbidities	13 (48.1%)	147 (60.7%)	160 (59.5%)	
Medication Treatment				0.14[c]
Missing	0 (0.0%)	7 (2.9%)	7 (2.6%)	
Yes	9 (33.3%)	49 (20.3%)	58 (21.6%)	
No	18 (66.7%)	186 (76.9%)	204 (75.8%)	
Antibiotics Treatment During Pregnancy				0.28[c]
Missing	0 (0.0%)	1 (0.4%)	1 (0.4%)	
Yes	0 (0.0%)	10 (4.1%)	10 (3.7%)	
No	27 (100.0%)	231 (95.5%)	258 (95.9%)	
Antibiotics Treatment During First Trimester				0.89[c]
Missing	0 (0.0%)	1 (0.4%)	1 (0.4%)	
Yes	0 (0.0%)	1 (0.4%)	1 (0.4%)	
No	27 (100.0%)	239 (98.8%)	266 (98.9%)	
Possible	0 (0.0%)	1 (0.4%)	1 (0.4%)	
Early Pregnancy Fasting Glucose (mg/dL), Mean (SD)	89.19 (12.05)	83.21 (7.22)	83.85 (8.06)	**0.02**[d]
Fasting Glucose Level Category [h]				**0.01**[c]
Missing	0 (0.0%)	7 (2.9%)	7 (2.6%)	
Not Tested	0 (0.0%)	8 (3.2%)	8 (3.0%)	
Normal	21 (77.8%)	212 (87.6%)	233 (86.6%)	
High and Very High	6 (22.2%)	15 (6.2%)	21 (7.8%)	
Smoking Status				**0.02**[c]
Missing	1 (3.7%)	16 (6.6%)	17 (6.3%)	
Yes	3 (11.1%)	21 (8.7%)	24 (8.9%)	
No	18 (66.7%)	194 (80.2%)	212 (78.8%)	
Past Smoking	5 (18.5%)	11 (4.5%)	16 (6.0%)	
Education (Years), Mean (SD)	14.6 (2.4)	15.3 (2.4)	15.2 (2.4)	0.22[d]
Education Category				0.44[c]
Missing	7 (25.9%)	71 (29.3%)	78 (28/9%)	
High School (Up to 12 Years)	7 (25.9%)	42 (17.4%)	49 (18.2%)	
First Degree (13–16 Years)	9 (33.3%)	74 (30.6%)	83 (30.9%)	
Second Degree or Above (17+ Years)	4 (14.8%)	55 (22.7%)	59 (21.9%)	
Ethnicity				0.27[c]
Missing	5 (18.5%)	70 (28.9%)	75 (27.9%)	
Ashkenazi	13 (48.1%)	64 (26.5%)	77 (28.6%)	
Sephardic	5 (18.5%)	57 (23.6%)	62 (23.0%)	
At Risk (Arab, Yemen, Ethiopian)	1 (3.7%)	14 (5.8%)	15 (5.6%)	
Other	3 (11.1%)	37 (15.3%)	40 (14.9%)	
Socioeconomic Status Level, Mean (SD)	7.93 (1.36)	7.76 (1.82)	7.78 (1.78)	0.99[d]
Socioeconomic Status Category				0.91[c]
Low Socioeconomic Status (1–7)	9 (33.3%)	78 (32.2%)	87 (32.3%)	
High Socioeconomic Status (8+)	18 (66.7%)	164 (67.8%)	182 (67.7%)	
Mean NDVI Level At 100m Buffer (RapidEye), Mean (SD)	0.151 (0.057)	0.152 (0.050)	0.152 (0.050)	0.97[b]
Mean NDVI Level Category At 100m Buffer (RapidEye)				0.41[c]
Low Mean NDVI Level	10 (37.0%)	80 (33.1%)	90 (33.5%)	
Medium Mean NDVI Level	6 (22.2%)	84 (34.7%)	90 (33.5%)	
High Mean NDVI Level	11 (40.7%)	78 (32.2%)	89 (33.1%)	
Mean NDVI Level At 300m Buffer (RapidEye), Mean (SD)	0.16 (0.04)	0.16 (0.04)	0.16 (0.042)	0.70[b]
Mean NDVI Level Category At 300m Buffer (RapidEye)				0.33[c]
Low Mean NDVI Level	12 (44.4%)	78 (32.2%)	90 (33.5%)	
Medium Mean NDVI Level	6 (22.2%)	84 (34.7%)	90 (33.5%)	
High Mean NDVI Level	9 (33.3%)	80 (33.1%)	89 (33.1%)	

(*continued on next page*)

Table 1 (*continued*)

	GDM group (N = 27)	Non-GDM group (N = 242)	Total population (N = 269)	*p* values[a]
Mean NDVI Level At 500m Buffer (RapidEye), Mean (SD)	0.16 (0.03)	0.16 (0.040)	0.16 (0.04)	0.73[b]
Mean NDVI Level Category At 500m Buffer (RapidEye)				0.34[c]
Low Mean NDVI Level	9 (33.3%)	81 (33.5%)	90 (33.5%)	
Medium Mean NDVI Level	12 (44.4%)	78 (32.2%)	90 (33.5%)	
High Mean NDVI Level	6 (22.2%)	83 (34.3%)	89 (33.1%)	

BMI- Body Mass Index; GDM- Gestational Diabetes Mellitus; LMP- Last Menstrual Period; NDVI- Normalized Difference Vegetation Index; SD- Standard Deviation; Categorical variables are presented as count (%) and continuous variables as mean (SD).
Significant differences (*p* value < 0.05) are marked in bold. Missing reported as count (%).
[a] *p* values were calculated according to variables' type and distribution.
[b] Student's T test for normal distributed continuous variables.
[c] Chi-squared Test for categorical variables.
[d] Mann-Whitney Test for non-normal distributed continuous variables.
[e] Hot season between March 31st through September 22nd, cold season between September 23rd through March 30th.
[f] BMI category- underweight <18.5 kg/m^2, normal 18.5–24.9 kg/m^2, overweight 25–29.9 kg/m^2, obesity >30 kg/m^2.
[g] GDM related comorbidities such as past or current preeclampsia, obesity, polycystic ovary syndrome, chronic or gestational hypertension and hyperemesis.
[h] Fasting glucose level- normal <95 mg/dL, high 95–126 mg/dL, very high >126 mg/dL.

index (Supplemental Table 2), which was lower in the GDM group. Most of the cytokines measured were also similar between the two groups, except for interleukin-6 and granulocyte-macrophage colony-stimulating factor which were significantly higher in the GDM group.

Supplemental Table 1 also presents the same covariates and outcome assessments, characterized by NDVI tertiles derived from RapidEye at 300 m buffer. Most covariates did not demonstrate significant differences between the tertiles, with the exception of higher body mass index at first trimester and lower levels of interferon gamma at the lowest tertile of NDVI exposure, and lower values of Shannon's diversity index (stool samples) at the highest tertile of exposure (Supplemental Table 1).

Sensitivity analysis using the entire population including non-urban living participants (N = 354) demonstrated similar sociodemographic characteristics (Supplemental Table 3). GDM diagnosis-related variables and possible mediators were vastly the same, with the exception of observed species index that was similar between the groups, and interleukin-8 concentration which was significantly higher in the GDM group compared to non-GDM.

3.2. Associations between residential greenness and GDM

We observed no statistically significant associations between residential greenness and GDM odds. Although statistical analysis demonstrated weak evidences, women who were exposed to medium tertile levels of mean NDVI at 300 m buffer, had lower GDM odds (OR = 0.46, 95% CI: 0.17, 1.29, p = 0.14) compared to women exposed to lowest tertile levels of mean NDVI (Fig. 3, detailed in Supplemental Table 4). Although also not significant, medium tertile levels of mean NDVI demonstrated lower GDM odds at 100 m buffer and higher odds at 500 m buffer, compared to lowest tertile levels of NDVI (*p* values = 0.30 and 0.49, respectively). Comparing the highest tertile levels to lowest tertile demonstrated higher GDM odds at 100 m buffer and lower odds at 500 m buffer, also without statistical significance (*p* values = 0.80 and 0.43, respectively).

A similar pattern of association was observed after adjusting for SES and season at LMP (OR = 0.45, 95% CI: 0.16, 1.26, p = 0.13) with lower GDM odds in the medium NDVI tertile levels at 300 m buffer compared to the lowest tertile (Fig. 3, detailed in Supplemental Table 4). Adjusted models for 100 and 500 m buffers also presented very similar results, yet again without statistically significant values (*p* values > 0.30).

Sensitivity analyses performed using Sentinel-2 data for urban population (Supplemental Table 4) and using both satellites for the entire population including non-urban living participants (Supplemental Table 5) demonstrated similar results.

3.3. Associations between residential greenness and glucose levels at GCT

For women with GCT measurements (N = 242) some beneficial associations were also observed, yet again without sufficient statistical significance (Fig. 4, detailed in Supplemental Table 6). The strongest effect estimate was observed for women exposed to medium tertile levels of mean NDVI at 300 m buffer, who demonstrated lower (though non-significant) mean glucose levels at GCT compared to the lowest tertile levels of mean NDVI (β = −5.82, 95% CI: 14.43, 2.79, p = 0.19). A stronger, yet still non-significant, effect estimate was observed when adjusting for season at LMP and SES (β = −6.28, 95% CI: 14.91, 2.34, p = 0.15). For the 100 m and 500 m buffers, no significant associations were observed, as indicated by the high *p* values.

Similar results were observed in the sensitivity analyses using Sentinal-2 data for the urban population (Supplemental Table 6), and using both satellites for the entire population including non-urban living participants (Supplemental Table 7).

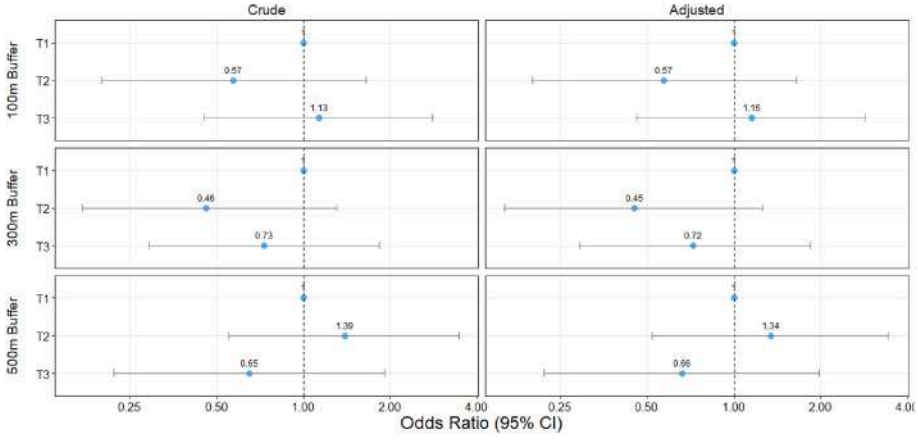

Fig. 3. Generalized Linear Models with binary family log link function evaluating the association between mean NDVI, abstracted from RapidEye data, and Odds Ratio for GDM in urban population (N = 269). Data is represented as crude and adjusted for SES and season at LMP at 100, 300 and 500 m buffers. Medium and highest tertiles were compared to lowest tertile of exposure. CI- Confidence interval; GDM- Gestational Diabetes Mellitus; NDVI- Normalized Difference Vegetation Index; T- tertiles of exposure; T1- reference tertile, low mean NDVI tertile; T2- medium mean NDVI tertile; T3- highest mean NDVI tertile; Values are detailed at Supplemental Table 4.

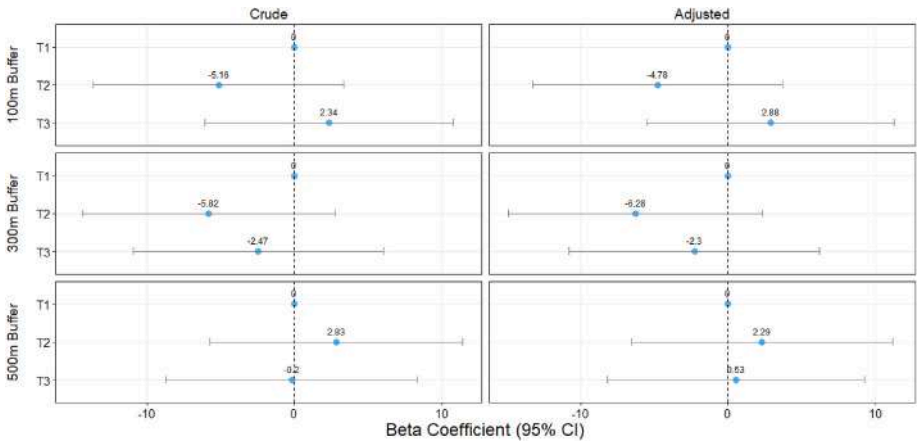

Fig. 4. Generalized linear models with normal distribution and identity link evaluating the association between mean NDVI, abstracted from RapidEye data, and change in mean glucose at GCT in urban population (N = 242). Data is represented as crude and adjusted for SES and season at LMP at 100, 300 and 500 m buffers. Medium and highest tertiles were compared to lowest tertile of exposure. CI- Confidence interval; GCT- Glucose Challenge Test; NDVI- Normalized Difference Vegetation Index; T- tertiles of exposure; T1- reference tertile, low mean NDVI tertile; T2- medium mean NDVI tertile; T3- highest mean NDVI tertile; Values are detailed at Supplemental Table 6.

3.4. Microbiome as a mediator

The results of the mediation analysis are reported in Supplemental Tables 8–11. The total effects of the mean NDVI tertiles at 300 m buffer on GDM (Supplemental Tables 8–9) and glucose levels at GCT (Supplemental Tables 10–11) are generally similar to the results reported in Supplemental Tables 4 and 6

3.4.1. Mediation on GDM

For those with microbiome data (N = 107 with saliva samples, N = 122 with stool samples), analyzing the possible mediation of microbiome on the crude association between NDVI and GDM odds did not reveal statistically significant change of the total effects in the highest and medium tertiles of mean NDVI at 300 m buffer compared to the lowest tertile (Supplemental Table 8). In each of the mediators inspected for stool and saliva samples, the direct effects were nearly the same as the total effects, hence there was practically no mediation effect of first trimester microbiome on the association between residential greenness and GDM. Similar patterns were observed in the adjusted models (Supplemental Table 9).

3.4.2. Mediation on glucose levels at GCT

Generally, for those with both GCT and microbiome data (N = 95 and 109 with saliva and stool samples in the medium tertile levels, and N = 98 and 113 with saliva and stool samples in the highest tertile levels, respectively), mediation of first trimester microbiome in the association between NDVI and glucose levels at GCT was not observed. However, although non-significant, in the crude model of the highest versus lowest tertile of mean NDVI at 300 m buffer for Faith's phylogenetic diversity index (stool sample), the direct effect of NDVI on the change in mean glucose levels had beta coefficient of −7.20 (95% CI: 17.39, 3.46, p = 0.23), whereas the total effect comprising the mediation effect had beta coefficient of −8.32 (95% CI: 19.31, 2.29, p = 0.12), with proportion mediated of 13.5% (95% CI: 42.31%,210.60%, p = 0.27) as presented in Supplemental Table 10. A similar pattern for the same index was observed in the adjusted model for SES and season at LMP (Supplemental Table 11).

4. Discussion

Although the associations we found were not statistically significant, lower odds of GDM and lower mean glucose levels were observed in women exposed to medium levels of residential greenness compared to low levels (mean NDVI at 300 m buffer). This pattern of association was maintained (though still not significant) after adjusting for SES and seasonality at LMP. To the best of our knowledge, this is the first study that evaluated mediation through first trimester microbiome, using four alpha diversity indices in stool and saliva samples. The mediation analysis did not reveal significant evidence of mediation of first trimester microbiome in the associations between residential greenness and GDM and glucose levels at GCT.

Our results are consistent with current equivocal literature, with several studies that could not identify significant associations between residential greenness, glucose intolerance and risk of GDM such as Rammah et al. performed in Spain amongst 2263 participants, and Lin et al. performed in China amongst 587 participants (Lin et al., 2021; Rammah et al., 2021). On the contrary, larger studies did find significant associations (Liao et al., 2019; Qu et al., 2020; Yu et al., 2023). Liao et al. reported reduced incidence of impaired glucose tolerance and GDM amongst 6807 participants in China. In their study, impaired glucose tolerance was defined as participants demonstrating some level of abnormal glucose tolerance, though not fully meeting the GDM diagnosis criteria (Liao et al., 2019). Qu et al. reported lower risk for GDM with higher NDVI exposure amongst 5327 participants in China (Qu et al., 2020). Yu et al. reported similar results during second trimester amongst 46,665 participants in China, with no protective effect of first trimester NDVI exposure on GDM risk (Yu et al., 2023). Both Qu et al. and Yu et al. used different exposure buffers than those used in our study. Furthermore, most of the previous studies reported linear associations between NDVI and blood glucose levels and GDM risk, with higher values of NDVI associated with the strongest beneficial effects (Liao et al., 2019; Lin et al., 2021; Yu et al., 2023). In our study, although non-significant, the patterns of associations between greenness and the outcomes were non-linear. It is rather hard to compare these finding to our results, since both the outcome and exposure were evaluated differently.

Our study population was much smaller compared to the studies described above. In addition, unlike our study, Qu et al., Yu et al. and Liao et al. all used GDM diagnosis using a one-step diagnosis approach with 75 g oral glucose tolerance test, which presumably identifies less women as GDM-positive compared to the 100 g oral glucose tolerance test performed in our study (Yogev et al., 2009). Liao et al. also used an impaired glucose tolerance diagnosis for participants who presented moderate glucose intolerance. Therefore, these studies included the more explicit GDM diagnosed participants (Liao et al., 2019; Qu et al., 2020; Yu et al., 2023). It is possible that stratifying the GDM-positive group to different glucose intolerance levels, might have made the differences in greenness exposure levels more distinguishable. These studies also used a lower resolution satellite (500 m resolution by Qu et al. and 250 m resolution by Yu et al. and Liao et al.) compared to the high resolution RapidEye (5 m × 5 m) and Sentinel-2 (10 m × 10 m) satellites that were used in our study. Using lower resolution satellite may cause exposure misclassification. However, this exposure misclassification is presumably random and therefore through the null and thus it cannot explain these gaps. Although we did not find statistically significant results in our study, in future studies, it might be beneficial to

use high resolution satellite data as in our study.

Although the possible interactions between the host, the environment, and the microbiome were suggested to affect human health (Panthee et al., 2022), our study did not find a role when considering GDM. Recent studies reported associations between microbiome diversity and greenness, changes in microbial compositions and specific species domination at different levels of NDVI exposure, and higher soil bacterial diversity in green spaces with higher plant diversity (Baruch et al., 2021; Gacesa et al., 2022; Wu et al., 2022). On the contrary, a previous study performed by Bowyer et al. amongst 2443 non-pregnant twin participants in the United Kingdom, could only find small compositional differences in stool samples across greenness quartiles at 3000 m buffers, whilst differences in abundancies of specific genera were observed for all measures according to urban-rural status (Bowyer et al., 2022).

To the best of our knowledge, our study is the first that aimed to evaluate mediation of microbiome on the possible beneficial association between greenness and GDM amongst pregnant women. We could not demonstrate sufficient evidence regarding the mediating effect of first trimester microbiome in the associations between residential greenness and GDM and glucose levels at GCT. First trimester microbiome diversity was vastly similar between GDM and healthy pregnant women in the urban population, except a significantly lower number of species in the GDM group, as represented by the observed species index in stool samples. It is rather possible that evaluating the more complex microbial composition (abundance of specific species comprising the microbiome) in future studies is needed, using samples from the pre-pregnancy period and from each trimester during pregnancy. This might be supported by Crusell et al., demonstrating only minor aberration in salivary microbiome during late pregnancy amongst 213 GDM participants in Denmark (Crusell et al., 2020), and by Pinto et al., who demonstrated a change in specific bacterial species amongst GDM and non-GDM participants, using the same database that was used in our study (Pinto et al., 2023). Nevertheless, inconsistent results regarding species abundancies amongst GDM-diagnosed participants were demonstrated in recent reviews, and therefore further studies should be performed (Ionescu et al., 2022; Rold et al., 2022).

Our study has a few limitations that should be mentioned. Evaluation of exposure to residential greenness was based on surrounding NDVI. NDVI is measured by satellite and it is a general measure of greenness, disregarding plant richness types, quality and use of green spaces. The residential address used for geocoding, seasonality and microbiome were all evaluated during first trimester of pregnancy. Hence, we did not have any knowledge about residential address or microbiome diversity prior to pregnancy. It is fairly possible that evaluating these variables before pregnancy and during the first and second trimesters, might have revealed a more comprehensive picture of the association. Furthermore, we did not have data on whether the residential address has changed for participants during later stages of pregnancy in which GDM diagnosis is made, which might have tipped the scales regarding exposure.

The study population was not sufficiently diverse. The majority of the participants are educated, from higher SES, living in cities in central Israel, without sufficient representation of minorities and periphery-living participants. Furthermore, the questionnaires given during recruitment were not translated into different languages such as Russian, Arabic, and Amharic. Since no compliance percentages were calculated during cohort recruitment, there may be a compliance bias that affects our external validity. A larger population size would have probably been helpful in determining significant associations. Another significant selection bias that was unintentionally present in our study was that participants with higher risk for GDM such as previous GDM diagnosis or polyhydramnios (N = 28), did not perform the GCT but only oral glucose tolerance test and therefore were excluded from the analysis of association between residential greenness and glucose levels at GCT. We can only assume that including these participants, with presumably higher risk for higher glucose levels at GCT, might have increased the strength of the associations observed.

In spite of the limitations present in our study, to the best of our knowledge, this is the first study that examined the associations between residential greenness and GDM and glucose levels at GCT with microbiome as a mediator, and the first to ever explore these associations among the Israeli population. Another strength is that microbiome was evaluated using both saliva and stool samples, with data collected prospectively. We had information on many personal, behavioral and biological variables that were considered in detailed DAGs and helped determine the minimal set of confounders for our models. In addition, we performed several sensitivity analyses. Albeit not statistically significant, our results suggest a non-linear association between residential greenness and risk for GDM, but did not demonstrate sufficient evidence for mediation by microbiome. This study provides a novel approach regarding the well investigated association between residential greenness and health benefits, and GDM in particular, that should be further evaluated in future studies.

5. Conclusions

Although not statistically significant, our study suggests possible associations between residential greenness and glucose intolerance and risk of GDM during pregnancy, with no sufficient evidence for mediation thorough microbiome. Our study does not provide sufficient evidence to support the hypothesis that the beneficial effect of residential greenness is mediated through microbiome, and further studies should examine this possible association, with emphasis on population size and diversity, and microbiome composition.

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CRediT authorship contribution statement

Ofir Avizemel: Data Curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - Original Draft, Writing - Review & Editing. **Sigal Frishman:** Formal analysis, Investigation, Resources. **Yishay Pinto:** Formal analysis, Investigation, Resources. **Yaron Michael:** Methodology, Data Curation. **Sondra Turjeman:** Formal analysis, Writing - Review & Editing. **Kinneret Tenenbaum-Gavish:** Resources. **Or Yariv:** Resources. **Yoav Peled:** Resources. **Eran Poran:** Resources. **Joseph Pardo:** Resources. **Rony Chen:** Resources. **Moshe Hod:** Resources. **Betty Schwartz:** Supervision. **Eran Hadar:** Resources, Supervision. **Omry Koren:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition. **Keren Agay-Shay:** Conceptualization, Methodology, Data Curation, Software, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare they have no actual or potential competing financial interest or any other conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114191.

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Sex-specific effect of perfluoroalkyl substances exposure on liver and thyroid function biomarkers: A mixture approach

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ABSTRACT

Although studies have investigated the effects of perfluoroalkyl substances (PFASs) on liver and thyroid function, little is known about its combined and sex-specific effect. A total of 688 participants were interviewed and serum PFASs concentration was measured using liquid chromatography/mass spectrometry. Five biomarkers of liver and thyroid function (ALT, GGT, TSH, FT3 and FT4) were chosen as outcomes. A restriction cubic spline function was applied to capture the dose-response relationship between PFASs and liver enzymes and thyroid hormones. Multivariable regression and Bayesian kernel machine regression (BKMR) models were performed to assess the single and overall associations of PFASs with targeted biomarkers. Single-pollutant analyses indicated that increased PFASs concentrations were associated with elevated ALT and GGT levels. BKMR models suggested positive dose-response relationships between PFASs mixtures and ALT and GGT levels. Significant associations were only detected between several PFASs and thyroid hormones, and joint effect of PFASs mixtures on FT3 levels was found at higher concentrations. Meanwhile, sex differences were found in the associations of PFASs with ALT and GGT levels, with significant results only in males. Our findings provide epidemiological evidence for combined and sex-specific effects of PFASs on ALT and GGT levels.

1. Introduction

Perfluoroalkyl substances (PFASs) are a group of man-made fluorinated organic compounds that are widely used in industrial and consumer products due to the thermal stability, and hydrophobic and oleophobic properties (Lindstrom et al., 2011). Nevertheless, perfluorooctane-sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), the two most widely used PFASs, have been listed as persistent organic pollutants since they were found to be highly toxic, bioaccumulative, long-distance migratory and environmentally persistent (Liu et al., 2022a). Humans are widely exposed to PFASs through contaminated food, drinking water, dust, and consumer products (Haug et al., 2011; Banzhaf et al., 2017; Akhbarizadeh et al., 2020), which results in a high detection rate of PFASs in biological samples.

Accumulating evidence has suggested that exposure to PFASs is associated with a range of adverse health conditions, and that the liver and thyroid are the target organs for PFASs (Coperchini et al., 2017; Costello et al., 2022). Rodent studies showed consistent evidence for PFASs hepatotoxicity, including increased liver weight and histopathological alterations (Costello et al., 2022). On the other hand, laboratory data suggested that PFASs may be thyroid toxicants that cause hypothalamic-pituitary-thyroid axis disorders by regulating the

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biosynthesis and transport of thyroid hormones and by interfering with thyroid receptors (Weiss et al., 2009; Ren et al., 2016; Coperchini et al., 2017). There are, of course, numerous epidemiological studies that have attempted to explore the effects of PFASs exposure on human liver and thyroid function, but the results are controversial (Olsen et al., 2003; Khalil et al., 2018; Mora et al., 2018; Salihovic et al., 2018; Nian et al., 2019; Xiao et al., 2020; Li et al., 2021; Derakhshan et al., 2022; Jensen et al., 2022; Li et al., 2022; Liu et al., 2022a). In addition to differences in study design, population characteristics and sample size, potential interactions between PFASs (additive or antagonistic) may account for the inconsistent results. As the study on mixtures in the field of environmental epidemiology continues to evolve, researchers are committed to quantify the risk of disease caused by environmental chemical mixtures, which may be helpful to identify modifiable exposures that are amenable for public health interventions (Braun et al., 2016).

To our knowledge, three studies reported the combined effects of mixed PFASs on liver enzymes with a positive association in general population of Chinese and Canadian adults (Borghese et al., 2022; Liu et al., 2022a) and a nonsignificant association in the European birth cohort (Stratakis et al., 2020). Furthermore, five population-based studies evaluated the overall association between PFASs exposure and thyroid hormones, including one study in the general adult population in China (Li et al., 2022) and four studies in pregnant women or newborns in China, Boston, and the United States (Aimuzi et al., 2020; Lebeaux et al., 2020; Preston et al., 2020; Guo et al., 2021). Thus, the epidemiological evidence for joint effects of PFASs mixtures on liver and thyroid function remains insufficient, especially for adults.

Sex differences in the rate of elimination of PFASs have been reported. Breastfeeding and menstruation were one of the pathways of PFASs excretion (Thomsen et al., 2010; Mondal et al., 2014; Wong et al., 2014), which may lead to rapid elimination of PFASs in young women (Attanasio, 2019). Moreover, the liver was considered a sexually dimorphic organ, since it could express sex hormone receptors (Attanasio, 2019). Thyroid-related disorders showed a sex-specific prevalence, with a 5-20-fold higher susceptibility in women than in men (Tunbridge et al., 1977; Vanderpump et al., 1995; Gietka-Czernel, 2017), for which the sex steroid environment may be a key determinant (Baksi and Pradhan, 2021). Therefore, the sex-specific effects of PFASs exposure on liver and thyroid function deserve profound consideration.

The purpose of this study was to assess single and overall associations of PFASs with biomarkers of liver and thyroid function and to explore potential modified effect of sex in a Chinese adult population.

2. Methods

2.1. Study population

The study was originally initiated to explore whether PFASs exposure was associated with risk of type 2 diabetes (T2DM), applying a case-control study design. T2DM cases were patients from the endocrinology department who donated the blood sample. We excluded patients with severe respiratory and cardiac failure, metabolic disease crises (e.g., diabetes and thyroid), type 1 diabetes, infection, surgery, or bed rest. Diabetes cases were matched (1:1) according to sex and age (±2) with controls that recruited from Medical Examination Center. A total of 688 participants recruited at two hospitals in Tianjin, China, were interviewed from April 2021 to March 2022. In current analyses, participants with missing data on hepatic enzymes (n = 24) and thyroid hormone (n = 94) were excluded. Thus, a total of 664 and 594 individuals were ultimately used to investigate the association between PFASs and liver and thyroid function biomarkers, respectively. All participants provided informed consent, and the study protocol was reviewed and approved.

2.2. Serum PFASs measurement

A total of 24 PFASs were identified and quantified in serum samples using a liquid chromatography-mass spectrometry (Shimadzu 8050, Japan). The limit of detection (LOD) and limit of quantification (LOQ) were considered, as the peak of compounds reached the signal-to-noise ratio of 3 and 10, respectively. In present study, 8 PFASs with detection rates higher than 70% were used for statistical analysis, including potassium perfluoro-1-octanesulfonate (PFOS), perfluoro-n-octanoic acid (PFOA), potassium perfluoro-1-hexanesulfonate (PFHxS), perfluoro-n-nonanoic acid (PFNA), perfluoro-n-decanoic acid (PFDA), perfluoro-n-undecanoic acid (PFUnDA), sodium 1H,1H,2H,2H-perfluoro-1-octanesulfonate (6:2 FTS), sodium perfluoro-1-heptanesulfonate (PFHpS), and serum concentrations of compounds below the LOD were replaced by LOD/$\sqrt{2}$. Detailed information was given in the Supplement, as reported in previous study (Liu et al., 2022b).

2.3. Outcome assessment

Serum alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) levels were measured by enzymatic methods. Concentrations of thyroid stimulating hormone (TSH), free triiodothyronine (FT3), and free thyroxine (FT4) were measured by a chemiluminescent immunoassay. When the liver enzyme level exceeded its upper limit of normal, the biomarkers of liver function were further classified into dichotomous outcomes, i.e., abnormal ALT and abnormal GGT. The clinical reference values of ALT and GGT in this study were ≤41 U/L in men and ≤31 U/L in women, and ≤60 U/L in men and ≤40 U/L in women, respectively (Liu et al., 2022a). Similarly, the thyroid functional status of the study participants was classified according to the thyroid hormone levels (Cappola et al., 2006), but further analysis was not performed because more than 90% of the participants had normal thyroid function.

2.4. Covariates

All participants were interviewed by two well-trained researchers and information on covariates was collected using a structured questionnaire. Confounders adjusted for in this study were as follows: age (year), sex (male or female), BMI (kg/m^2), drinking (yes or no), smoking (yes or no), diabetes (yes or no), hypertension (yes or no), and dyslipidemia (yes or no). Drinking was defined as consuming alcohol at least once a week for one year. Participants who self-reported smoking ≥1 cigarette per day for at least 6 months were considered to be smokers. Diabetes was considered if fasting glucose was ≥7.0 mmol/L, or self-reported on glucose-lowering therapy. Hypertension was defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg, or taking anti-hypertensive medication. Dyslipidemia was defined as having one or more of the following conditions: total cholesterol ≥6.2 mmol/L (hypercholesterolemia), triglyceride ≥2.3 mmol/L (hypertriglyceridemia), low-density lipoprotein cholesterol ≥4.1 mmol/L (hyperbetalipoproteinemia), and high-density lipoprotein cholesterol <1.0 mmol/L (hypoalphalipoproteinemia), consistent with the Chinese national guidelines for the prevention and treatment of adult dyslipidemia (Kong et al., 2022).

2.5. Statistical analysis

The normality of the data was examined using Q-Q plots. The subject characteristics were presented as mean (standard deviations, SDs) for normally distributed variables, median (interquartile range, IQR) for skewed variables, and as number (percentage) for categorical variables, respectively. Student t-test/Wilcoxon rank-sum test and chi-square test were used to compare sex differences in participant characteristics. Q-Q plots showed skewed distributions of PFASs and biomarkers of liver and thyroid function; therefore, natural log-transformation was performed for the above continuous variables.

Restricted cubic spline (RCS) regression was developed to assess the dose-response curves of PFASs with liver and thyroid biomarkers. 3

knots located at the 10th, 50th and 90th percentiles were selected for the RCS function according to the lowest Akaike Information Criterion. Multivariable linear regression models were established to assess the difference percentage of liver and thyroid biomarkers associated with ln-unit increment in PFASs, since most of the relationships between liver and thyroid biomarkers and PFASs concentration were linear (Fig. S1 to Fig. S5). Regression coefficients (β) and standard errors (SE) were used to calculate the percentage differences in the dependent variables and corresponding 95% confidence intervals (95% CI) based on the following two formulas: [exp (β)-1] × 100% and [exp (β ± 1.96 × SE)-1] × 100% (Nian et al., 2019). Multivariable logistic regression models were additionally applied to explore the associations between PFASs and abnormal liver functions. Furthermore, the mixed effect of PFASs on biomarkers of liver and thyroid function was evaluated using BKMR models with 10,000 iterations by a Markov chain Monte Carlo algorithm. The overall effect was evaluated when all PFASs mixtures were fixed at a certain percentile concentration (in the range of 25th to 75th percentile, with 5% increments), and compared to the mixture concentration that remained at the median. The posterior inclusion probabilities (PIPs) were calculated to identify the main components contributing to the effect, using 0.5 as the threshold.

A sex-stratified analysis was conducted to evaluate whether sex modified the relationship of PFASs exposure with biomarkers of liver and thyroid function. In addition, Bayesian kernel machine regression (BMKR) models were further developed in men and women, respectively, to assess the combined effects of PFASs mixtures on targeted biomarkers. Several sensitivity analyses were additionally performed to assess the robustness of the main models. (1) Considering that renal failure may confuse the associations between PFASs and liver enzymes and thyroid hormones (Jain and Ducatman, 2019b), the participants with chronic kidney disease (CKD), defined as an estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m^2, were excluded. In current study, the Japanese coefficient-modified Chronic Kidney Disease Epidemiology Collaboration equation was used to calculate eGFR (Horio et al., 2010). (2) Given the bias stemmed from missing data of thyroid function, multiple imputation by chained equations was applied to interpolate the missing thyroid hormone values. (3) Additional adjustment was performed in the sensitivity analyses for medication of diabetes and hypertension.

Data analyses were carried out by R software (version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria). $P < 0.05$ in the main analyses and $P < 0.1$ in the subgroup analyses were considered statistically significant (both two-tailed) (Tian et al., 2019; Webster et al., 2016; Zeng et al., 2019).

3. Results

3.1. Population characteristics

Table 1 presents the general characteristics of the 688 subjects, with 325 of them being male. The average age of all participants was 57.58 ± 12.57 years. Among the 8 PFASs with a detection rate higher than 70%, the highest serum concentration of PFASs was PFOS (median: 16.79 ng/ml), followed by PFOA (median: 9.40 ng/ml), PFNA (median: 1.86 ng/ml), PFHxS (median: 1.63 ng/ml), PFDA (median: 1.33 ng/ml), PFUnDA (median: 0.75 ng/ml), PFHpS (median: 0.43 ng/ml), and 6:2 FTS (median: 0.42 ng/ml).

3.2. Associations of single-PFASs exposure with liver and thyroid function

Table 2 presents the individual associations of PFASs with estimated changes of liver enzymes and thyroid hormones. It was observed that serum PFASs was positively associated with ALT and GGT. For example, per ln-unit increment in PFOA and PFOS exposure was associated with 5.98% (95% CI: 2.10%, 10.01%) and 6.16% (95% CI: 0.44%, 12.22%) higher GGT levels in serum, respectively. Likewise, as illustrated in

Table 1
Characteristics of the study population (n = 688).

	Total	Males	Females	P value
PFOA, ng/ml (M, IQR)	9.40 (5.32, 15.10)	9.42 (5.61, 15.46)	9.33 (5.06, 14.77)	0.431
PFHxS, ng/ml (M, IQR)	1.63 (1.21, 2.20)	1.78 (1.31, 2.33)	1.53 (1.07, 2.03)	< 0.001
PFOS, ng/ml (M, IQR)	16.79 (10.00, 28.67)	18.80 (11.73, 33.71)	14.80 (9.15, 24.94)	< 0.001
PFNA, ng/ml (M, IQR)	1.86 (1.15, 2.73)	1.93 (1.24, 2.83)	1.72 (1.10, 2.57)	0.025
PFDA, ng/ml (M, IQR)	1.33 (0.72, 2.94)	1.52 (0.79, 3.47)	1.21 (0.70, 2.41)	0.001
PFUnDA, ng/ml (M, IQR)	0.75 (0.46, 1.23)	0.82 (0.45, 1.36)	0.71 (0.47, 1.10)	0.056
6:2 FTS, ng/ml (M, IQR)[a]	0.42 (0.16, 0.64)	0.48 (0.29, 0.67)	0.34 (0.16, 0.59)	< 0.001
PFHpS, ng/ml (M, IQR)	0.43 (0.22, 0.71)	0.48 (0.29, 0.74)	0.36 (0.17, 0.68)	< 0.001
Age, years	57.58 ± 12.57	55.36 ± 12.36	59.58 ± 12.44	< 0.001
BMI, kg/m^2	25.79 ± 3.86	26.20 ± 3.45	25.41 ± 4.16	0.009
Smoking, n (%)	241 (35.0%)	195 (60.0%)	46 (12.7%)	< 0.001
Drinking, n (%)	199 (28.9%)	167 (51.4%)	32 (8.8%)	< 0.001
Hypertension, n (%)	424 (61.6%)	207 (63.7%)	217 (59.9%)	0.289
Diabetes, n (%)	344 (50.0%)	159 (48.9%)	185 (51.1%)	0.621
Hyperlipidemia, n (%)	335 (48.7%)	185 (56.9%)	150 (41.4%)	< 0.001
ALT, U/L (M, IQR)[b]	19.10 (14.10, 27.63)	21.90 (15.75, 31.35)	16.85 (13.00, 24.42)	< 0.001
GGT, U/L (M, IQR)[b]	21.95 (15.97, 34.00)	25.60 (19.10, 40.40)	19.00 (14.07, 28.40)	< 0.001
TSH, mIU/L (M, IQR)[c]	1.85 (1.21, 2.76)	1.69 (1.15, 2.45)	2.02 (1.35, 3.10)	< 0.001
FT3, pmol/L (M, IQR)[c]	3.57 (1.04, 4.68)	2.07 (1.04, 4.68)	3.88 (1.04, 4.68)	0.617
FT4, pmol/L (M, IQR)[c]	10.55 (8.47, 13.66)	10.50 (8.46, 13.74)	10.66 (8.57, 13.51)	0.650

[a] The concentrations below the LOD were replaced by LOD/$\sqrt{2}$.
[b] 664 individuals were used for analysis.
[c] 594 individuals were used for analysis.

Table S4, significant associations of PFOA (OR = 1.23, 95% CI: 1.00, 1.56), PFNA (OR = 1.70, 95% CI: 1.18, 2.48), PFDA (OR = 1.51, 95% CI: 1.17, 1.98), and PFUnDA (OR = 1.66, 95% CI: 1.18, 2.39) with greater odds of abnormal GGT were also detected in multivariable logistic regression models. In addition, several PFASs were found to be associated with decreased TSH and FT4 levels and increased FT3 levels. A ln-unit increase in PFHxS was associated with decreasing of 12.74% (95% CI: 3.23%, 21.31%) TSH level.

The results of subgroup analyses stratified by sex are described in Table 3. Sex-specific associations of serum PFASs concentrations with liver function biomarkers were found in present study. For instance, exposure to PFOA, PFHxS and PFOS significantly increased GGT concentrations in males (PFOA: 9.43%, 95% CI: 4.54%–14.55%; PFH_XS: 18.38%, 95% CI: 5.91%–32.32%; PFOS: 8.74%, 95% CI: 0.82%–17.27%), but not in females (PFOA: -1.07%, 95% CI: -6.62%–4.80%; PFHxS: 1.10%, 95% CI: -10.63%–14.37%; PFOS: 1.83%, 95% CI: -5.80%–10.08%) (all P for interaction <0.1). However, no modified effect of sex on the associations of PFASs concentrations with thyroid hormones levels was seen in our results.

3.3. Associations of PFASs mixtures with liver and thyroid function

The PIPs identified PFNA as a significant chemical associated with liver function, while PFNA and PFHxS were recognized as important chemicals linked to thyroid function (Table S5). Fig. 1 depicts the overall effect of an increase in PFASs mixtures on ALT and GGT levels when the

Table 2
The estimated percentage difference (95% CI) for liver and thyroid function biomarkers associated with per ln-PFASs increment in multivariable linear regression models.

Ln-PFASs (ng/ml)	ALT[a]	GGT[b]	TSH[c]	FT3[d]	FT4[e]
PFOA	2.99 (−0.55, 6.64)	**5.98 (2.10, 10.01)**	−2.78 (−7.13, 1.78)	**2.65 (0.77, 4.56)**	**−2.63 (-4.31, -0.92)**
PFHxS	**10.09 (1.78, 19.07)**	**11.44 (2.44, 21.24)**	**−12.74 (-21.31, -3.23)**	0.27 (−3.87, 4.58)	−2.27 (−6.07, 1.68)
PFOS	4.62 (−0.65, 10.17)	**6.16 (0.44, 12.22)**	−5.33 (−11.49, 1.25)	1.34 (−1.39, 4.15)	**−2.93 (-5.38, -0.41)**
PFNA	**10.58 (3.44, 18.20)**	**14.33 (6.45, 22.79)**	**−10.64 (-18.12, -2.47)**	**4.96 (1.31, 8.74)**	−2.14 (−5.37, 1.20)
PFDA	**6.31 (1.39, 11.46)**	**8.84 (3.46, 14.50)**	**−6.92 (-12.68, -0.79)**	−1.47 (−4.00, 1.11)	−1.57 (−3.94, 0.87)
PFUnDA	**7.95 (1.65, 14.63)**	**11.00 (4.08, 18.38)**	**−8.56 (-15.58, -0.95)**	−0.43 (−3.62, 2.86)	−1.17 (−4.15, 1.91)
6:2 FTS	**9.95 (2.19, 18.31)**	**9.42 (1.13, 18.39)**	−4.11 (−13.27, 6.02)	1.79 (−2.27, 6.01)	−1.61 (−5.31, 2.24)
PFHpS	**7.02 (0.88, 13.53)**	0.57 (−5.65, 7.19)	−5.16 (−12.53, 2.84)	2.23 (−1.07, 5.64)	**−3.17 (-6.11, -0.14)**

Adjusted for age, sex, BMI, smoking, drinking, diabetes, hypertension, and hyperlipidemia.
Bold indicates $P < 0.05$.
[a] Ln-ALT (U/L).
[b] Ln-GGT (U/L).
[c] Ln-TSH (mIU/L).
[d] Ln-FT3 (pmol/L).
[e] Ln-FT4 (pmol/L).

8 PFASs mixtures are fixed at the 50th percentile. The BKMR models indicated positive dose-response relationships between PFASs mixtures and ALT and GGT in the total population. Further analyses of BKMR models run separately in males and females revealed that significant joint effect of PFASs mixtures on liver function biomarkers was only found in males, consistent with the results of multivariable linear regression models. When holding all PFASs at the higher levels (65th, 70th and 75th percentile) compared to their median concentrations, PFASs mixtures were negatively associated with the FT3 levels (Fig. S6). However, the joint effect of PFASs on FT3 levels was nonsignificant in both men and women (Fig. S6). Furthermore, the overall associations of PFASs mixtures with TSH and FT4 levels were not significant across population subgroups (Fig. S6). As illustrated in Fig. S7, it was found that an IQR increase in the ln-transformed concentration of PFDA and PFHxS was negatively associated with ln-FT3 level when other chemicals were set at the 25th, 50th and 75th percentile, respectively. However, for PFNA, the results were in the opposite direction (Fig. S7). In addition, the bivariate exposure-response function for PFASs did not exhibit interactions, as evidenced by the parallel curves with equal slopes at the 10th, 50th, and 90th percentiles (Figs. S8–S12).

3.4. Sensitivity analyses

Table S6 shows the associations of single PFASs exposure with interested biomarkers estimated using multivariate linear regression models after excluding participants with CKD. Overall, positive associations between PFASs and ALT and GGT were still detected and several PFASs were positively or negatively associated with FT3, TSH and FT4, similar to the main analyses. Consistent results were also found in the BKMR models (Figs. S13–S14). Because missing data on thyroid hormones in this study may bias the association of PFASs with thyroid

Table 3
The estimated percentage difference (95% CI) for liver and thyroid function biomarkers associated with per ln-PFASs increment in multivariable linear regression models, stratified by sex.

Biomarkers	Ln-PFASs (ng/ml)	Males	Females	P inter
ALT[a]	PFOA	**4.95 (0.39, 9.72)**	−0.65 (−5.97, 4.97)	**0.019**
	PFHxS	**12.82 (1.38, 25.55)**	5.96 (−5.79, 19.18)	0.241
	PFOS	5.81 (−1.58, 13.76)	2.67 (−4.69, 10.59)	0.295
	PFNA	**13.37 (3.16, 24.60)**	7.72 (−2.00, 18.40)	0.432
	PFDA	**6.75 (0.06, 13.89)**	4.83 (−2.25, 12.42)	0.169
	PFUnDA	7.41 (−0.75, 16.24)	8.42 (−0.99, 18.73)	0.319
	6:2 FTS	**15.27 (4.08, 27.67)**	3.07 (−7.56, 14.92)	**0.080**
	PFHpS	**13.13 (4.25, 22.77)**	1.13 (−7.38, 10.42)	**0.082**
GGT[b]	PFOA	**9.43 (4.54, 14.55)**	−1.07 (−6.62, 4.80)	**0.000**
	PFHxS	**18.38 (5.91, 32.32)**	1.10 (−10.63, 14.37)	**0.054**
	PFOS	**8.74 (0.82, 17.27)**	1.83 (−5.80, 10.08)	**0.081**
	PFNA	**24.47 (12.98, 37.13)**	3.07 (−6.68, 13.84)	**0.003**
	PFDA	**10.66 (3.46, 18.36)**	5.33 (−2.11, 13.34)	**0.018**
	PFUnDA	**12.06 (3.22, 21.65)**	9.85 (−0.11, 20.80)	**0.030**
	6:2 FTS	**18.33 (6.36, 31.64)**	−2.68 (−13.17, 9.07)	**0.013**
	PFHpS	4.09 (−4.57, 13.53)	−4.85 (−13.21, 4.31)	0.334
TSH[c]	PFOA	0.14 (−5.87, 6.54)	−4.63 (−11.03, 2.22)	0.231
	PFHxS	−8.28 (−20.79, 6.20)	**−14.67 (-26.56, -0.84)**	0.105
	PFOS	−7.87 (−16.49, 1.64)	−2.53 (−11.21, 7.00)	0.956
	PFNA	**−12.55 (-23.07, -0.60)**	−6.60 (−17.26, 5.43)	0.778
	PFDA	**−11.39 (-18.87, -3.23)**	−2.79 (−11.34, 6.59)	0.569
	PFUnDA	**−10.87 (-20.07, -0.62)**	−7.43 (−17.66, 4.07)	0.946
	6:2 FTS	−2.82 (−15.66, 11.97)	−1.15 (−14.84, 14.74)	0.292
	PFHpS	−1.41 (−12.01, 10.47)	−5.81 (−16.38, 6.10)	0.134
FT3[d]	PFOA	**2.90 (0.95, 4.90)**	2.84 (−0.55, 6.35)	0.561
	PFHxS	3.99 (−0.68, 8.89)	−2.27 (−9.16, 5.15)	0.422
	PFOS	−0.48 (−3.52, 2.65)	3.27 (−1.27, 8.02)	**0.074**
	PFNA	0.75 (−3.26, 4.92)	**8.44 (2.34, 14.90)**	**0.031**
	PFDA	−2.02 (−4.72, 0.76)	−0.57 (−4.90, 3.96)	0.202
	PFUnDA	−1.41 (−4.75, 2.04)	0.64 (−4.92, 6.52)	0.189
	6:2 FTS	0.79 (−3.60, 5.39)	4.22 (−3.01, 11.99)	0.233
	PFHpS	−0.37 (−3.87, 3.26)	**6.11 (0.21, 12.35)**	**0.070**
FT4[e]	PFOA	**−3.42 (-5.77, -1.00)**	−2.14 (−4.55, 0.34)	0.658
	PFHxS	−3.28 (−8.84, 2.63)	−2.19 (−7.38, 3.29)	0.688
	PFOS	**−4.20 (-7.92, -0.33)**	−2.19 (−5.41, 1.15)	0.498
	PFNA	−3.38 (−8.27, 1.78)	−1.80 (−6.00, 2.58)	0.778

(continued on next page)

Table 3 (*continued*)

Biomarkers	Ln-PFASs (ng/ml)	Males	Females	P inter
	PFDA	−2.02 (−5.48, 1.57)	−1.15 (−4.38, 2.19)	0.831
	PFUnDA	−1.39 (−5.66, 3.08)	−0.71 (−4.83, 3.58)	0.994
	6:2 FTS	−3.59 (−8.94, 2.08)	−0.63 (−5.83, 4.85)	0.355
	PFHpS	−3.80 (−8.10, 0.70)	−3.80 (−7.82, 0.40)	0.788

Adjusted for age, sex, BMI, smoking, drinking, diabetes, hypertension, and hyperlipidemia.
Bold indicates $P < 0.05$ or $P < 0.1$.
[a] Ln-ALT (U/L).
[b] Ln-GGT (U/L).
[c] Ln-TSH (mIU/L).
[d] Ln-FT3 (pmol/L).
[e] Ln-FT4 (pmol/L).

function, single- and multi-pollutant analyses were performed again after multiple interpolation of the missing data, and the results were comparable to those in the main models (Table S7 and Fig. S15). The results found in the sensitivity analyses with additional adjustment for medication were consistent with the main findings (Table S8 and Figs. S16-S17).

4. Discussion

The results of current analyses suggested positive associations of single and joint PFASs exposure with ALT and GGT levels by using multivariable linear regression and BKMR models. Whereas for thyroid function, several PFASs were inversely correlated with TSH and FT4 levels and positively correlated with FT3 levels in the single-pollutant analyses. However, the BKMR models only showed that PFASs mixtures were negatively correlated with FT3 at higher levels. Additionally, the sex-specific effects of PFASs on ALT and GGT were observed, with significant associations only in males. Our findings provide epidemiological evidence for combined and sex-specific effects of PFASs on ALT and GGT levels in Chinese adults.

PFOS (median: 16.79 ng/ml) and PFOA (median: 9.40 ng/ml) were the most contaminated PFASs, which were comparable to levels reported in previous studies conducted in Chinese populations (Li et al., 2022). For example, a cross-sectional investigation of general adult population in Guangzhou, China reported the concentrations of PFOS and PFOA were 14.85 ng/ml and 8.97 ng/ml, respectively. Another case-control study conducted in Tianjin, China found the serum concentrations were 11.64 ng/ml for PFOS and 12.28 ng/ml for PFOA (Duan et al., 2021). However, PFOS and PFOA concentrations in this study were higher than those reported in studies undertaken in Canada (3.3 ng/ml for PFOS; 1.3 ng/ml for PFOA) (Cakmak et al., 2022), the United States (6.3 ng/ml for PFOS; 2.2 ng/ml for PFOA) (Jain and Ducatman, 2019a), Australia (1.8 ng/ml for PFOS; 2.3 ng/ml for PFOA) (Eriksson et al., 2017), and Sweden (0.57 ng/ml for PFOS; 2.53 ng/ml for PFOA) (Salihovic et al., 2018).

Numerous epidemiological studies have investigated the individual associations of PFASs with liver and thyroid function. Overall, positive (Salihovic et al., 2018; Nian et al., 2019; Borghese et al., 2022; Cakmak et al., 2022; Liu et al., 2022a) or null (Mundt et al., 2007; Sakr et al., 2007; Khalil et al., 2018; Nilsson et al., 2022) associations were found for PFASs-ALT and PFASs-GGT analyses in published studies. For instance, Nian et al. found positive associations between PFASs and liver function biomarkers, including ALT and GGT, in the general adult population (Nian et al., 2019). Positive associations were also found for PFASs-ALT and PFASs-GGT in a nationally representative sample of 6768 individuals from the Canadian Health Measures Survey (Cakmak et al., 2022). However, there was no statistically significant relationship between PFASs and ALT levels in Australian firefighters (Nilsson et al., 2022). Similarly, no significant association of PFASs with ALT levels was observed in obese children (Khalil et al., 2018). In addition, epidemiological findings for PFASs-thyroid hormones were inconsistent, and pregnant women and newborns were priority populations of concern. For example, previous evidence showed that PFASs were positively (Webster et al., 2014; Webster et al., 2016; Byrne et al., 2018), negatively (Aimuzi et al., 2019; Liang et al., 2020; Li et al., 2022), or insignificantly (Preston et al., 2018; van Gerwen et al., 2020) correlated with TSH levels.

In current analyses, we also found that PFASs were associated with elevated ALT and GGT levels, which was similar to the documented results. On the other hand, multivariable linear regression models suggested that several PFASs were negatively correlated with TSH and FT4 levels and positively correlated with FT3 levels. The controversial results may be attributed to differences in study population, study design,

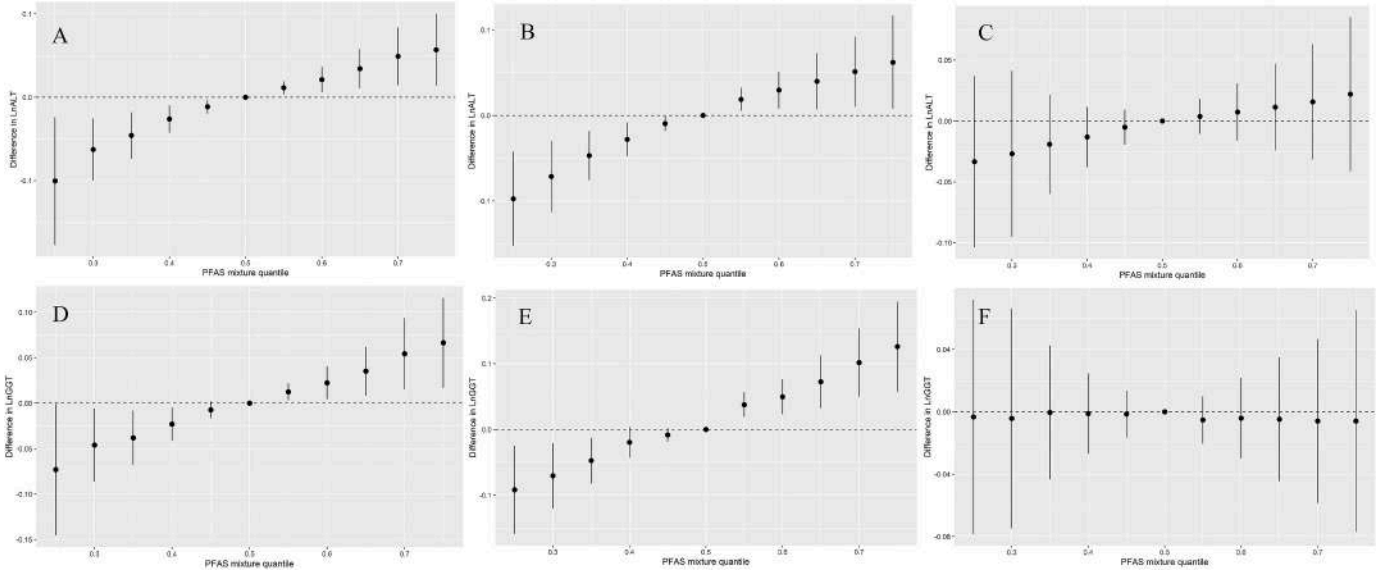

Fig. 1. Overall effects of PFASs mixtures on hepatic enzymes in (A, D) total population, (B, E) males, and (C, F) females using BKMR model. Models were adjusted for age, sex, BMI, smoking, drinking, diabetes, hypertension, and hyperlipidemia.

concentration and composition of PFASs, and genetic background. Exposure assessment of PFASs in environmental media such as soil, river and atmosphere showed a distinct spatial distribution of PFASs (Lin et al., 2020; Lin et al., 2021; Shi et al., 2021; Du et al., 2022; Mattias et al., 2022), which may be related to the fluoridation industry sites and human activities. Meanwhile, accumulating evidence suggested that the health effects of PFASs may be non-monotonic and non-linear (Aimuzi et al., 2020; Skogheim et al., 2021; Li et al., 2022; Liu et al., 2022a), suggesting that differences in PFASs concentrations across studies may lead to complex and inconsistent results (Han et al., 2021).

To date, little is known about joint effects of PFASs mixtures on liver and thyroid function. After an extensive search of previous studies, we found three population studies that assessed the overall associations of combined PFASs exposure with liver enzymes levels (Stratakis et al., 2020; Borghese et al., 2022; Liu et al., 2022a). Liu et al. observed a positive dose-response pattern of PFASs mixtures with ALT and GGT levels by applying BKMR models in a general population of Chinese adults (Liu et al., 2022a). Data from the Canadian Health Measures Survey suggested that one-quartile increment in the PFASs mixtures was positively associated with GGT, as assessed by the quantile g-computation (Borghese et al., 2022). Another study by Stratakis et al. evaluated the overall association of prenatal PFASs exposure with liver function, and the results showed a positive association between PFASs mixtures and liver injury risk, but the combined effects on ALT and GGT levels were almost insignificant (Stratakis et al., 2020). In this study, after fully adjusting for potential confounders in BKMR models, significant associations between PFASs mixtures and elevated ALT and GGT levels were obtained, consistent with prior studies.

In addition, to our knowledge, five studies investigated the combined effects of PFASs mixtures on thyroid hormones (Aimuzi et al., 2020; Lebeaux et al., 2020; Preston et al., 2020; Guo et al., 2021; Li et al., 2022). Li et al. found a nonlinear combined effect of PFASs mixtures on TSH and FT4 levels in the Chinese adult population (Li et al., 2022). Guo et al. evaluated the overall association between cord serum PFASs concentration and neonatal thyroid function using weighted quantile sum (WQS) regression models and results showed that PFASs mixtures were positively associated with FT4 and negatively associated with TSH, but not with FT3 (Guo et al., 2021). Aimuzi et al. observed a negative overall association of PFASs mixtures with maternal FT4 level, and null associations with TSH and FT3 levels (Aimuzi et al., 2020). Moreover, two studies explored the mixed effects of prenatal PFASs exposure on maternal and neonatal thyroid function. Only maternal free T4 index was inversely associated with PAFSs mixtures in the study by Preston et al. (Preston et al., 2020), while another study showed non-significant associations between PFASs mixtures and biomarkers of thyroid function in both mothers and newborns (Lebeaux et al., 2020). In the current study undertaken in Chinese adult population, BKMR models showed that high concentrations of PFASs mixtures were inversely correlated with FT3 level, but not with either TSH or FT4. The available evidence for the effects of PFASs mixtures on thyroid hormones is contradictory, and well-designed studies are encouraged to address this confusion.

Given the sex differences in PFASs elimination and the sexually dimorphic effect of liver and thyroid, this study further assessed whether the association of PFASs with liver and thyroid biomarkers was sex-specific. First, we established stratified analyses by sex and found that the single associations of PFASs with ALT and GGT levels were stronger in males with significant interaction terms ($P < 0.1$). Further, BKMR models run across sex also demonstrated that the significant joint effects of PFASs mixtures on ALT and GGT levels were observed only in males. However, we did not find the sex-specific association between PFASs and thyroid hormones. Stratified analyses by BMI, smoking, drinking, diabetes, hypertension, and dyslipidemia were conducted to explore potential modifiers of the association between PFASs and thyroid function biomarkers, because growing evidence has shown that smoking, obesity, hyperglycemia, and pre-existing cardiometabolic diseases can worsen TSH in the circulation. The results showed decreased TSH levels associated with PFASs were only found in overweight individuals and those with dyslipidemia (Table S9). Published studies suggested controversial results for sex differences in the association between PFASs and liver function. Analysis of data from the general adult population in China did not show sex differences in the associations of PFASs with GGT and ALT levels (Liu et al., 2022a). Similarly, a study by Mora et al. investigated the relationship between prenatal and mid-childhood PFASs concentrations and mid-childhood ALT levels stratified by child sex and showed a non-significant interaction term between PFASs and child sex (Mora et al., 2018). Using data from NHANES 2013–2016, another study was conducted to explore the sex differences in the association of PFASs and liver function in adolescents aged 12–19 years (Attanasio, 2019). The authors reported the sex differences in the association of PFASs and liver function, such that PFOA was correlated with increased ALT level and a higher risk of elevated ALT in females, while the reversed results were found in males.

The underlying mechanisms of the hepatotoxicity associated with PFASs exposure are unclear. In vitro experiments showed that PFOA treatment could induce endoplasmic reticulum stress and mitochondrial-mediated apoptosis in human liver L02 cells via endoplasmic reticulum-mitochondria communication (Wang et al., 2022b). Animal models suggested that PFOS could form an ion pair with choline, which in turn caused hepatic steatosis and oxidative stress (Zhang et al., 2016). Zebrafish liver cells exhibited inhibition of cell viability and massive accumulation of autophagic vacuoles after PFOA exposure, and further transcriptome analysis revealed significant changes in autophagy, signal transduction, tight junctions, endocrine system, immune system, and metabolism-related pathways (Wu et al., 2022). Lin et al. reported that exposure to environmentally relevant concentrations of PFOS and PFOA could result in liver damage and oxidative stress in male black-spotted frogs, which might be associated with dysregulation of the gut microbiota (Lin et al., 2022b). In addition, it has been shown that the PFASs-induced abnormalities in hepatic lipid metabolism are mediated in part through the peroxisome proliferator-activated receptor (Jiang et al., 2021; Lin et al., 2022a; Wang et al., 2022a; Wang et al., 2022c). Sex differences in tissue distribution and pharmacokinetics of PFASs may explain the sex-specific effect of PFASs on liver function. Research conducted by Kim et al. found that the tissue-plasma partition coefficient (Kp) values of PFOS and PFOA in the liver showed significant differences between male and female rats (Kim et al., 2016). Specifically, the study reported that male rats had Kp values for PFOS and PFOA that were approximately 1.9 and 2.9 times higher, respectively, than those in female rat. Additionally, a review of literature revealed that female rats exhibited higher urinary excretion of PFASs compared to males, which could potentially account for the observed sex differences in the distribution of specific PFASs, such as PFHxS and PFOA, in rats (Pizzurro et al., 2019).

Several limitations in this study must be acknowledged. First, non-longitudinal data cannot make causal inferences about PFASs exposure and targeted biomarkers. Second, while adjusting for several important covariates, the confounding effects of other factors, such as medications, diet, and unmeasured environmental contaminants, could not be completely eliminated, similar to published studies (Attanasio, 2019; Cakmak et al., 2022; Liu et al., 2022a). In present study, additional adjustment for medication of hypertension and diabetes was performed in the sensitivity analyses, and the results were similar to the main analyses. Third, the missing data of thyroid hormones may cause bias in the results. However, sensitivity analysis of multiple interpolations of thyroid hormones showed comparable results to those obtained by the main models. Fourth, participants were from a case-control study assessing the association of PFASs with risk of T2DM, which may lead to the sample size of the current study is inadequate to provide sufficient statistical power for certain analyses and limit the generalization of the findings. Fifth, whether differences in PFASs concentrations across sex are responsible for the sex-specific effects of PFASs on ALT and GGT warrants further investigation.

5. Conclusion

In conclusion, we found positive associations between single and joint exposure to PFASs and ALT and GGT levels in Chinese adults, and the significant associations were found only in males. Our findings provide population-based evidence for the hepatotoxicity of combined PFASs exposure and the sex-specific adverse effects of PFASs on liver function.

Author contribution

Ze Yang: Conceptualization, Methodology, Formal analysis, Data Curation, Writing-Original Draft; **Ruifang Liu:** Formal analysis, data Curation, Writing-Original Draft; **Hongbo Liu**: Methodology, Formal analysis; **Jiemin Wei**: Conceptualization, Validation; **Xiaohui Lin**: Methodology; **Mingyue Zhang**: Methodology; **Yu Chen**: Conceptualization; **Jingyun Zhang**: Data Curation; **Meiqing Sun**: Methodology; **Zhe Feng**: Methodology; **Jian Liu**: Methodology; **Xiangyang Liu**: Methodology; **Xiaoxu Huo**: Data Curation, **Kun Men**: Resources; **Qiaoyun Yang**: Methodology, Data Curation, **Xi Chen**: Data Curation; **Nai-jun Tang**: Writing-Review & Editing, Supervision, Funding acquisition.

Declaration of competing interest

Authors declare no actual or potential competing financial interests.

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Appendix A. Supplementary data

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Urinary neonicotinoid insecticides and adiposity measures among 7-year--old children in northern China: A cross-sectional study

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ABSTRACT

Background: Neonicotinoid insecticides (NEOs) are emerging synthetic insecticides used in various pest management regimens worldwide. Toxicology studies have indicated the obesogenic potential of NEOs, but their associations with adiposity measures are largely unknown.
Objectives: We aimed to assess urinary levels of NEOs/metabolites and their associations with children's adiposity measures, and to further investigate the potential role of oxidative stress.
Methods: This study included 380 children who participated in the 7th year's follow-up of the Laizhou Wan Birth Cohort in northern China. Urinary levels of seven NEOs and two metabolites and a biomarker of lipid peroxidation named 8-iso-prostaglandin-F2α (8-iso-PGF2α) were detected. A total of nine indicators of adiposity were measured. Body mass index (BMI) z-score ≥85th percentile was defined as overweight/obesity, and waist-to-height ratio (WHtR) ≥0.5 was considered as abdominal obesity. Multiple linear regression, binary logistic regression and mediation analysis were performed.
Results: Six NEOs [imidacloprid (IMI, 99.7%), clothianidin (CLO, 98.9%), dinotefuran (DIN, 97.6%), thiamethoxam (THM, 95.5%), acetamiprid (ACE, 82.9%), thiacloprid (THD, 77.6%)] and two metabolites [N-desmethyl-acetamiprid (N-DMA, 100.0%), 6-chloronicotinic acid (6-CINA, 97.9%)] exhibited high detection rates. Multiple linear regressions showed positive associations of waist circumference with urinary levels of IMI and THM, of WHtR with IMI and THM levels, and of body fat percentage with 6-CINA levels. In contrast, exposure to N-DMA was negatively associated with body fat percentage and fat mass index. Binary logistic regressions further revealed that higher IMI levels were associated with overweight/obesity (OR = 1.556, 95% CI: 1.100, 2.201) and abdominal obesity (OR = 1.478, 95% CI: 1.078, 2.026) in children. 8-iso-PGF2α demonstrated 27.92%, 69.52% and 35.37% mediating effects in the positive associations of IMI, THD and THM with WHtR, respectively. Sex modified the associations of DIN with body fat mass (p_{int} = 0.032), body fat percentage (p_{int} = 0.009), fat mass index (p_{int} = 0.037) and the overweight/obesity rate (p_{int} = 0.046), with negative associations in girls and nonsignificant positive associations in boys.
Conclusions: School-age children in northern China were widely exposed to NEOs/metabolites. Urinary levels of NEOs/metabolites were associated with adiposity measures through the mediating role of 8-iso-PGF2α. These associations were mixed, and a sex-specific effect might exist.

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1. Introduction

Neonicotinoid insecticides (NEOs) are emerging synthetic insecticides serving as a replacement for organophosphorus and pyrethroid insecticides (Lu et al., 2018; Oya et al., 2021). Due to their high insecticidal activities and low mammalian toxicities, NEOs are widely used in agriculture, garden forestry, and animal husbandry for pest control (Matsuda et al., 2005; Ospina et al., 2019; Wang et al., 2019). The usage of NEOs has dramatically increased worldwide since the early 1990s, and it accounted for more than 25% of the global insecticide market share in 2014 (Bass et al., 2015; Cimino et al., 2017). Until now, NEOs/metabolites have been ubiquitous in ecosystems and they have been broadly detected in various media, such as air, soil and water (Bonmatin et al., 2015, 2021; Casillas et al., 2022). Human exposure to NEOs mainly occurs via the intake of contaminated drinking water and food (Chen et al., 2020; Mahai et al., 2021). China is an important producer and consumer of NEOs (Zhang et al., 2019b). The general population in China have increased exposure to NEOs due to their wide application for pest control for planting and residential purposes (Pan et al., 2022; Song et al., 2020); in particular, the elevated levels of NEOs among Chinese children have raised significant concerns in public health (Wang et al., 2020a).

Childhood obesity is considered to be a global public health problem, and the prevalence of overweight/obesity in children has been increasing worldwide over the past decades (Fan and Zhang, 2020; NCD-RisC, 2017; Torres-González et al., 2020; Wang et al., 2020b). Notably, children with obesity are more likely to become obese in adulthood and develop cardiometabolic complications, metabolic disorders, and cancer later in life (Gurnani et al., 2015; Weihrauch-Blüher et al., 2019). Although the underlying factors are far from fully clear, growing evidence has proposed endocrine disrupting chemicals (EDCs) as emerging risk factors for childhood obesity (Heindel et al., 2015; Muscogiuri et al., 2017; Yang et al., 2018). In recent years, an increasing body of evidence has revealed that NEOs possess endocrine disrupting properties (Caron-Beaudoin et al., 2018; Mesnage et al., 2018; Mikolić and Karačonji, 2018) that may disturb adipose metabolism and thereby increase fat accumulation (Mesnage et al., 2018; Sun et al., 2017). Limited epidemiological studies on the influence of NEOs/metabolites on adiposity measures are available, and more research is needed to investigate these associations.

The underlying mechanisms of NEOs' toxicity have not been clearly elucidated, but *in vivo* and *in vitro* studies have suggested that oxidative stress may play an important role, e.g., the generation of reactive oxygen/nitrogen species (ROS/RNS) (El-Gendy et al., 2010; Sheets et al., 2016). Previous epidemiological studies reported that exposure to NEOs was associated with increased oxidative stress biomarkers of lipid damage (Li et al., 2020; Zhang et al., 2021). Elevated levels of oxidative stress were shown to be correlated with children's adiposity measures (Arogbokun et al., 2021; Loy et al., 2014). Moreover, animal studies have shown that NEOs cause oxidative stress, which may further induce lipid accumulation and obesity (Lu et al., 2021; Yan et al., 2020). However, it remains unclear whether oxidative stress plays a role in the associations between NEOs and adiposity measures in epidemiological studies. Therefore, we explored the mediating effect of oxidative stress in these associations among children.

Based on the information collected from the follow-up survey in the 7th year of the Laizhou Wan Birth Cohort (LWBC) in northern China, we aimed to assess urinary levels of NEOs/metabolites in children, their associations with adiposity measures and the role of 8-iso-PGF2α in these associations. Additionally, we further examined whether sex modified the relationships between NEOs/metabolites and adiposity measures in children.

Abbreviations

NEOs	neonicotinoid insecticides
8- iso-PGF2α	8-iso-prostaglandin F2α
BMI	body mass index
WHtR	waist-to-height ratio
IMI	imidacloprid
CLO	clothianidin
DIN	dinotefuran
THM	thiamethoxam
ACE	acetamiprid
THD	thiacloprid
N- DMA	N-desmethyl-acetamiprid
6- CINA	6-chloronicotinic acid
EDCs	endocrine disrupting chemicals
LWBC	Laizhou Wan Birth Cohort
NIT	nitenpyram
HPLC–MS/MS	high-performance liquid chromatography coupled with triple quadrupole mass spectrometry
RSDs	relative standard deviations
LODs	limits of detection
SD	standard deviation
ORs	odds ratios
CIs	confidence intervals
TE	total effect
IE	ndirect effect
DE	direct effect

2. Materials and methods

2.1. Study population

The Laizhou Wan Birth Cohort was established in 2010–2013 in the southern coastal area of Laizhou Wan (Bay) of the Bohai Sea, Shandong Province, northern China. Detailed information about the cohort was published elsewhere (Ding et al., 2013; Han et al., 2018; Yao et al., 2019). Briefly, the eligibility criteria for recruitment included expectant mothers with aged ≥18 years old, singleton pregnancy, residence in the area for ≥3 years and having no report of assisted reproduction, chronic or pregnancy-associated hypertension and diabetes, HIV or AIDS infection, and illicit drug use. In total, 773 pregnant women with an average gestational week of 39.47 ± 1.39 weeks met the recruitment criteria and participated in the study (baseline population). Of these 773 subjects, a total of 456 children with their mothers participated in the 7th year follow-up survey. We excluded 55 children without sufficient urine samples for NEOs and oxidative stress measures, 10 children with creatinine concentrations <0.1 g/L, which were considered to be too dilute for accurate analysis (Ding et al., 2017; Eskenazi et al., 2004), and 11 children who were missing important confounder variables. Ultimately, 380 children were included in this study. This research was approved by the Medical Ethics Committee of Xinhua Hospital affiliated with Shanghai Jiao Tong University School of Medicine.

2.2. Data collection and adiposity measures

At the baseline survey, pregnant women were interviewed by trained nurses using standardized questionnaires to collect information on social demographics, living habits, and perceived environmental exposures. Medical and obstetric histories were obtained from medical

records. At the 7th year's follow-up survey, main caregivers of the participating 7-year-old children were asked to complete the follow-up questionnaires, including information on children's dietary habits, sleep quality, and disease history. Every participant provided written informed consent prior to participating in this study.

Children's adiposity measures were measured by trained investigators during the 7-year follow-up. Height (cm) was measured while the children were not wearing hats or shoes with their heels against the wall and standing straight; weight (kg) was measured while the children were wearing thin clothes, without shoes, and with an empty bladder; waist circumference (cm) was measured using a plastic measuring tape, which was placed around the abdomen and close to the skin (Hu et al., 2022). Body fat mass (kg), body fat percentage (%), and visceral fat area (cm^2) were estimated using a body composition analyzer (S10, Inbody Co. Ltd) while the children were wearing thin clothes with bare hands and feet, and had fasted or eaten at least 2–3 h previously (Bukowska et al., 2021).

Body mass index (BMI), waist-to-height ratio (WHtR) and fat mass index were calculated using the following formulas: BMI = weight (kg)/height^2 (m^2); WHtR = waist circumference (cm)/height (cm); and fat mass index = body fat mass (kg)/height^2 (m^2). Sex- and age-specific height z-scores, weight z-scores and BMI z-scores were calculated according to the 2007 World Health Organization growth standard (De Onis et al., 2009). Children with BMI z-scores ≥85th percentile were classified as overweight/obesity (Grace et al., 2021), and children with WHtR ≥0.5 were defined as abdominal obesity (Gibson and Ashwell, 2020).

2.3. Urinary NEOs/metabolites measurements

Spot random urine samples were collected under the guidance of the children's main caregivers and then aliquoted and stored in polypropylene (PP) tubes at −80 °C until further analysis. During measurement, analytical standards of acetamiprid (ACE), imidacloprid (IMI), thiamethoxam (THM), thiacloprid (THD), clothianidin (CLO), dinotefuran (DIN), nitenpyram (NIT) (Sigma, USA), N-desmethyl-acetamiprid (N-DMA), and 6-chloronicotinic acid (6-CINA) (Dr. Ehrensorfer, GER) and isotopically labeled internal standards of ACE-d_3, IMI-d_4, THM-d_3, THD-d_4 (Sigma, USA), NIT-d_5, 6-CINA-$^{13}C_6$, and N-DMA-$^{13}C_2$ (Cambridge Isotope Laboratories, USA) were used. NEOs/metabolites were detected based on the modified method reported by Pan et al. (2022) via high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (HPLC−MS/MS, Agilent 1290 infinity, Sciex Triple Quad™4500, USA). Briefly, a 3-mL urine sample was spiked with 10 μL of 100 ng/mL internal standards and 300 μL of β-glucuronidase solution (100 Unit/mL). After incubating at 37 °C for 12 h, the urine was extracted and purified by solid-phase extraction (SPE) and was subsequently concentrated to near dryness. After resuspension in 100 μL acetonitrile, the sample was fully vortexed and centrifuged (13200 rpm, 5 min). A total of 10 μL of sample extract was injected into an Eclipse Plus 95A-C18 column (5 μm, 2.1*150 mm; Agilent, USA) with a flow rate of 0.3 mL/min. The mobile phase consisted of phase A (0.1% formic acid solution) and phase B (acetonitrile solution), starting with 70% and 30%, respectively. In 5 min, phases A and B reached 50%, then dropped to 10% and rose to 90%, and ultimately returned to the initial percentage. Strict quality control was conducted during analysis. At intervals of every 20 samples, two filed blanks and two low-concentration quality control samples (0.5 ng/mL) were measured repeatedly. The recovery of spike samples ranged from 80% to 120%. The intra-day and inter-day relative standard deviations (RSDs) were 2.21–9.40% and 7.98–16.63%, respectively. The limits of detection (LODs) ranged from 0.001 to 0.040 ng/mL in this study. Concentrations below the LODs were replaced with the LOD divided by the square root of 2 (Furukawa et al., 2010).

2.4. Oxidative stress measurements

Among the multiple accepted biomarkers for oxidative stress, 8-iso-PGF2α is considered one of the best due to its availability and stability in biological fluids (Gołąb et al., 2022; Mure et al., 2015). 8-iso-PGF2α is produced by the nonenzymatic peroxidation of arachidonic acid in membrane phospholipids (Basu, 2010), which is closely related to childhood obesity as a valid biomarker of lipid peroxidation (Arogbokun et al., 2021). Hence, we used 8-iso-PGF2α to represent the oxidative stress levels in this study. Urinary concentrations of 8-iso-PGF2α were detected using an 8-iso-PGF2α ELISA Kit (Cayman, USA) according to the manufacturers' instructions. The 8-iso-PGF2α values were calculated based on calibration sigmoid plots (ELISA Calc Software) of the absorbance at 405 nm of a standard at various concentrations. In addition, urinary creatinine concentrations were measured by an automated chemistry analyzer (7100, Hitachi, Japan) using a clinically validated enzymatic method.

2.5. Statistical analysis

Descriptive statistics were calculated for the maternal sociodemographic characteristics and childhood adiposity measures using the mean ± standard deviation (SD) or n (%). Distributions of NEOs/metabolites are described with detection rate, range, and quartiles. Independent t tests and chi-square tests were conducted to compare the differences in the baseline characteristics between the included population and the excluded population. Given the skewed distributions, urinary levels of NEOs/metabolites and 8-iso-PGF2α were ln-transformed as continuous variables before analyses. Statistical analyses were limited to NEOs/metabolites with detection rates above 75%.

The associations of NEOs/metabolites with continuous adiposity measures were analyzed by multiple linear regressions, and binary logistic regressions were conducted to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for overweight/obesity and abdominal obesity. To explore the mediating effects of 8-iso-PGF2α on the relationship between NEOs/metabolites and adiposity measures, a mediation analysis was conducted using the R package 'regmedint' if the following criteria were met (Valeri and Vanderweele, 2013; VanderWeele, 2016): 1) exposure significantly affected the mediator; and 2) the mediator significantly affected the outcome. The total effect (TE) of NEOs/metabolites on adiposity measures was decomposed into the indirect effect (IE; i.e., the effect of NEOs/metabolites on adiposity measures that is mediated by 8-iso-PGF2α) and the direct effect (DE; i.e., the estimated effect after controlling for 8-iso-PGF2α), and the estimated proportion mediated by 8-iso-PGF2α was calculated as the β coefficient of the IE divided by the β coefficient of the TE. Considering the potential sex-specific effects of NEOs (Arslan et al., 2016; Godbole et al., 2022), interaction terms between NEOs/metabolites and sex were included in the previously described models, and stratified analysis by sex was further performed.

Directed acyclic graph (DAG) (Fig. S1) was used to select the potential covariates according to our prior knowledge and published literature (Pan et al., 2022; Zhao et al., 2022), including sex (boy, girl), pediatric age (continuous, years), maternal education level (less than high school, high school, college and above), and household monthly income (<3000 CNY, 3000–5000 CNY, >5000 CNY; the median household monthly income of residents in Shandong, China was about 4000 CNY, as a reference). Pre-pregnancy BMI (continuous, kg/m^2), parity (primiparous, multiparous), and method of delivery (vaginal delivery, caesarean) were also included as confounding factors due to their influences on childhood obesity (Gaillard et al., 2014; Huang et al., 2022; Önnestam et al., 2022). Urinary creatinine levels were adjusted as continuous covariates in the final models to control for urine dilution (Jacobson et al., 2019; Liu et al., 2019). Multicollinearity was not detected, as the variance inflation factor was <2 for all covariates.

Several sensitivity analyses were conducted to confirm the

robustness of our results. First, considering that vegetables and fruits are the main sources of dietary NEOs exposure (Chen et al., 2020), and that sleep duration is strongly associated with childhood obesity (Glasgow et al., 2022), the frequency of food (vegetables, fruits) consumption (<1 time/week, 1–3 times/week, ≥4 times/week) and sleep duration (continuous, hours) were included as additional covariates in the models. Second, children with preterm delivery (<37 weeks of gestation, n = 7) or low birth weight (<2500 g, n = 7) were further excluded to verify our findings, and 366 children were ultimately included in the sensitivity analyses. Moreover, the restricted cubic spline (RCS) model was used to evaluate the nonlinear relationship between NEOs/metabolites and adiposity measures. Statistical evaluations were carried out using SPSS v.22 and R software (version 4.1.3). All statistical tests were two-sided tests and $P < 0.05$ was considered significant.

3. Results

3.1. Population characteristics and adiposity measures

The characteristics and adiposity measures of the study population are shown in Table 1 (Table S1). A total of 68.2% of the mothers were primiparous, 56.5% had a high school education or higher, and 37.9% had a household monthly income of more than 3000 CYN. No significant difference was observed in demographic characteristics between the included population (n = 380) and the excluded population (n = 393) based on the LWBC (Table S1). The average age of the 380 participating children was 7.68 ± 0.62 years old, and 53.2% of them were boys. The mean values (SDs) of various adiposity measures were 128.78 (6.44) cm for height, 28.01 (6.81) kg for weight, 16.73 (3.04) kg/m^2 for BMI, 58.79 (8.23) cm for waist circumference, 0.46 (0.05) for WHtR, and 21.93 (8.51)% for body fat percentage. Of these children, 57 (15.0%) were considered as overweight/obesity, and 67 (17.6%) were considered as abdominal obesity. The adiposity measures were significantly higher in boys than in girls ($P < 0.05$), except for height (Table 1).

Table 1
Adiposity measures of 7-year-old children in this study based on the Laizhou Wan Birth Cohort.

Adiposity measures	Mean ± SD or n (%)			*P* value[a]
	Total (n = 380)	Boys (n = 202)	Girls (n = 178)	(Boys vs Girls)
Height (cm)	128.78 ± 6.44	129.61 ± 6.28	127.84 ± 6.50	0.704
Weight (kg)	28.01 ± 6.81	29.37 ± 7.40	26.47 ± 5.71	0.001
BMI (kg/m^2)	16.73 ± 3.04	17.32 ± 3.35	16.07 ± 2.50	<0.001
Height z-score	0.65 ± 0.96	0.71 ± 0.93	0.59 ± 0.99	0.592
Weight z-score	0.67 ± 1.35	0.92 ± 1.45	0.40 ± 1.18	0.004
BMI z-score	0.37 ± 1.49	0.64 ± 1.65	0.06 ± 1.22	<0.001
Waist circumference (cm)	58.79 ± 8.23	60.45 ± 8.78	56.91 ± 7.13	0.002
WHtR	0.46 ± 0.05	0.47 ± 0.06	0.45 ± 0.05	0.005
Body fat mass (kg)	6.61 ± 4.23	7.05 ± 4.84	6.10 ± 3.35	<0.001
Body fat percentage (%)	21.93 ± 8.51	22.04 ± 9.54	21.82 ± 7.19	<0.001
Fat mass index (kg/m^2)	3.90 ± 2.30	4.10 ± 2.62	3.66 ± 1.85	<0.001
Visceral fat area (cm^2)	30.25 ± 21.58	32.73 ± 25.02	27.42 ± 16.47	<0.001
Categorized by BMI z-scores				
Normal (<85th)	323 (85.0%)	155 (76.7%)	168 (94.4%)	<0.001
Overweight/obesity (≥85th)	57 (15.0%)	47 (23.3%)	10 (5.6%)	
Categorized by WHtR				
Normal (<0.5)	313 (82.4%)	155 (82.4%)	158 (82.4%)	0.002
Abdominal obesity (≥0.5)	67 (17.6%)	47 (17.6%)	20 (17.6%)	

[a] Independent samples *t*-test for continuous variables; Chi-square test for categorical variables.

3.2. Urinary levels of NEOs/metabolites and 8-iso-PGF2α

The urinary levels of NEOs/metabolites and 8-iso-PGF2α are summarized in Table 2. High detection rates were found for six NEOs, including IMI (99.7%), CLO (98.9%), DIN (97.6%), THM (95.5%), ACE (82.9%), and THD (77.6%), with median concentrations of 0.241, 0.258, 0.111, 0.044, 0.003, and 0.006 ng/mL, respectively. The detection rate of NIT was only 36.6%, which was regarded as too low to perform further meaningful analyses. For the two metabolites of NEOs, the median concentrations of N-DMA (detection rate: 100.0%) and 6-CINA (97.9%) were 0.565 and 0.496 ng/mL, respectively. Moreover, the creatinine-unadjusted and creatinine-adjusted median concentrations of 8-iso-PGF2α in our study were 0.725 ng/mL and 1.328 μg/g creatinine (Table 2), which were at a moderate level compared to those of children aged 6–10 years in other countries (Kordas et al., 2018; Selvaraju et al., 2019; Yamano et al., 2015).

3.3. Associations of NEOs/metabolites with adiposity measures and effect modification by sex

The associations of NEOs/metabolites levels with continuous adiposity measures in 7-year-old children are presented in Fig. 1 (Table S2). For NEOs, exposure to IMI was associated with a 0.986-cm increase in waist circumference (95% CI: 0.111, 1.861) and an increase of 0.007 in WHtR (95% CI: 0.001, 0.013). Similarly, exposure to THM was associated with a 0.927-cm increase in waist circumference (95% CI: 0.186, 1.669) and an increase of 0.006 in WHtR (95% CI: 0.001, 0.011). For the two metabolites of NEOs, exposure to 6-CINA was positively associated with body fat percentage (β = 0.565, 95% CI: 0.047, 1.083), while significant negative associations were observed between N-DMA exposure and body fat percentage (β = −1.189, 95% CI: -1.965, −0.414) and fat mass index (β = −0.231, 95% CI: -0.440, −0.022). The results showed that there were sex differences in the associations between NEOs/metabolites and continuous adiposity measures (Table S2). Specifically, sex modified the association between CLO and height z-score ($p_{int} = 0.043$) with a positive association in girls (β = 0.161, 95% CI: 0.032, 0.289), and an insignificant inverse association in boys. Sex differences were also observed in the associations of DIN exposure with body fat mass ($p_{int} = 0.032$), body fat percentage ($p_{int} = 0.009$) and fat mass index ($p_{int} = 0.037$), with negative associations in girls (body fat mass: β = −0.658, 95% CI: -1.143, −0.173; body fat percentage: β = −1.468, 95% CI: -2.512, −0.424; fat mass index: β = −0.352, 95% CI: -0.622, −0.082) and nonsignificant positive associations in boys.

The risk of NEOs/metabolites levels on childhood obesity was further explored, and the ORs and 95% CIs are shown in Table 3. Eeposure to IMI was found to be associated with a higher risk of childhood overweight/obesity (OR = 1.556, 95% CI: 1.100, 2.201) and abdominal obesity (OR = 1.478, 95% CI: 1.078, 2.026). After stratification by sex, similar significant associations were only pronounced among boys, showing that IMI exposure was positively associated with overweight/obesity (OR = 1.504, 95% CI: 1.013, 2.232) and abdominal obesity (OR = 1.498, 95% CI: 1.005, 2.231), and sex modified the association between DIN exposure and the rate of overweight/obesity ($p_{int} = 0.046$), with a significant negative association among girls (OR = 0.356, 95% CI: 0.135, 0.939), and a null association among boys.

3.4. Relationships of 8-iso-PGF2α with NEOs/metabolites and adiposity measures

The relationships of NEOs/metabolites with 8-iso-PGF2α and the relationships of 8-iso-PGF2α with adiposity measures are presented in

Table 2
Concentrations of urinary NEOs/metabolites and 8-iso-PGF2α among 7-year-old children in northern China.

	LOD (ng/mL)	Detection rate (%)	Unadjusted (ng/mL)				Creatinine adjusted (μg/g creatinine)			
			Range	Percentile			Range	Percentile		
				25th	50th	75th		25th	50th	75th
ACE	0.0017	82.9	< LOD - 0.601	0.002	0.003	0.007	< LOD[a] - 1.125	0.003	0.007	0.014
IMI	0.0133	99.7	< LOD - 9.957	0.137	0.241	0.480	< LOD[a] - 12.707	0.249	0.454	0.822
THD	0.0033	77.6	< LOD - 0.071	0.004	0.006	0.009	< LOD[a] - 0.272	0.007	0.010	0.017
CLO	0.0333	98.9	< LOD - 17.004	0.122	0.258	0.664	< LOD[a] - 28.162	0.206	0.488	1.159
THM	0.0067	95.5	< LOD - 5.312	0.021	0.044	0.091	< LOD[a] - 8.798	0.036	0.074	0.150
DIN	0.0133	97.6	< LOD - 2.027	0.054	0.111	0.239	< LOD[a] - 3.508	0.108	0.193	0.376
NIT	0.0067	36.6	< LOD - 1.010	< LOD	< LOD	0.011	< LOD[a] - 1.325	< LOD[a]	< LOD[a]	0.021
N-DMA	0.0067	100.0	0.031–11.364	0.265	0.565	1.114	0.059–36.031	0.434	0.977	2.129
6-CINA	0.0167	97.9	< LOD - 18.724	0.129	0.496	1.887	< LOD[a] - 29.446	0.238	0.967	3.109
8-iso-PGF2α	–	–	0.008–6.371	0.423	0.725	1.528	0.015–51.916	0.985	1.328	1.866

[a] Below the limits of detection for the urinary concentrations were not corrected for creatinine.

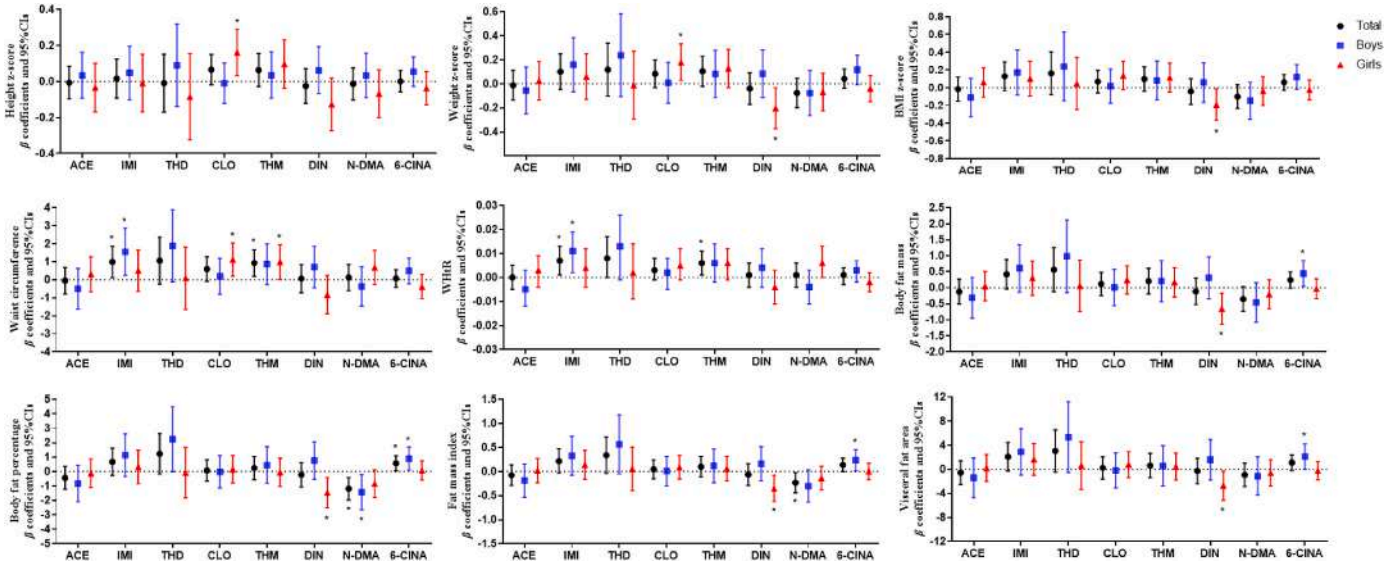

Fig. 1. Changes (95%CI) in adiposity measures associated with NEOs/metabolites based on linear regression models. Concentrations of NEOs/metabolites were ln-transformed. Models in the total population (n = 380) were adjusted for urinary creatinine levels, sex, pediatric age, maternal education, household monthly income, pre-pregnancy BMI, parity and method of delivery. Models in boys (n = 202) or girls (n = 178) were adjusted for urinary creatinine levels, pediatric age, maternal education, household monthly income, pre-pregnancy BMI, parity and method of delivery.
*Significant at $P < 0.05$.

Table S3 and Table S4, respectively. IMI, THD, CLO, THM, DIN and N-DMA were positively associated with 8-iso-PGF2α ($P < 0.001$) (Table S3). Concentrations of 8-iso-PGF2α were associated with increased BMI z-score, waist circumference, WHtR, body fat mass, body fat percentage and fat mass index ($P < 0.05$). A null association of 8-iso-PGF2α with the risk of overweight/obesity and abdominal obesity was observed in the logistic regression model (Table S4).

3.5. Mediation analysis

Mediation analysis was used to estimate the proportions of the effects of NEOs/metabolites (except ACE and 6-CINA) on adiposity measures (BMI z-score, waist circumference, WHtR, body fat mass, body fat percentage and fat mass index) mediated by 8-iso-PGF2α (Table S5). The results showed that a significant mediation effect of 8-isoPGF2α was observed for the associations of IMI (IE: β = 0.002, 95% CI: 0.000, 0.005), THD (IE: β = 0.006, 95% CI: 0.000, 0.012) and THM (IE: β = 0.003, 95% CI: 0.000, 0.006) with WHtR, and the proportions were 27.92%, 69.52% and 35.37%, respectively.

3.6. Sensitivity analyses

Sensitivity analyses demonstrated the robustness of our results, as the overall findings were not affected by adjustments for additional covariates (the frequency of vegetables consumption, the frequency of fruits consumption, and sleep duration), or by removing children with preterm delivery or low birth weight from the total population (Table S6) and the sex-stratified population (Table S7) in all models. However, removing children with preterm delivery or low birth weight resulted in some findings that were no longer significant in the mediation analysis, including the mediation effect of 8-isoPGF2α in the associations of IMI, THD and THM with WHtR (Table S8). Moreover, the RCS model depicted the dose–response relationship between NEOs/metabolites and adiposity measures (Fig. S2). Statistically significant nonlinear associations were found in the relationship of ACE with height z-score and weight z-score, in the relationship of IMI with weight z-score, waist circumference, WHtR, body fat mass, fat mass index and visceral fat area, and in the relationship of CLO with height z-score ($P < 0.05$ for the nonlinear test).

Table 3
Odds ratios (OR) and 95% confidence intervals (CIs) for urinary NEOs/metabolites concentrations and childhood obesity.

NEOs/ metabolites	Binary logistic regression OR (95% CI)							
	Overweight/obesity (BMI z-scores ≥85th, n = 57)			P_{int}	Abdominal obesity (WHtR ≥0.5, n = 67)			P_{int}
	Total[a]	Boys[b]	Girls[b]		Total[a]	Boys[b]	Girls[b]	
ACE	0.844 (0.624, 1.141)	0.728 (0.503,1.054)	1.309 (0.752,2.279)	0.112	0.909 (0.697, 1.184)	0.750 (0.522,1.077)	1.216 (0.805,1.837)	0.083
IMI	**1.556 (1.100, 2.201)***	**1.504 (1.013,2.232)***	1.979 (0.865,4.532)	0.800	**1.478 (1.078, 2.026)***	**1.498 (1.005,2.231)***	1.468 (0.871,2.474)	0.983
THD	1.262 (0.758, 2.102)	1.547 (0.851,2.812)	0.836 (0.236,2.960)	0.390	1.335 (0.850, 2.097)	1.670 (0.916,3.045)	1.021 (0.481,2.170)	0.482
CLO	1.074 (0.830, 1.390)	1.100 (0.824,1.469)	1.107 (0.623,1.966)	0.915	1.196 (0.940, 1.520)	1.145 (0.851,1.540)	1.329 (0.876,2.014)	0.486
THM	1.150 (0.864, 1.532)	1.217 (0.878,1.687)	0.994 (0.529,1.869)	0.706	1.284 (0.989, 1.666)	1.316 (0.944,1.835)	1.330 (0.854,2.070)	0.889
DIN	1.016 (0.756, 1.365)	1.236 (0.878,1.739)	**0.356 (0.135,0.939)***	**0.046**	1.119 (0.854, 1.466)	1.394 (0.981,1.981)	0.732 (0.450,1.190)	0.083
N-DMA	0.831 (0.621, 1.113)	0.879 (0.630,1.226)	0.659 (0.341,1.273)	0.488	0.961 (0.738, 1.251)	0.849 (0.605,1.192)	1.183 (0.768,1.824)	0.213
6-CINA	1.067 (0.888, 1.282)	1.156 (0.936,1.427)	0.860 (0.557,1.328)	0.302	1.037 (0.876, 1.228)	1.167 (0.940,1.448)	0.833 (0.616,1.126)	0.141

*Significant at $P < 0.05$.

[a] The models were adjusted for urinary creatinine levels, sex, pediatric age, maternal education, household monthly income, pre-pregnancy BMI, parity and method of delivery.

[b] The models were adjusted for urinary creatinine levels, pediatric age, maternal education, household monthly income, pre-pregnancy BMI, parity and method of delivery.

4. Discussion

In the present study, we detected urinary levels of NEOs/metabolites in 7-year-old children from northern China, estimated their associations with adiposity measures, investigated the role of oxidative stress in these associations, and explored whether sex modifies the effect. We observed relatively high detection rates and concentrations of NEOs/metabolites, positive associations of IMI, THM and 6-CINA with certain adiposity measures, such as waist circumference, WHtR and body fat percentage, and negative associations of N-DMA with body fat percentage and fat mass index. Furthermore, 8-iso-PGF2α may be a potential mediator regulating the relationships of IMI, THD and THM with increased WHtR, and effect modification by sex was observed.

Past studies of human exposure to NEOs/metabolites mainly focused on adults, and evidence is still limited for children. To date, only nine studies have reported urinary levels of NEOs/metabolites in children worldwide, including four Chinese studies (Wang et al., 2020a; Yue et al., 2022; Zhao et al., 2022; Zhou et al., 2021), three Japanese studies (Ikenaka et al., 2019; Osaka et al., 2016; Oya et al., 2021), one U.S. study (Ospina et al., 2019), and one European study (Laubscher et al., 2022) (Table S9). In general, relatively higher detection rates and concentrations of urinary NEOs/metabolites were observed in Chinese children than in children from other countries. For instance, a Japanese study reported the detection rates of IMI, ACE, THM and CLO as 13%, 9%, 28%, and 41% in 3- to 6-year-old children, respectively (Ikenaka et al., 2019). However, relatively higher detection rates of IMI (95.7%), ACE (95.1%), THM (99.7%) and CLO (93.4%) were reported in 305 Chinese children aged 8–11 years (Zhao et al., 2022), with levels similar to those in our study. Moreover, the median concentrations of CLO (0.258 ng/mL) detected among 7-year-old children in this study were slightly higher than those reported in American children aged 6–11 years (n = 416, <0.20 ng/mL) (Ospina et al., 2019) and in Japanese children (n = 1036, <0.13 ng/mL) (Oya et al., 2021). Similarly, compared with the Japanese study (Oya et al., 2021), our study had a relatively higher median concentration of IMI (China vs. Japan = 0.241 vs. < 0.07 ng/mL). Additionally, the median concentration of N-DMA (0.565 ng/mL) was highest in our study, which was higher than that in Japanese children (0.39 ng/mL) (Ikenaka et al., 2019) and that of Swedish children (0.324 ng/mL) (Laubscher et al., 2022). Compared with developed countries (i.e., the USA and Japan), China has higher NEOs usages for agricultural production (Li et al., 2019; Tao et al., 2019). Thus, Chinese children might have more chances for exposure to NEOs via the route of vegetables and fruits intake (Zhang and Lu, 2022; Zhang et al., 2019a), which could partly explain the higher urinary concentrations of NEOs/metabolites in the current study compared with studies in other areas.

The associations between NEOs/metabolites and children's adiposity measures were mixed in our study. At present, the results of associations between NEOs levels and obesity are inconsistent in mammalian experiments. Some animal studies have indicated positive relationships between NEOs and obesity (Tanaka, 2012; Yan et al., 2020). For example, after 30 days of exposure to NEOs (4 mg/kg bw/day), significantly increased body weight was observed in ICR mice compared with the control groups (Yan et al., 2020). However, some animal studies have reported inverse associations (Mosbah et al., 2018; Sheets et al., 2016). For instance, wistar rats aged 8–12 weeks old with ACE (27 mg/kg/day) in their diet for over 45 days exhibited a greater decrease in weight (Mosbah et al., 2018). Unlike existing animal studies, only four epidemiological surveys investigated the relationships between NEOs/metabolites and adiposity measures, with two focusing on children. A biomonitoring-based study reported that N-DMA concentrations were associated with higher odds of obesity in school-age children from Shanghai, China, though the association was only of marginal significance (β = 2.03, 95% CI: 0.99, 4.17) (Wang et al., 2020a). A null association between 6-CINA and BMI z-score was reported among 11- to 12-year-old children in Cyprus (Makris et al., 2019). Among US adults, detectable levels of ACE were associated with decreased BMI, waist circumference, body fat percentage and fat mass index, but exposure to the metabolite of IMI was associated with greater rates of overweight/obesity (Godbole et al., 2022). In addition, Peng et al. observed that concentrations of IMI in hair samples were positively associated with BMI (β = 0.03, 95% CI: 0.02, 0.04) among Chinese women (Peng et al., 2020). In short, exposure to NEOs may be closely correlated with adiposity measures, but the results of existing studies are mixed. Considering that the obesogenic effect of NEOs/metabolites remains uncertain, more studies should be conducted in the future.

Sex differences were observed in the influence of certain NEOs on adiposity measures in this study. Specifically, significant negative associations were observed between DIN exposure and body fat mass, body fat percentage, fat mass index and the risk of overweight/obesity among girls, while nonsignificantly positive associations were observed

among boys. Differences in these associations between girls and boys could be explained by some potential biological mechanisms. For example, a protective effect generated by estrogen (Sun et al., 2017) and the activation of PPARα (a major receptor involved in fatty acid metabolism) may be responsible for the decrease in the weight of female mice (Anderson et al., 2004). The adipogenic effect of NEOs in male mice might be related to the increased oxidative stress and higher levels of triglycerides in the liver (Lukowicz et al., 2018). Given the limited evidence and relatively small sample size of the present study, more research is warranted to verify the potential sexually dimorphic traits of NEOs.

We also observed that positive associations of NEOs/metabolites with adiposity measures were mediated by 8-iso-PGF2α. Several epidemiological studies have documented that EDCs (He et al., 2020; Steffensen et al., 2020; Tran et al., 2017) including NEOs could induce peroxidation of lipid and subsequent excessive generation of 8-iso--PGF2α (Li et al., 2020; Makris et al., 2019), which are consistent with our results. Moreover, previous studies reported that 8-iso-PGF2α levels were associated with increased adiposity measures, e.g., weight and BMI (Arogbokun et al., 2021; Jia et al., 2019). It is noteworthy that 8-iso--PGF2α has been documented to be associated with adipose tissue dysfunction, consequentially playing a critical role in the development of obesity and metabolic syndrome (Soldo et al., 2022). Therefore, it is biologically plausible that 8-iso-PGF2α may be involved in associations between NEOs/metabolites exposures and adiposity measures. However, due to the cross-sectional study design, our findings regarding the potential role of 8-iso-PGF2α in the aforementioned associations should be interpreted with caution and it should be confirmed by future prospective studies and experimental studies.

Our study examined the levels of a relatively large number of NEOs/metabolites and provides a possible clue for future research on the mechanism of their effect on adiposity measures. However, the limitations in this study should also be acknowledged. First, the half-lives of NEOs/metabolites (e.g., IMI, CLO, DIN and N-DMA) are short (0.17–1.45 days) in the human body (Harada et al., 2016); therefore, our collection of single-spot urine samples may cause exposure misclassification. Repeated or 24-h urine samples should be collected to better characterize average/integrated exposure of NEOs/metabolites over time in future studies (Xu et al., 2021). Second, we only used creatinine for urine dilution correction, and information about specific gravity was not available, which may be more preferable in children (Wang et al., 2015). Third, the cross-sectional study design in the current study restricted the ability to determine causal relationships between NEOs/metabolites and adiposity measures and the potential mediation of oxidative stress. Our results need to be further verified by longitudinal studies. Fourth, the influence of some confounding factors that are correlated with childhood obesity, such as other environmental chemicals (e.g., heavy metals, organochlorines, pyrethroids) (Nasab et al., 2022; Pinos et al., 2021) and nutritional status (e.g., breast feeding, calorie intake) (Ma et al., 2020) could not be excluded, but the consistent results in sensitivity analyses suggested the robustness of our findings. Finally, selection bias may be a concern due to the relatively high rate of loss to follow-up. However, no significant difference was observed in demographic characteristics between the study population and the excluded population, indicating that our study population could generally represent the whole cohort.

5. Conclusions

As shown in this study, 7-year-old children in northern China are widely exposed to NEOs/metabolites. Our results suggested mixed associations between NEOs/metabolites and adiposity measures in children, with a promoting effect of IMI, THM and 6-CINA and a reverse effect of N-DMA. Oxidative stress may be one of the underlying mechanisms of the effect of NEOs on childhood obesity. Moreover, there were sex differences in the influence of specific NEO exposure (such as DIN) on adiposity measures. Cautious interpretations are recommended until our findings can be replicated in other larger longitudinal studies.

Declaration of competing interest

The authors declare that they have no actual competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114188.

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Modification of low temperature-related hospital admissions for cardiovascular diseases by multiple green space indicators at multiple spatial scales: Evidence from Guangzhou, China

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ABSTRACT

Background: Extreme temperatures have an adverse effect on the occurrence of cardiovascular diseases (CVDs). Previous literatures tend to discuss the modification of CVDs occurrence by green space under high temperature. Relatively less attention is paid to the modification under low temperature. The variation of different attributes and spatial scales of green space in affecting CVDs occurrence are also overlooked.

Methods: This study collected a total of 4364 first-time admission cases due to CVDs in a tertiary hospital in Guangzhou from 2012 to 2018, measured the scale of green space by greening rate (GR) and percentage of landscape (PLAND), the distribution of green space by patch density (PD), mean nearest neighbor distance (ENN_MN) and largest patch index (LPI), and the accessibility of green space by green patch accessibility index (GPAI). Using the time stratified case crossover design method, the modification of low temperature-related CVDs occurrence by the above green space indicators is evaluated in an area with a radius of 100–1000 m which is further divided at an interval of 100 m.

Results: We found high GR, high PLAND, high PD, low ENN_MN, high LPI, and low GPAI corresponds to low risk of CVDs occurrence, the optimal modification scale of each green space indicator, which is radius corresponding to the maximum risk difference between high and low indicator subgroups, is around 800 m (GR), 600 m (PLAND and PD), 500 m (GPAI), and 300 m (LPI and ENN_MN), respectively. As the temperature decreases further, the health benefit from low GPAI at the optimal scale is weakened, whereas the benefits from the others are strengthened.

Conclusions: Low temperature related CVDs occurrence risk can be modified by multiple green space indicators, and these modifications have spatial scale effect. Our findings have important theoretical and practical significance for the formulation and implementation of local green space policies.

1. Introduction

Cardiovascular diseases (CVDs) are a serious threat to global public health (Jagannathan et al., 2019). In China, CVDs are the leading cause of death and premature death (Ma et al., 2020). According to statistics, over 40% of China's population deaths are attributable to CVDs. Existing studies have indicated that abnormal temperature is a key factor in inducing CVDs. Various abnormal temperature factors, such as extreme temperature, cold spells, and heat waves, impact the occurrence of CVDs, among which low temperature has a significant adverse effect on the occurrence of various CVDs subtypes, whereas the adverse effect of high temperature may insignificant in some CVDs subtypes and regions (Phung et al., 2016). Low temperature can induce CVDs through a variety of pathophysiological mechanisms (Freund and Sawka, 1996; Keatinge et al., 1984). Continued attention to the relationship between low temperature and CVDs is crucial (Chen et al., 2019).

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The urban built environment modifies the relationship between air temperature and health, the reason is that various built environment factors affect temperature distribution within the city, this disparity results in different health risks to the public in different parts of the city (Schinasi et al., 2018). Urban "green space" is any vegetation in the urban landscape—a place that provides leisure and recreation for residents and a habitat for nature (Bolund and Hunhammar, 1999; Kabisch and Haase, 2013). Modifying temperature is an important way of green space influencing the human health. It is well known that under high temperature weather, green space can produce cooling effects through shading and evapotranspiration (Davtalab et al., 2020; Yu et al., 2018). Several studies have focused on the modification of high temperature related disease by green space, especially in the context of global warming, for example, Burkart et al. (2016) selected Lisbon, Portugal as the study area to explore the impact of green spaces on heat-related mortality; Bao et al. (2021) studied modification of heat-related stroke by green space in Shenzhen, China. The results of the above studies all show that the high temperature related disease risk decreases with the increase of green space rate. However, as an important climate modification factor, the impact of green space on air temperature is not limited to high temperatures. In low temperatures, green space can protect against cold wind and release sensible heat (Hong et al., 2012; Song and Wang, 2015; Vogel, 1989), this may have a protective effect on human health. To date, little is known about the modification of low temperature-related diseases, including CVDs, by green space. A study conducted in Wuhan, China (a subtropical city) has found that the low temperature related hospital admission risk of ischemic stroke decrease with the increase of green space rate (Li et al., 2021). The modification of low temperature related CVDs by green space is a question worthy of further consideration (Meili et al., 2021; Zhang et al., 2018).

Most of previous studies involving modification of temperature-related CVDs by green space only construct the vegetation scale indicator (Bao et al., 2021; Li et al., 2021), and rarely discuss the spatial distribution pattern of green space. The vegetation density and its arrangement can affect urban microclimate (Hami et al., 2019; Pérez and Perini, 2018), and may affect the relationship between temperature and the occurrence of CVDs. Shen and Lung (2016) selected the Taipei metropolitan area as their study area, and chose the annual mean temperature as the mediating variable. The researchers found that the temperature is positively related to the green patch distance and the green patch fragmentation, and the increase in temperature would increase the mortality of CVDs. However, in this study, the abnormal temperature interval is not set, so the modification by the green space distribution pattern on the low temperature is not analyzed. In addition, the accessibility of green space has been used to study CVDs (Coutts et al., 2010; Ngom et al., 2016). However, there is still a lack of attention to accessibility when discussing the modification of temperature-related CVDs by green space. In terms of spatial scale, previous studies have involved large scales to explore the modification by green space, for example, Shen and Lung (2016) et al. used blocks as research units, and the unit area exceeded 4.3 km^2; Li et al. (2021) took the block divided by the main road network as the research unit, and the unit area could reach 3 km^2. For the elderly, who are most prone to CVDs, the scope may exceed the daily activity space of this group (World Health Organization, 2016). In addition, Previous studies focused on a single spatial scale of green space (Bao et al., 2021; Burkart et al., 2016; Li et al., 2021), the modification by green space may change if the scale is altered. In conclusion, exploring the modification of temperature-related CVDs by green space with different spatial attributes and scales is necessary.

Based on the previous literature (Coutts et al., 2010; Lee et al., 2021; Liu et al., 2022; Ngom et al., 2016; Rahnama and Shaddel, 2019), this study constructed six green space indicators from three aspects: green space scale, distribution pattern, and accessibility. The time-stratified case-crossover design (TSCC) is used to explore the modification of the occurrence of low temperature-related CVDs by each indicator in circular buffers with radii ranging between 100 and 1000 m with the patient's home address as the center, at intervals of 100 m (Liu et al., 2022; World Health Organization, 2016; Yeager et al., 2020). We chose Guangzhou, China as the study area. Guangzhou is a subtropical city with a developed economy in China. Previous studies have shown that low temperature has adverse effects on CVDs in Guangzhou (Yang et al., 2015). The purpose of this study is to solve three important questions: 1) Whether green space attributes affect the occurrence of CVDs under low temperature conditions? 2) What are the characteristics of this effect with the change of spatial scales, and which scale has the strongest modification effect? and, 3) How does the modification change as the temperature further decreases? In the context of rapid urbanization, the answers to the above questions are significant to urban planning and related policy formulation.

2. Data and methods

2.1. The study area and population

Guangzhou is located in southern China and is the capital of Guangdong Province. Guangzhou is a first-tier city in China with a Gross Domestic Product (GDP) of 2.5 trillion yuan in 2020, ranking fourth in China. Guangzhou located at longitude 112°57′ to 114°3′ east and latitude 22°26′ to 23°56′ north, covering an area of 7434.4 square kilometers (2870.4 square miles). Guangzhou belongs to the subtropical monsoon climate, with high temperatures and, rainy summer, mild and dry winter, it has an annual mean temperature of 21.5–22.2 °C (70.7–72.0 °F). According to the results of the seventh census, the population of permanent residents is 18.68 million (National Bureau of Statistics of China, 2020). The study area selected for this study is the central urban area of Guangzhou (Fig. 1), including Liwan District, Haizhu District, Yuexiu District, and Tianhe District. The total area of these districts is 279.63 square kilometers, accounting for 3.8% of the city's total area, with a permanent population of 6.239 million, accounting for 33.2% of the city's population, with a GDP of 1.2 trillion-yuan, accounting for 47.4% of city's GDP.

2.2. Cases and meteorological data

We collected the raw data from a well-known third-class hospital in Guangzhou, located in Yuexiu District. The data comprised information on patients admitted to the hospital for the first time due to CVDs; the disease type is I00–I99 under the ICD-10 coding standard. Only including cases from the central urban area of Guangzhou greatly ensures that the patient's residential address is consistent with the registered address for a period of time before admission. The registration information includes personal information such as age, sex, and place of residence, as well as treatment information such as admission time, admission cost, and number of operations. Baidu map and AMAP are two well-known mapping software in China, providing rich application program interfaces services (APIs), among which geocoding service can convert physical addresses into geographic coordinates, which has been applied in multiple previous studies (Hua, 2018; Li et al., 2019). To ensure the accuracy of the patients' address coordinates, we used both Baidu map and AMAP geocoding services to convert the patients' address into coordinates points and excluded the points in the two sets of results where the projected coordinates representing the same address differ by more than 100 m. The coordinate points with ambiguous addresses were also manually excluded. Finally, we conducted follow-up study based on the remaining Baidu coordinate points.

China Meteorological Data Network (http://data.cma.cn/) provides daily meteorological data for this study. We use data from Guangzhou Station (No. 59287), including information on mean temperatures (unit: 0.1 °C), mean wind speed (unit: 0.1 m/s), atmospheric pressure (unit: 0.1 hPa), and relative humidity (unit: 1%). The temperature indicator, converted to the unit of °C, is taken as the explanatory variable, whereas

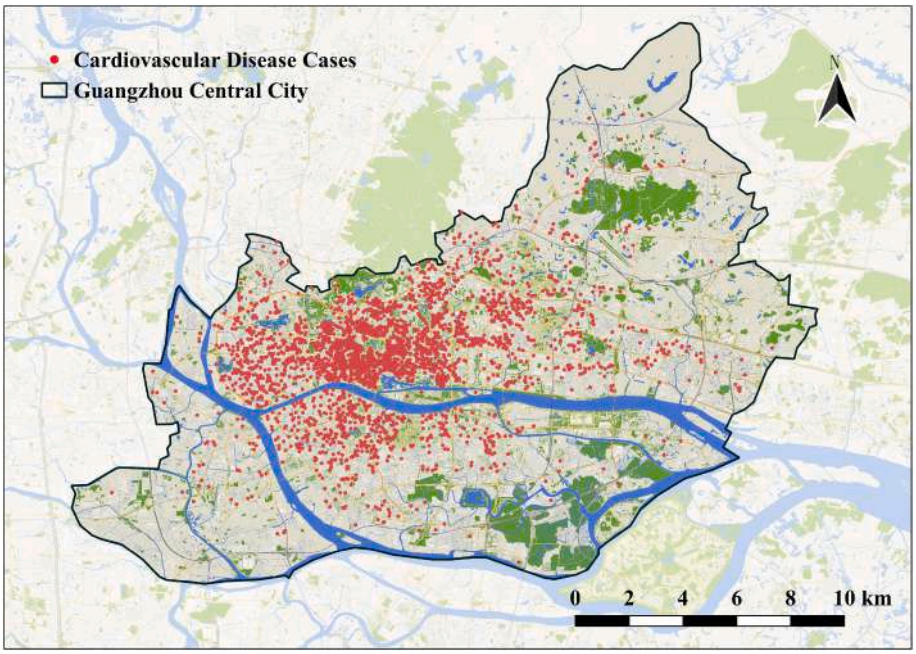

Fig. 1. Overview of the study area and the distribution of cases.

the remaining meteorological indicators are covariates.

2.3. Construction methods of green space indicators

This study reflects the scale, distribution pattern, and accessibility of green space by constructing six different indicators. Indicators reflecting the scale of green space include greening rate (GR) and percentage of landscape (PLAND). Specifically, this study uses the normalized vegetation index (NDVI) to stand for the greening rate. NDVI is widely used to explore the relationship between green space and health due to its simple calculation and good indication. The NDVI data comes from the Landsat spectral indices products over China (Peng et al., 2020) (http://databank.casearth.cn), with a spatial resolution of 30 m and a temporal resolution of 16 days. This study collected all NDVI data from 2012 to 2018. A total of 11 clear sky image products were selected, covering all research years. The pixel values of all images are averaged, and the average image is used as the basis for calculating the GR (Burkart et al., 2016). This study uses the 10-m resolution global land cover in 2017 to calculate the PLAND (Gong et al., 2019; Xu et al., 2017). Referring to previous studies, we select forest, grassland, shrub, wetland (Code: 20, 30, 40, 50) as green space (Bauwelinck et al., 2021), and then combine the four types of green space to calculate the ratio of total green space to buffer area. When constructing the green space distribution pattern and accessibility indicators, the same data as the PLAND is used. The indicators reflecting the layout of green space include: 1) Patch density (PD), the calculation formula is: N/A, where N is the total number of green patches in the buffer, and A is the buffer area. Since we calculate the risk of admission in a specific buffer area, the number of green spaces N is used instead of the density of green spaces. 2) The mean nearest neighbor distance (ENN_MN), the calculation formula is $\sum_{i=1}^{N} D_i/N$, where i represents the green patches in the buffer, D_i is the distance between a green patch and the nearest neighbor green patch, and N is the total number of green patches in the buffer. This indicator expresses the concentration of green space distribution. The larger the value, the more discrete the green space distribution and the lower the adjacency. 3) Largest patch index (LPI) is the ratio of the largest green space patch area in the buffer to the buffer area. The calculation formula of green patch accessibility index (GPAI) is: $\sum_{i=1}^{N} D_{ij}(A - A_i)/A(N-1)$, where j represents the patient's address, D_{ij} is the distance between a patch in the buffer and the patient's address, A_i is the area of a patch in the buffer, and N is the total number of green patches in the buffer. This formula expresses the average distance from the patient's address to each green patch (weighted by the patch area). The value increases with the increase of the distance between the home address and the green patch, and decreases with the increase of the patch area. The larger the value, the less accessible the public is to the surrounding green space.

All the above indicators are counted in the 100 m–1000 m circular buffer centered on the home address. The above-mentioned green space indicators construction and numerical calculation were implemented by ArcGIS10.5 software, QGIS3.16 software, and python language.

2.4. Statistical analysis

In this study, the time stratified case crossover design (TSCC) is used to construct an experimental group and a control group. The TSCC design adopts a self-matching method, taking several time points before and after the onset of the patient as a control, reducing the data volume requirements and eliminating potential confounding factors that do not change with time, such as individual's age, sex, basic health status, socio-economic situation, etc. This approach is widely used when studying the relationship between short-term environmental exposure factors and diseases (Mostofsky et al., 2018). For this study, all identical days of the week within the 30 days prior to admission were selected as controls (Liu et al., 2021). According to the protocol, each case had 3 or 4 controls. Conditional logistic regression is used to fit the TSCC, and we apply a cubic spline function to fit the mean temperature with 4 degrees of freedom (df) and a cubic spline function with 3 df to fit all covariates (Chen et al., 2021; Guo et al., 2017). The formula is as follows:

$$ln(h(t,\ X)) = ln(h_0(t)) + ns(TEM, df = 4)\beta_1 + ns(RHU, df = 3)\beta_2 + ns(WIN, df = 3)\beta_3 + ns(PRS, df = 3)\beta_4$$

Where t is the date of CVDs hospital admission; X represents independent variable, includes explanatory variables and covariates; $ln(h(t,X))$ represents the risk function of exposure to X on the day t; $ln(h_0(t))$ is the baseline risk function; $\beta_1 - \beta_4$ represents the variable coefficients. In our study, the OR (Odds Ratio) is expressed as the risk of a certain temperature value relative to the reference temperature at which admission is not affected, here we set the 50th percentile as the reference tem-

perature. The significance level is set as 0.05, and the 95% confidence interval is calculated. The 5th percentile is used as low temperature calculation point, and the 2.5th percentile is used as the extremely low temperature calculation point to explore the modification by green space when the temperature further decrease. We take 30 days as the preset maximum lag days (Huang et al., 2022; Zhao et al., 2022). To explore cumulative effect of temperature, we use the moving-average lag structure, which calculates the average multi-day temperature within a certain lag period (Zhang et al., 2021). We calculate the cumulative low temperature risk of each lag day, and select the best lag period based on the highest OR and significance of the OR for subsequent research on the modification by green space (Wichmann et al., 2012). In this study, the median of each green space indicator under each buffer radius is used to stratify all cases into two subgroups, by comparing the difference of low temperature-related CVDs admission risk between high and low indicator subgroups, to explore the modification strength by the indicator. For any specific green space indicator, the corresponding radius with the largest OR difference and smallest confidence interval overlap is selected as the optimal modifying scale.

In the sensitivity analysis, we observe the OR and its significance of each cumulative lag period under different dfs (3–7) of the temperature variable, which further help us choose the best lag period for the following study on the modification by green space. This study used the R language (version 4.2.1) Survival package (version 3.3–1) and the DLNM package (version 2.4.7) to achieve the above statistical analysis. The copyright of the R language belongs to the entire open-source community, and anyone is free to use, modify and distribute the code of the R language.

3. Result

3.1. Descriptive statistics

After the above screening process, a total of 4364 cases were included in this study, with a time span of 2012/9/3–2018/9/3. Male cases accounted for 56% of the total number of cases, and elderly cases aged 65 and above accounted for 52% of the total number of cases. There were slight annual variations in admission counts despite the absence of a clear-cut downward or upward trend over the study period (Fig. 2a). The mean temperature indicated an obvious V-shaped fluctuation trend with seasonal changes (Fig. 2b), the temperature ranged from 3.4 °C to 31.1 °C throughout the study time. In exploring the relationship between low temperature and admission to CVDs, the reference temperature is set to 23.4 °C (50th percentiles), the low temperature is set to 10.9 °C (5th percentiles), and the extremely low temperature is set to 9.3 °C (2.5th percentiles).

The GR and the PLAND, the GR and the LPI, and the PLAND and the LPI all have a high positive correlation, especially the correlation coefficients between the PLAND and the LPI in all radius buffers are above 0.86, indicating that the buffer with a larger percentage of landscape is more likely to have a larger single green space patch. The correlation coefficients of PD and ENN_MN in all radii are below −0.66, indicating that the higher the number of green patches, the better the adjacency of the green patches (Table S1). The stratification standards of each green space indicator under different radius buffers are shown in Table 1.

3.2. The hospital admission risk of low temperature-related CVDs in each cumulative lag period

With the increase in the cumulative lag period, the admission risk of low temperature-related CVDs goes through a decline process from the cumulative lag of 0 days to the cumulative lag of 16 days (Fig. 3). The peak OR is reached in the cumulative lag of 2 days (OR: 1.3 [95%CI: 1.03, 1.65]), and there is a OR extreme value which is greater than the ORs on the left and right lags (OR: 1.28 [95%CI: 0.99, 1.64]) in the cumulative lag of 7 days. After the cumulative lag of 16 days, the risk curve becomes stable, and the confidence interval widens. When changing the dfs (3–7) of the explanatory variables, the cumulative lag 2-day and 7-day hospital admission ORs also reach extreme values, and the lag 2-day ORs are significant in all dfs, and the lag 7-day ORs are significant in all dfs except 4 (Table S2). Based on the above results, the modification by green space on low temperature-related CVDs is expressed in terms of cumulative lag of 2 days, and the result of lag of 7 days is used as auxiliary analysis.

3.3. The modification by green space indicators on admission risk of low temperature-related CVDs

3.3.1. The risk modification trend by each green space indicator with the change of radius

In terms of green space scale, results with a cumulative lag of 2 days indicate that low GR and PLAND corresponds to higher admission risk in all radius buffers. For the low GR (Fig. 4a), In the radius of 100 m–600 m, the risk changes to a “V" shape, and 400 m is the turning point of the risk from falling to rising. In the radius of 600 m–800 m, the risk also changes to a “V" shape, and 700 m is the turning point. After 800 m, the modification by GR tends to be stable. In the radius of 800 m, the risk reaches its maximum (OR: 1.52 [95%CI: 1.09, 2.11]), which increases by 42% relative to high GR. For the low PLAND (Fig. 4b), the risk curve on both sides of the 600 m radius changes to a “U" shape, but the change

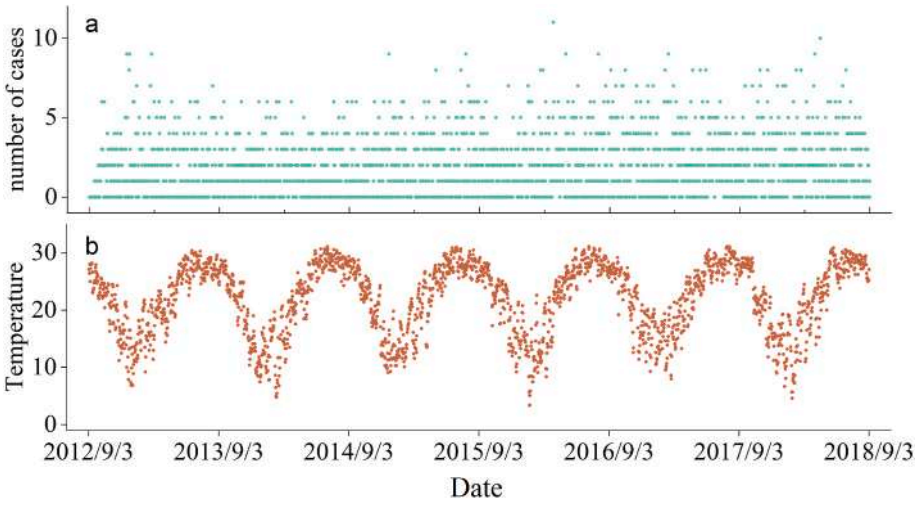

Fig. 2. The daily CVDs cases and daily mean temperature changes.

Table 1
Stratifications for high and low indicator subgroups of each green space indicator under different radius buffers. Abbreviations: Greening rate (GR), Percentage of landscape (PLAND), Patch density (PD), Largest patch index (LPI), Mean nearest neighbor distance (ENN_MN), Green patch accessibility index (GPAI).

Radius(m)	Low GR	High GR	Low PLAND (%)	High PLAND (%)	Low PD	High PD
100	0.11–0.27	0.27–0.6	0–7.26	7.26–78.97	0–6	6–19
200	0.14–0.28	0.28–0.62	0–9.23	9.23–86.14	0–21	21–47
300	0.16–0.28	0.28–0.63	0.19–9.6	9.6–87.6	1–45	45–90
400	0.17–0.28	0.28–0.63	0.88–11.16	11.16–87.01	6–78	78–132
500	0.18–0.28	0.28–0.62	1.47–11.23	11.23–86.05	11–119	119–185
600	0.19–0.29	0.29–0.61	1.65–12.39	12.39–82.29	21–172	172–245
700	0.2–0.29	0.29–0.58	1.88–13.13	13.13–75.35	40–234	234–312
800	0.2–0.29	0.29–0.57	2.09–12.93	12.93–67.12	77–301	301–385
900	0.21–0.29	0.29–0.57	2.44–14.22	14.22–66.33	99–377	377–468
1000	0.21–0.29	0.29–0.57	2.33–13.36	13.36–66.14	115–456	456–571
Radius (m)	Low LPI (m^2)	High LPI (m^2)	Low ENN_MN (m)	High ENN_MN (m)	Low GPAI (m)	High GPAI (m)
100	0–1008	1008–24755	13.56–47.2	47.2–200	0–65.73	65.73–99.93
200	0–3206	3206–107957	19.93–43.6	43.6–226	0–130.47	130.47–198.5
300	184–6291	6291–248295	23.82–42.67	42.67–130	118.79–196.39	196.39–276.45
400	916–9617	9617–436235	30.2–42.29	42.29–120.93	156.03–261.39	261.39–336.88
500	1465–14654	14654–668867	31.14–42.08	42.08–77.29	235.08–326.28	326.28–402.46
600	1557–21533	21533–914535	32.53–41.73	41.73–78.96	298.37–393.16	393.16–470.73
700	1557–32493	32493–1137489	33.22–41.46	41.46–79.1	342.83–459.06	459.06–544.56
800	2656–46066	46066–1307173	34.08–41.33	41.33–66.05	415.24–525	525–642.81
900	2931–61707	61707–1556269	34.34–41.26	41.26–64.97	487.03–591.23	591.23–718.76
1000	3114–84253	84253–1882368	33.9–41.28	41.28–65.59	538.05–657.65	657.65–778.44

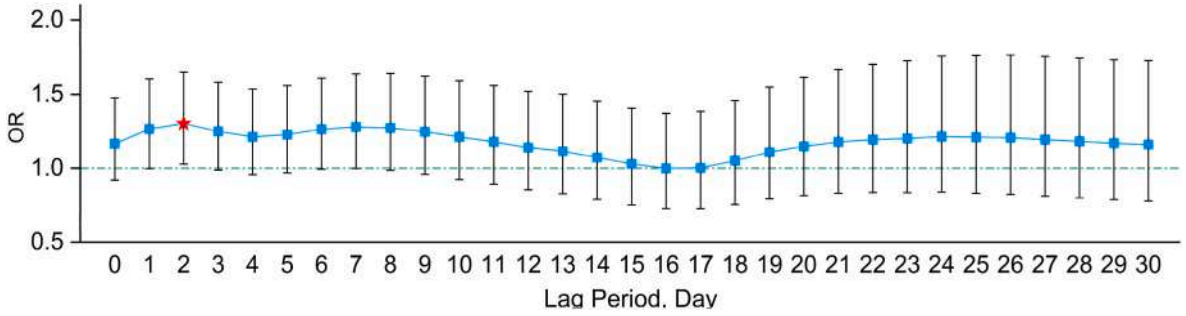

Fig. 3. The lag risk curve of low temperature-related CVDs admission (5th percentiles of temperature distribution). The red star indicates the highest and significant OR with lag 2 days. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

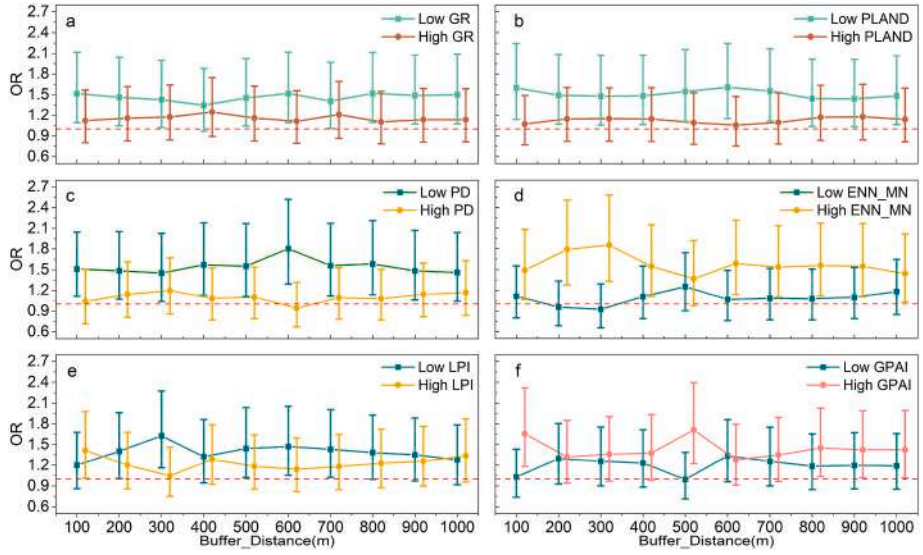

Fig. 4. The admission risk of low temperature-related CVDs corresponding to the subgroups of six green space indicators in all radii (cumulative lag of 2 days). Abbreviations: Greening rate (GR), Percentage of landscape (PLAND), Patch density (PD), Largest patch index (LPI), Mean nearest neighbor distance (ENN_MN), Green patch accessibility index (GPAI). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

process is more gradual than GR. Here, 300 m and 800 m are the turning points of the risk change. In the radius of 600 m, the risk reaches its maximum (OR: 1.61 [95%CI: 1.15, 2.24]), which increases by 56% relative to the high PLAND. The cumulative 7-day lag results also show that the larger green space scale is associated with a lower admission risk (Table S3). As the radius increases, the risk trend is similar to the cumulative lag of 2 days. The modification by GR is the strongest at 900 m, but the degree of modification is not obviously different from that at 800 m. The PLAND also has the strongest modification at 600 m.

In terms of spatial distribution pattern of green space, results with a cumulative lag of 2 days indicate that low PD, high ENN_MN and low LPI correspond to higher risk in vast majority radius buffers. 1) For the low PD (Fig. 4c), The risk in the radius of 100 m–600 m shows an overall upward trend. After 600 m, the admission risk gradually decreases. In the radius of 600 m, the risk reaches its highest (OR: 1.8 [95%CI: 1.29, 2.52]), which is increases by 86% compared to the high PD. 2) For the high ENN_MN (Fig. 4d), the risk is on the rise in the radius of 100 m–300 m. In radius of 300 m–600 m, the risk changes to a "V" shape. The risk gradually weakens after 600 m. In the radius of 300 m, the risk reaches its maximum (OR: 1.85 [95%CI: 1.33, 2.58]), which increases by 93% relative to the low ENN_MN. 3) Except for the radius of 100 m, the low LPI in all radius buffers correspond to a higher admission risk (Fig. 4e). For low LPI, As the radius changes from 100 m to 300 m, the risk gradually increases. In the radius of 300 m–400 m, the risk decrease. After 400 m, the risk shows a slow upward-downward trend. In the radius of 1000 m, the modification by LPI basically disappears. In the radius of 300 m, the risk reaches its maximum (OR: 1.63 [95%CI 1.16, 2.27]), which increases by 58% relative to the high LPI. The results of the cumulative lag of 7 days (Tables S4 and S5) are similar to the results of the cumulative lag of 2 days. The modification by PD is the strongest in 600 m, the ENN_MN has the strongest modification in 300 m, and the LPI has the strongest modification in 300 m.

The results with a cumulative lag of 2 days shows that high GPAI corresponds to a higher admission risk of low temperature-related CVDs in all radii except 600 m (Fig. 4f). For high GPAI, In the radius of 100 m–200 m, the admission risk decrease, and the GPAI has a weak modification in the radius of 200 m–400 m. The risk increases in the radius of 400 m–500 m and decrease in the radius of 500 m–600 m. After 600 m, the risk increases slowly. After 800 m, the risk tends to stabilize. In 500 m radius buffer, the modification reaches its peak (OR: 1.71 [95% CI: 1.22, 2.39]), which increases by 72% relative to low GPAI. The results of the cumulative lag of 7 days (Table S5) show that with the increase of radius, the OR value change trend is similar to that of the cumulative lag of 2 days. The modification by GPAI is the strongest at 900 m, but the degree of modification is not obviously different from that at 500 m.

3.3.2. *Analysis of the modification by green space indicators with the temperature further decreases (from the 5th percentile to the 2.5th percentiles)*

Based on the results of cumulative lag of 2 days and 7 days, the optimal scales are selected, and the modification by each green space indicator is analyzed when the temperature further decreases (Fig. 5). Specifically, a radius of 800 m is selected for the GR, a radius of 600 m for the PLAND and the PD, a radius of 300 m for the LPI and the ENN_MN, and a radius of 500 m for the GPAI.

Regarding the results with a cumulative lag of 2 days, for the low GR, the OR value of the 2.5th percentiles is 1.69 [1.17, 2.43], which increases the risk by 17% compared to the 5th percentiles; for the high GR, the OR value of the 2.5th percentiles is 1.13 [0.77, 1.66], which increases the risk by 3% compared to the 5th percentiles (Fig. 5a). For the low PLAND, the OR value of the 2.5th percentiles is 1.77 [1.22, 2.56], which increases the risk by 16% compared to the 5th percentiles; for the high PLAND, the OR value of the 2.5th percentiles is 1.10 [0.76,1.60], which increases the risk by 5% compared to the 5th percentiles (Fig. 5b). For low PD, the OR value of the 2.5th percentiles is 2.00 [1.38, 2.90], which increases the risk by 20% compared to the 5th percentiles; for the high PD, the OR value of the 2.5th percentiles is 0.97 [0.67, 1.42], which is a 3% increase in risk compared to the 5th percentiles (Fig. 5c). For the low ENN_MN, the OR value of the 2.5th percentiles is 0.99 [0.67, 1.44],

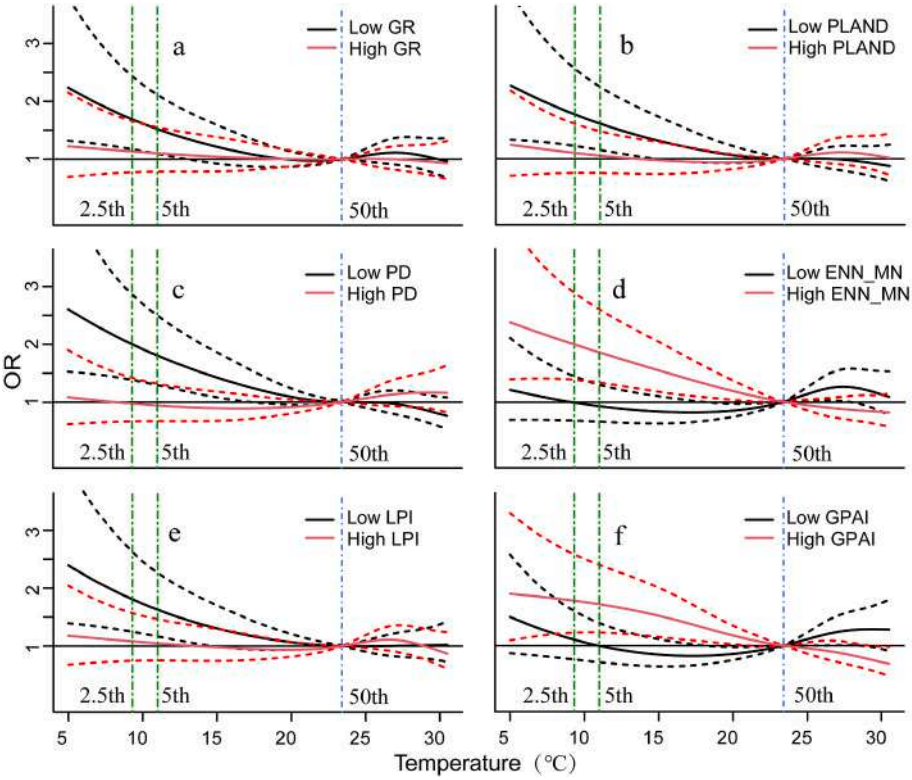

Fig. 5. The admission risk curve of temperature-related CVDs corresponding to the subgroups of each green space indicator at the optimal modification scale (cumulative lag of 2 days). The solid curves represent estimated ORs, and the dashed curves represent upper or lower bounds of 95%CI. The 50th, 5th, and 2.5th respectively represent reference temperature, low temperature, and extremely low temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

which increases the risk by 7% compared to the 5th percentiles; for the high ENN_MN, the OR value of the 2.5th percentiles is 1.99 [1.38, 2.88], which increases the risk by 14% compared to the 5th percentiles (Fig. 5d). For the low LPI, the OR value of the 2.5th percentiles is 1.81 [1.25, 2.62], which increases the risk by 18% compared to the 5th percentiles; for the high LPI, the OR value of the 2.5th percentiles is 1.08 [0.74, 1.57], which increases the risk by 3% compared to the 5th percentiles (Fig. 5e). For the low GPAI, the OR value of the 2.5th percentiles is 1.10 [0.76, 1.60], which increases the risk by 11% compared to the 5th percentiles; for the high GPAI, the OR value of the 2.5th percentiles is 1.77 [1.22, 2.58], which increases the risk by 6% compared to the 5th percentiles (Fig. 5f).

From the above results, it can be concluded that with a further decrease in temperature, the high GR, the high PLAND, the high PD, the high LPI, and the low ENN_MN will produce a smaller incremental risk of hospital admissions, indicating that their degree of risk-modifying increases as temperatures continue to drop. As for the GPAI, although the risk of high GPAI is still higher than that of the low GPAI at extremely low temperatures, the growth rate of the overall risk curve slows down, while the risk curve corresponding to the low GPAI still maintains a certain growth rate, indicating that the health benefit from the low GPAI weakens as the temperature continues to decrease. The changing trend of each risk curve corresponding to the cumulative lag of 7 days and the cumulative lag of 2 days is consistent (Fig. S1).

4. Discussion

4.1. The modification mechanism of the occurrence of low temperature-related CVDs by green space

This study explores the modification by various green space indicators on the occurrence of low temperature-related CVDs, compares the modification strengths at different spatial scales, and then explores the risk-modifying effects when the temperature is further lowered.

This study finds that high GR has a protective effect on low temperature-related CVDs. Li et al. (2021) found that high GR corresponds to a lower admission risk of low temperature-related stroke. The study was carried out in Wuhan, which shares the subtropical monsoon climate with Guangzhou, corroborating our findings. The PLAND, GR, and LPI used in this study can reflect the scale and growth of trees. In addition, the PD and the ENN_MN can reflect the compactness of tree arrangement. Our results express the benefit of dense and compact green space on somatosensory temperature at low temperatures, which is reflected in the reduction in the admission risk of low temperature-related CVDs. Zhang et al. (2018) constructed a Wuhan residential community model to explore the effect of trees on body temperature under low temperature conditions. The researchers found that closely arranged evergreen vegetation is more conducive to creating a good body temperature than deciduous vegetation (the zonal vegetation in Guangzhou is South subtropical monsoon evergreen broad-leaved forest). Meili et al. (2021) researched four cities in different climate zones as study areas and comprehensively evaluated the effects of vegetation and solar radiation interaction, evapotranspiration, and changes in surface roughness on surface temperature. Among them, the warming effect of vegetation can be summarized into two aspects. First, the canopy can absorb more short-wave radiation and release a large amount of sensible heat to the surface. Second, vegetation can reduce the surface roughness of urban neighborhoods, hinder the energy interaction of turbulent flow, and increase the temperature during the day. This warming effect increases with the increase of vegetation density and coverage area. The temperate oceanic climate type cities have an obvious warming effect in winter. The mean temperature in Guangzhou is also above 0 °C in winter, and it is expected that this warming effect will also be obvious in the Guangzhou area. No research has been found on the impact of accessibility on the admission risk of low temperature-related CVDs. The possible explanation is that the better the access to green space, the more direct the public is affected by green space, and thus better able to obtain the benefits of green space (Tamosiunas et al., 2014). When analyzing the modification of each green space with the further decrease of temperature, we find that at the optimal modification scale, the benefits of the high GR, the high PLAND, the high PD, the low ENN_MN, and the high LPI are enhanced. These conclusions further demonstrate the benefits of large-scale, high-density vegetation. However, the GPAI results show that with further reductions in temperature, low GPAI corresponds to a larger increase in risk than high GPAI. That is, the health benefit from the low GPAI gradually decrease with the decrease in temperature. A possible explanation is that at low temperatures, the public with high access to vegetation is more susceptible to the adverse effects of vegetation, as well as its benefits, because vegetation blocks direct sunlight, making the already low temperature even low (Xing et al., 2019), which is not conducive to cardiovascular and cerebrovascular health. The modification of green space on the low temperature-related CVDs may also be related to the time, frequency, and way of public participation in green space. Different accessibility indicators need to be tried in the future.

4.2. The scale effect of green space modification

This study discovers that each green space indicator has a scale effect on the modification of low temperature-related CVDs, and there is a spatial scale with the strongest modification. Our study shows that the optimal modification radii of GR and PLAND are around 800 m and 600 m. Similar to the results of previous studies, for example, Su et al. (2019) selected Barcelona, Spain, as their research area, randomly choosing survey subjects in each block, and studying whether the GR around the participants' home addresses impacted their health. The study includes circular buffers with radii of 50 m, 100 m, 250 m, 500 m, and found that the green space within a radius of 500 m has the greatest health benefits. Browning and Lee (2017) reviewed the impact of green space on the public health at all ages, and found that 500 m–1000 m radius buffer GR centered on home addresses were better predictors of physical health. Liu et al. (2022) reviewed the effect of green space on the occurrence of CVDs, and found that the GR in the 500 m radius buffer has a stronger protective effect on cardiovascular and cerebrovascular health than at 300 m and 1000 m. The above studies all show a scale effect on the impact of GR on physical health, and the optimal modification scale may occur between 500 m and 1000 m, which supports our results.

The "Green Space and Health" document published by the World Health Organization lists several criteria for the selection of green space radii (World Health Organization, 2016), all of which are based on walking elements. Among them, the "Green Infrastructure Planning Standard" in England recommends that there is at least one green space not less than 2 hectares within 300 m around the residence. The European Union suggests the elderly walk a distance of 15 min as the standard, which corresponds to a walking distance of about 500 m and a straight-line distance of about 300 m. The online tool of the US Environmental Protection Agency provides calculation services for green space-related indicators within walking distance of 500 m. Yeager et al. (2020) reviewed the relationship between green space and cardiovascular and cerebrovascular health and found that previous studies mostly calculated green space indicators in a 200 m–500 m radius buffer. The above common radius selection methods are based on two reasons. First, the radius range is more in line with the public's green vision range, and second, the green space within this radius is more likely to be frequently used by the public. Our study constructed the GPAI to express the use opportunity of green space. The results with a cumulative lag of 2 days showed that GPAI modification is strongest within a 500 m radius. The optimal modification scale for results with a cumulative lag of 7 days is larger, and these distances are all larger than the standard value of the World Health Organization. First, the benefits of physical and mental pleasure, emotion regulation, and increased exercise time obtained by the public participating in green space are not within the scope of our

study. Green space may have a larger spatial scope for the modification of low temperature-related CVDs admissions. Second, the 300 m straight-line distance standard is relatively conservative. Millward et al. (2013) chose Halifax, Canada, as the research area, collected a large number of individual behavioral trajectory data, analyzed the public walking mode, and found that most of the public are willing to participate in urban space within a range of 600 m. Finally, farther green spaces with greater recreational opportunities and unique significance can motivate the public to break through distance barriers to participate (Ekkel and de Vries, 2017).

The optimal modification radius of each green space distribution pattern indicator is within the optimal modification radius of green space scale. Specifically, the optimal modification radius of high PD is around 600 m, and the optimal modification radius of high LPI and high ENN_MN is around 300 m. It shows that when urban green space planning is carried out, sufficient vegetation scale and density should be maintained at a larger spatial scale, while at a relatively small scale, more attention should be paid to the compact layout of green space, and a single larger area should be provided within 300 m as much as possible. At the same time, reasonably adjusting the area and the total number of green space patches at the intermediate scale to provide greater accessibility to green space. These measures will be beneficial in protecting the public's cardiovascular and cerebrovascular health under low temperature conditions.

4.3. Limitations

In this study, relative humidity, wind speed, and air pressure are controlled in the process of constructing the relationship between temperature and CVDs admission, but there may still be some confounding factors that are not controlled. In addition, due to the limitation of data acquisition, the number of cases involved in this study is relatively small, and it is necessary to be expanded further to improve the statistical results of the risk model. Our conclusion should be used with caution in an area which the climate, population, economy and other characteristics are very different from us. More empirical research is needed to draw conclusions applicable to different areas. When looking into the future, firstly, in the field of research on the relationship between green space and physical health, more attention should be paid to the influence of green space attributes such as green space distribution patterns and vegetation types, not limited to the scale of green space, especially the relationship between air temperature and CVDs. Secondly, when constructing a model of the relationship between green space and health, attention should not only be paid to spatial scale effects, but also to geographic issues such as variable unit area and uncertainty of spatial background. Finally, in terms of mechanism exploration, it is necessary to clarify the specific path of vegetation in modifying low temperature-related cardiovascular and cerebrovascular health and carry out corresponding empirical research.

5. Conclusion

In this study, the time-stratified case-crossover design method is used to explore the modification of admissions risk of low temperature-related CVDs by green space scale, green space distribution pattern, and accessibility, six specific green space indicators were constructed. It is found that high GR, high PLAND, high PD, low ENN_MN, high LPI, and low GPAI correspond to low risk of CVDs occurrence, the optimal scales are around 800 m (GR), 600 m (PLAND and PD), 500 m (GPAI), and 300 m (LPI and ENN_MN), respectively, with the further decrease in temperature, the health benefit from the low GPAI is weakened, while the benefits from the others are strengthened. For countries and regions where the morbidity or mortality of CVDs is significantly affected by low temperature, the conclusions of this study have important theoretical and practical significance when forming and implementing local green space policies.

Ethical approval

Medical Ethics Committee for Clinical Research and Animal Trials of the First Affiliated Hospital of Sun Yat-sen University.

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Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114193.

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Monitoring ambient air pollution and pulmonary function in asthmatic children by mobile applications in COVID-19 pandemic

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ABSTRACT

Background: Several public health measures were implemented during the COVID-19 pandemic. However, little is known about the real-time assessment of environmental exposure on the pulmonary function of asthmatic children. Therefore, we developed a mobile phone application for capturing real-time day-to-day dynamic changes in ambient air pollution during the pandemic. We aim to explore the change in ambient air pollutants between pre-lockdown, lockdowns, and lockdowns and analyze the association between pollutants and PEF mediated by mite sensitization and seasonal change.

Method: A prospective cohort study was conducted among 511 asthmatic children from January 2016 to February 2022. Smartphone-app used to record daily ambient air pollution, particulate matter (PM2.5, PM10) Ozon (O_3), nitrogen dioxide (NO_2), Carbon Monoxide (CO), sulfur dioxide (SO_2), average temperature, and relative humidity, which measured and connected from 77 nearby air monitoring stations by linking to Global Positioning System (GPS)-based software. The outcome of pollutants' effect on peak expiratory flow meter (PEF) and asthma is measured by a smart peak flow meter from each patient or caregiver's phone for real-time assessment.

Results: The lockdown (May 19th, 2021, to July 27th, 2021) was associated with decreased levels of all ambient air pollutants aside from SO_2 after adjusting for 2021. NO_2 and SO_2 were constantly associated with decreased levels of PEF across lag 0 (same day when the PEF was measured), lag 1 (one day before PEF was measured), and lag 2 (two days prior when the PEF was measured. Concentrations of CO were associated with PEF only in children who were sensitized to mites in lag 0, lag 1, and lag 2 in the stratification analysis for a single air pollutant model. Based on the season, spring has a higher association with the decrease of PEF in all pollutant exposure than other seasons.

Conclusion: Using our developed smartphone apps, we identified that NO_2, CO, and PM10 were higher at the pre- and post-COVID-19 lockdowns than during the lockdown. Our smartphone apps may help collect personal air pollution data and lung function, especially for asthmatic patients, and may guide protection against asthma attacks. It provides a new model for individualized care in the COVID era and beyond.

1. Introduction

Approximately three million people worldwide have asthma, and the burden of this disease is substantial as asthmatic individuals may experience frequent night-waking, loss of work productivity, increased healthcare expenses, and increased time spent on treatment (Dierick et al., 2020). Although treatment for asthma control is currently available, poor asthma control leads to emergency visits and hospitalization

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(Fergeson et al., 2017). Pulmonary function tests such as peak expiratory flow (PEF), forced vitality capacity (FVC), and forced expiratory volume in the first second (FEV1) may be used to monitor respiratory function in asthmatic patients (Enright et al., 1994). Asthma is a multifactorial disease attributable to genetic susceptibility factors, host factors, and environmental exposures. Therefore, avoiding environmental pollutants/irritants besides asthma treatment is crucial to prevent asthma exacerbations (Dharmage et al., 2019).

Previous studies have demonstrated that exposure to hazardous air pollutants may decline pulmonary function (Altman et al., 2023). Various studies have provided evidence that sulfate (SO_4^{2-}), ammonium (NH_4^+), nitrogen dioxide (NO_2), sulfur dioxide (SO_2), particle matter (PM10 and PM2.5), and ozone (O_3) exposure in asthmatic patients were associated with decreased PEF levels, (Edginton et al., 2021; Kim et al., 2021a; Gehring et al., 2020). Additionally, the existing biological hazards in air pollutants such as mites have previously been reported to reduce PEF levels because of their allergy sensitization (Okasha et al., 2021). However, the vivid mechanism remains unclear and needs to be addressed.

Concerning the literature on air pollution and asthma surveillance, previous studies did not amass air pollutants based on geo-located locations. Instead, data were measured in fixed settings (Gehring et al., 2020). These data collection methods may be subject to measurement error because participants may move across areas, and the association observed may have been biased.

To overcome this issue, collecting air pollution data via smartphone applications (apps) may be optimal because smartphones have built-in Global Positioning System (GPS) functions and can personally collect air pollutant exposure based on one's location. Therefore, the collected data may be more precise compared with previous methods. In addition, smartphone apps can assess the dynamic change of air pollutants without burdening the participants and study investigators (Johnston et al., 2018; Loh et al., 2017). In pandemics such as COVID-19, this has been a crucial advantage of smartphone apps, as lockdowns may occur and impact the progress of data collection. Smartphone apps also provide an opportunity to understand the dynamic air quality change between pre-post and during lockdowns. These gadgets are also beneficial in collecting data more efficiently from longitudinal cohorts across multiple years and provide valuable information about asthma and its symptoms (Ambrosini et al., 2017).

Few studies used smartphone applications to amass environmental data and asthma outcomes. A study in Australia collected pollen concentration and respiratory symptoms using smartphone apps (Jones et al., 2021). However, the environmental data collected were not specifically on air pollutants. Another study in the United States developed an app to monitor air pollutants for asthmatic children. Nevertheless, the app still needs to be evaluated in a real-world setting (Kim et al., 2021b). Currently, studies on collecting air pollutants using smartphone apps and assessing their association with asthma outcomes in the pediatric population still need to be made available. Therefore, we developed a smartphone app to collect real-time personal pollutants to explore the change in the ambient air pollutants between pre-lockdown, lockdowns, and lockdowns; and to analyze the association between pollutants and PEF among asthmatic children and observe if the association is modified by mite sensitization and season.

2. Method and material

2.1. Study design, setting, and population

This was a prospective cohort study employing repeated measurements of the outcome and exposure. This study was conducted between January 2016 and February 2022. A total of 511 children were recruited with eligibility criteria of 18 years of age, diagnosed with asthma, and who visited the Taipei Hospital. During the follow-up period, a lockdown (May 19th, 2021, to July 27th, 2021) was imposed due to the COVID-19 pandemic. The hospital's Institutional Review Board approved the study (TH-IRB-0016-0038), and parents provided written consent before their child was enrolled.

2.2. Smartphone app and exposure collection

We collected real-time personal exposure of particulate matter $\leq$10 m and $\leq$2.5 m in aerodynamic diameter (PM10 and PM2.5), sulfur dioxide (SO_2), nitrogen dioxide (NO_2), ozone (O_3), and carbon monoxide (CO) from air monitoring station by the Environmental Protection Administration. There were a total of 77 stations located throughout Taiwan. PM 2.5 ($\mu g/m^3$) and PM10 ($\mu g/m^3$) were detected using beta ray, O_3 (ppb) was measured using ultraviolet absorbance, CO (ppm) was measured using infrared absorbance, SO_2 (ppb) was measured using an ultraviolet fluorescence spectrometer, and NO_2 (ppb) was measured by gas-phase chemiluminescence detection. The relative humidity and temperature were also collected. Participants or their caregivers were required to download a smartphone app that can automatically record the personal air pollution parameters from the closest air pollution stations in real-time using the in-built Global Positioning System (GPS) function. This smartphone app can also send in-time reminders of avoidance of unhealthy air quality to the participants or caregivers when it detects poor air quality. The sensitization to mite allergens was defined as levels of specific IgE $\geq$0.7 U/mL.

2.3. Outcome measurement

Forced vital capacity (FVC), forced expiratory volume in 1 s (FEV1), and FEV1/FVC ratio were measured by spirometry in the baseline assessment. A smart peak expiratory flow meter from each patient or caregiver's phone was used for real-time assessment. PEF is a measure used by asthma patients to record their asthma control conditions. The instrument is constructed with a unique air tube that was 3D printed and a small number of electronic parts that communicate with a smartphone using a unique app. The headphone jack is used to transmit a pressure-dependent frequency signal created when the user blows into the tube to a smartphone app. The specifically designed app reads the signal, transforms the frequency to a flow rate, and displays this information for the patients to see, as well as logging it for condition monitoring.

2.3.1. Quality assurance and control

We adapted the QA and quality control (QA/QC) procedures for PFT that were in place in our laboratory for oscillometry in addition to the suggestions from the ERS guidelines and the Tremoflo user manual (Wu et al., 2020). The ERS guidelines and the Tremoflo user manual suggest that measurements should have a minimum duration of 6 s, valid data points $\geq$70%, and a minimum of 3 valid measurements with a coefficient of variation $\leq$15%. However, these suggested guidelines were not automatically excluded and must be assessed by the operator. In our QA/QC procedures, we gave subjects 3 min of rest before the first oscillometry measurement to stabilize lung volumes because it is a 60-m walk from the waiting area to the laboratory. A minimum of 3 tidal breaths must be observed before each oscillometry recording to ensure subjects are breathing at resting functional residual capacity level to avoid mechanical drifts. A 30 s rest is also given to subjects between each measurement to avoid short-term variability. We increased the minimum rest time from 3 to 10 min to stabilize the lung volumes if forced respiratory maneuvers were performed before the oscillometry test. Biological calibration was also added to ensure the two oscillometry devices function correctly.

2.4. Statistical analysis

We tested the P for the trend of air pollution levels in different periods of the follow-up: before the COVID-19 lockdown (2016 Jan 4th −2021 May 14th), during lockdown (2021 May 15th-July 27th), and

after lockdown (2021 July 28th - 2022 Feb 25th). Interrupted time series analysis assessed the association between air pollutants and the COVID-19 lockdown. Associations between repeated measures of air pollutants at lag 0 lag 1- and lag 2- and PEF were assessed using generalized estimate equations (GEE) (lag 0: on the same day when the PEF was measured: lag1: 1 day before PEF outcome measurements; lag 2: 2 days before PEF outcomes measurement).

Age, gender, body mass index, environmental tobacco smoke, family income, temperature, and relative humidity were adjusted in the regression models. Effect modification was explored by mite sensitization, and stratified associations were presented. For these analyses, two-pollutant models were also examined. Associations between repeated measures of air pollutants and emergency room visits were assessed using GEE with a Poisson distribution, and adjusted results were presented. Because mite is a vital aeroallergen, which may confound the effects of air pollutants, the stratification results by mite sensitization were also presented. All analysis was performed on SPSS software version 22.0 (IBM, Armonk, NY, USA) and R program version 3.5.3 (R Fountin for Statistical Computing, Vienna, Austria).

3. Results

3.1. Baseline characteristics of the study subjects

At baseline, the mean age was 8.1 years, and 213 (41%) participants were male (Table 1). The mean FVC was 3263 mL, and the mean FEV1 was 2680 mL. The mean PEF was 177.2 L/minute, and the mean ACT score was 18.8.

3.2. The change in ambient air pollution between pre-lockdown, lockdowns, and lockdowns

3.2.1. Ambient air pollutant distribution during the follow-up period

The daily mean concentration of air pollutants at lag 0, lag 1, and lag2 during the follow-up period are presented in (Table 2). The mean of each air pollutant did not vary mainly across the three lags. There was evidence of correlations among the air pollutants (Table A.1).

3.2.2. Ambient air pollution levels and COVID-19 lockdowns

There was a trend that the concentrations of NO_2, CO, O_3, and PM10 were lower during the COVID-19 lockdown compared to pre-and post-lockdown periods (Fig. 1). COVID-19 lockdowns (May 19th, 2021, to July 27th, 2021) were associated with decreased levels of all air pollution aside from SO_2 after adjusting for the year 2021 (Table A.2).

3.3. Associations between ambient air pollutants and PEF among asthmatic children

3.3.1. Associations between air pollutants and PEF by single pollutant model

NO_2 and SO_2 were constantly associated with decreased levels of PEF across lag 0 (same day when the PEF was measured), lag 1 (one day before PEF was measured), and lag 2 (two days prior when the PEF was measured) (NO_2: lag 0: 1.01, 95%CI, −1.72 to −0.27, p = 0.03; lag1: 1.22, 95%CI: −1.96 to −0.48, p = 0.005; lag2: 0.89, 95% CI: −1.59 to −0.19, p = 0.031; SO_2: lag 0: 5.23, 95%CI, −9.99 to −0.43, p = 0.017; lag1: 4.83, 95%CI: −9.04 to −0.61, p = 0.025; lag2: 6.10, 95% CI: −11.07 to −1.13, p = 0.005). While PM 2.5 was only associated with lag 2 (−0.79, 95%CI: −1.45 to −0.12, p = 0.031), PM10 was associated with lower levels of PEF at lag 0 (−0.44, 95%CI: −0.77 to −0.10, p = 0.030) and lag 2 (−0.39, 95%CI: −0.76 to −0.02, p = 0.023). CO was associated with decreased levels of PEF only at day 0 (−49.0, 95%CI: −77.97 to −18.33, p = 0.008) and at day 1 (−43.1, 95%CI: −71.81to −14.34, p = 0.01). O_3 was not associated with PEF on either of the days (Table 3).

Table 1
Study characteristics.

	Total (N = 511)
BMI, mean (SD)	18.8 (4.7)
Age, mean (SD)	8.1 (5.2)
Sex (male)	213
Mite (>2vs. ≤ 2) (N = 202)	156 vs.48
FVC(mL)	3263 (817)
FEV1 (mL)	2680 (663)
FEV1/FVC(%)	83.2 (10.6)
FeNO	29.4 (15.6)
PEF	177.2 (98.9)
ACT score	18.8 (6.5)

Table 2
Daily concentrations of ambient air pollutants (N = 511).

	Min	Max	IQR	SD	Mean
Lag 0					
$PM_{2.5}$ (μg/m^3)	2	42	10	14.48	7.43
PM_{10} (μg/m^3)	6	85	19	31.02	13.85
NO_2(ppb)	5	43	4.4	17.30	6.41
CO (ppm)	0	1	0.19	0.46	0.17
O_3 (ppb)	9	68	13.8	30.89	10.82
SO_2 (ppb)	0	8	1.5	2.82	1.33
Lag 1					
$PM_{2.5}$ (μg/m^3)	1	42	10	14.24	7.57
PM_{10} (μg/m^3)	5	77	20	30.37	15.01
NO_2(ppb)	1	40	7.86	15.74	6.58
CO (ppm)	0	1	0.10	0.43	0.17
O_3 (ppb)	8	61	7.83	30.56	11.12
SO_2 (ppb)	0	9	1.11	2.69	1.26
Lag 2					
$PM_{2.5}$ (μg/m^3)	1	45	9	14.19	7.56
PM_{10} (μg/m^3)	7	112	17	29.93	15.80
NO_2(ppb)	4	40	7.78	15.57	6.48
CO (ppm)	0	1	0.20	0.43	0.16
O_3 (ppb)	7	74	14.2	30.02	10.76
SO_2 (ppb)	1	9	1.10	2.74	1.29

Lag 0 : Same day; Lag 1 : 1 day before; Lag 2 : 2 day before.

3.3.2. Associations between air pollutants and PEF by two pollutant model

When including O_3 as a co-pollutant, the association for all pollutants was similar to the results observed in the single pollutant models. However, when having NO_2 or CO as a co-pollutant, the association was slightly different from the effects observed in the single air pollutant models. Only the association between SO_2 and PEF at lag 2 remained significant in these co-pollutant models. When including PM2.5 or PM10 as co-pollutants in the models, only the association between CO, NO_2, and PEF at lag 0 and the association between SO_2 and PEF at lag 2 remained significant (Table A3).

3.3.3. Effect modifications by mite sensitization for PEF outcomes

There was evidence that mite sensitization may modify the effect of specific air pollutants on PEF. In the single air pollutant models, concentrations of CO was associated with PEF only in children who were sensitized to mite in lag 0, lag 1, and lag 2 in the stratification analysis (lag 0: −48.15, 95%CI: 77.97, −18.33, P = 0.002; lag 1: −43.07, 95% CI: −71.81, −14.34, P = 0.003, lag 2: −33.85, 95%CI: −61.75, −5.94, P = 0.017). Additionally, PM10 and PM 2.5 were only associated with PEF in children sensitized to mites in lag 0 and lag2. No effect modification was observed for O_3 (Table 3).

3.3.4. Effect modifications by seasons for PEF outcomes

There was evidence that seasons may modify the effect of specific air pollutants on PEF. In the single air pollutant models, concentrations of CO was associated with PEF only in children who were sensitized to mite in lag 0, lag 1, and lag 2 in the stratification analysis (lag 0: −48.15, 95%

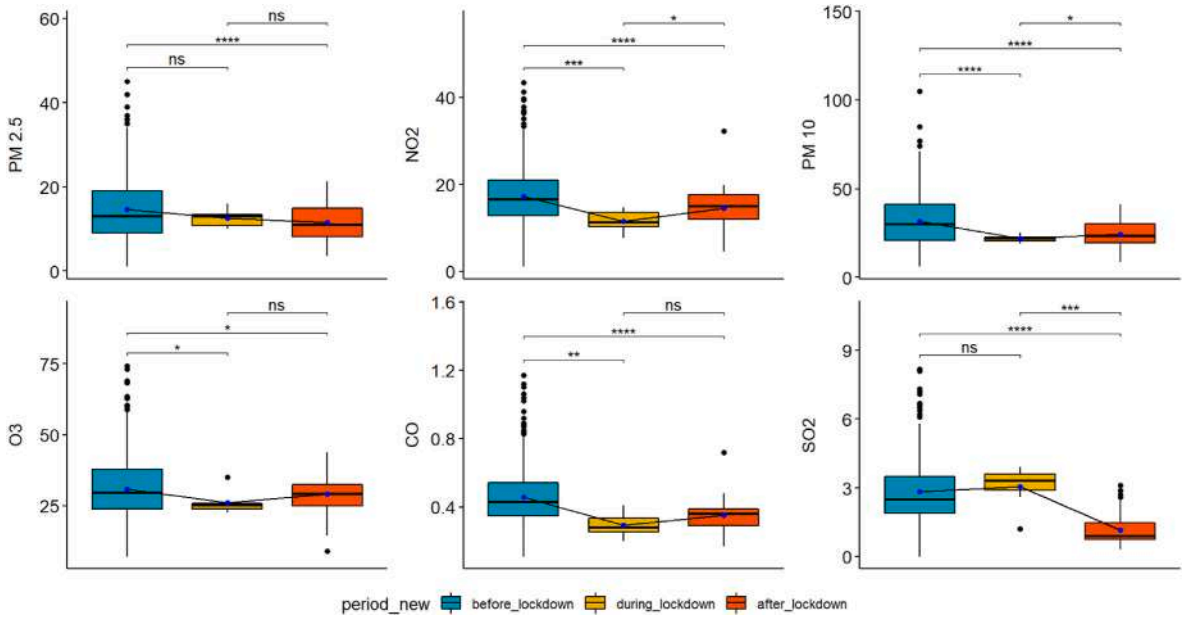

Fig. 1. Air pollution levels during pre-post lockdowns and lockdowns.

Table 3
Single-pollutant models of associations between ambient air pollution exposure and PEF stratified by mite sensitization.

PEF	Total (N = 511)			Mite sensitization (−)			Mite sensitization (+)		
	B	95%CI	P-value	B	95%CI	P-value	B	95%CI	P-value
Lag 0									
$PM_{2.5}$ (μg/m^3)	−0.67	−1.25, −0.09	0.081	0.60	−4.61, 5.82	0.820	**−0.67**	**−1.25, -0.09**	**0.025***
PM_{10} (μg/m^3)	**−0.44**	**−0.77, -0.10**	**0.030***	−0.54	−4.52, 3.43	0.788	**−0.44**	**−0.77, -0.10**	**0.011***
NO_2(ppb)	**−1.01**	**−1.72, -0.27**	**0.031***	0.37	−3.48, 8.22	0.427	**−1.00**	**−1.72, -0.27**	**0.007***
CO (ppm)	**−49.0**	**−77.97, -18.33**	**0.008***	59.5	−149, 268	0.576	**−48.15**	**−77.97, -18.33**	**0.002***
O_3 (ppb)	−0.06	−0.50, 0.39	0.823	−0.32	−3.89, 3.25	0.860	−0.06	−0.50, 0.39	0.802
SO_2 (ppb)	**−5.23**	**−9.99, -0.43**	**0.017***	−6.87	−28.4, 14.6	0.531	**−5.21**	**−9.99, -0.43**	**0.032***
Lag 1									
$PM_{2.5}$ (μg/m^3)	−0.54	−1.18, 0.09	0.128	−1.28	−5.89, 3.34	0.587	−0.54	−1.18, 0.09	0.094
PM_{10} (μg/m^3)	−0.34	−0.74, 0.06	0.081	−0.32	−3.11, 2.47	0.823	−0.34	−0.74, 0.06	0.097
NO_2(ppb)	**−1.22**	**−1.96, -0.48**	**0.005***	−1.76	−9.80, 7.08	0.752	**−1.22**	**−1.96, -0.48**	**0.001***
CO (ppm)	**−43.1**	**−71.81, -14.34**	**0.010***	−32.0	−294, 230	0.811	**−43.07**	**−71.81, -14.34**	**0.003***
O_3 (ppb)	0.36	−0.06, 0.78	0.153	1.41	−1.24, 4.06	0.298	0.36	−0.06, 0.78	0.090
SO_2 (ppb)	**−4.83**	**−9.04, -0.61**	**0.025***	−6.18	−27.9, 15.6	0.578	−4.83	−10.49, 0.84	0.095
Lag 2									
$PM_{2.5}$ (μg/m^3)	**−0.79**	**−1.45, -0.12**	**0.031***	−2.93	−7.67, 1.82	0.227	**−0.79**	**−1.45, -0.12**	**0.021***
PM_{10} (μg/m^3)	**−0.39**	**−0.76, -0.02**	**0.023***	−0.67	−2.20, 0.87	0.393	**−0.39**	**−0.76, -0.02**	**0.040***
NO_2(ppb)	**−0.89**	**−1.59, -0.19**	**0.031***	**−6.47**	**−12.5, -0.40**	**0.037***	**−0.89**	**−1.59, -0.19**	**0.013***
CO (ppm)	−33.8	−61.75, −5.94	0.052	−168.2	−412, 76.1	0.177	**−33.85**	**−61.75, -5.94**	**0.017***
O_3 (ppb)	0.21	−0.27, 0.68	0.422	1.76	−1.31, 4.83	0.262	0.21	−0.27, 0.68	0.398
SO_2 (ppb)	**−6.10**	**−11.07, -1.13**	**0.005***	**−19.4**	**−37.0, -1.82**	**0.030***	**−6.10**	**−11.07, -1.13**	**0.016***

Lag 0 : Same day; Lag 1 : 1 day before; Lag 2 : 2 day beforeModels were adjusted for age, gender, body mass index, environmental tobacco smoke, family income, temperature, and relative humidity.*$p < 0.05$.[a]

[a] Bold font indicates statistical significance

CI: 77.97, −18.33, P = 0.002; lag 1: −43.07, 95% CI: −71.81, −14.34, P = 0.003, lag 2: −33.85, 95%CI: −61.75, −5.94, P = 0.017). Additionally, PM10 and PM 2.5 were only associated with PEF in children sensitized to mites in lag 0 and lag2. No effect modification was observed for O_3 (Table 4).

4. Discussion

In the present study, lower levels of NO_2, CO, O_3, and PM10 were observed during the COVID-19 lockdown compared to pre-and post-lockdown periods in Taiwan, but this trend was not observed in SO_2 and PM2.5. Our findings that real-time monitored NO_2, CO, O_3, and PM10 were lower during COVID-19 lockdown compared with pre/post lockdowns are primarily consistent with previous studies in different settings (Adam et al., 2021), which can be explained by the reduced transportation emission during lockdowns. The trend of SO_2 levels during lockdown is interesting, and the evidence is contraindicated from the literature (Adam et al., 2021; Kenawy et al., 2021). Our findings may be explained by the fact that SO_2 levels are generally higher in summer or spring throughout the year (Lee et al., 2018). Our observation may be possible due to seasonal factors rather than lockdown interventions, as the lockdown occurred in the summer seasons (May–July). Future studies examining the effect of lockdowns and air pollutants should consider seasonal factors.

Table 4
Single-pollutant models of associations between ambient air pollution exposure and stratified by seasons.

PEF score												
Spring				Summer			Autumn			Winter		
	B	95%CI	P-value	B	95%CI	P-value	B	95%CI	P-value	B	95%CI	P-value
Lag 0												
PM2.5	−1.89	−3.68, −0.09	0.039*	−0.38	−1.62, 0.85	0.545	−1.48	−2.72, −0.24	0.019*	1.54	0.43, 2.65	0.006*
PM10	−0.62	−1.72, 0.47	0.263	−0.14	−0.87, 0.59	0.702	−0.76	−1.35, −0.16	0.013*	0.34	−0.19, 0.88	0.209
NO2	−3.20	−5.01, −1.39	0.001*	−1.42	−3.04, 0.19	0.085	−0.86	−2.60, 0.88	0.332	0.70	−0.43, 1.84	0.224
CO	−166.5	−232.2, −100.9	0.000*	−61.00	−130.4, 8.44	0.085	0.72	−64.1, 65.5	0.983	51.9	3.64, 100.1	0.035*
O3	0.23	−0.82, 1.27	0.672	0.21	−0.91, 1.32	0.716	−0.76	−1.58, 0.05	0.067	0.20	−0.50, 0.90	0.568
SO2	−2.19	−14.21, 9.83	0.721	−6.34	−16.41, 3.74	0.218	−4.00	−13.45, 5.46	0.407	0.33	−6.37, 7.04	0.923
Lag 1												
PM2.5	−1.108	−2.476, 0.260	0.112	−0.268	−1.568, 1.032	0.686	−1.212	−2.337, −0.088	0.035*	1.042	0.062, 2.021	0.037*
PM10	−0.286	−1.240, 0.669	0.557	−0.032	−0.876, 0.812	0.940	−0.734	−1.465, −0.004	0.049*	0.406	−0.098, 0.909	0.114
NO2	−2.679	−4.907, −0.487	0.017*	−0.864	−2.650, 0.922	0.343	−1.062	−2.421, 0.297	0.125	0.184	−1.098, 1.466	0.778
CO	−120.047	−193.382, −46.711	0.001*	−48.593	−114.642, 17.457	0.149	−20.622	−78.979, 37.735	0.489	20.353	−26.950, 67.656	0.399
O3	0.456	−0.700, 1.162	0.439	0.694	−0.296, 1.683	0.169	0.101	−0.736, 0.938	0.813	0.385	−0.416, 1.187	0.346
SO2	−3.583	−19.883, 12.716	0.667	−2.904	−10.576, 4.768	0.458	−0.974	−6.120, 8.068	0.788	−0.958	−6.644, 4.728	0.741
Lag 2												
PM2.5	−1.394	−2.801, 0.014	0.052	−0.911	−2.340, 0.517	0.211	−0.730	−2.376, 0.916	0.385	−0.66	−1.39 , 0.08	0.079
PM10	−0.355	−0.974, 0.265	0.262	−1.144	−3.018, 0.731	0.232	−0.361	−1.411, 0.688	0.500	−0.137	−0.68, 0.407	0.622
NO2	−2.349	−4.277, −0.420	0.017*	−1.144	−3.018, 0.731	0.232	−1.038	−2.375, 0.300	0.129	0.625	−0.548, 1.797	0.296
CO	−99.308	−183.504, −15.112	0.021*	−59.880	−137.624, 17.864	0.131	−26.504	−74.586, 21.578	0.280	29.620	−15.303, 74.543	0.196
O3	0.994	−0.064, 2.052	0.066	0.087	−0.808, 0.982	0.849	0.261	−0.813, 1.344	0.634	−0.092	−0.852, 0.669	0.813
SO2	−0.148	−12.202, 11.906	0.981	−3.151	−11.788, 5.486	0.475	−2.405	−9.104, 4.293	0.482	−6.088	−12.162, −0.015	0.049*

Lag 0 : Same day; Lag 1 : 1 day before; Lag 2 : 2 day beforeModels were adjusted for age, gender, body mass index, environmental tobacco smoke, family income, temperature, and relative humidity.*$p < 0.05$.

While PM10, NO_2, CO, and SO_2 were associated with lower levels of PEF in the lag 0 model, PM2.5 was only associated with lower levels of PEF in the lag 2 model in the follow-up period. We found that personal exposure to air pollutants reduced lung function in single- and two-way air pollutant models. The association between air pollutants and respiratory function has been explored by several studies (Kim et al., 2021a; Gehring et al., 2020). Although previous studies also explored other respiratory function parameters such as FEV_1 and FVC, and we only explored PEF, our results were akin to most previous findings (Kim et al., 2021a), (Gehring et al., 2020), (Ye et al., 2021). Such findings may be explained by the oxidative stress produced after exposure to pollutants, which may lead to airway hyper-reactivity and lung injury (Ghio et al., 2012). Alternatively, a possible explanation is that these air pollutants may promote Th2 inflammation (Bleck et al., 2006).

Interestingly, a research group in the UK used a small portable sensor to capture personal air pollutant levels and investigated the association between personal air pollution and respiratory parameters (PEF, FENO, FEV_1, and FVC), but they did not find an association. Although this study also used a personal approach to collect exposure data, the results contradict ours. This may be because of the different methods used for collecting individual measurements. We used a smartphone app linked to the closest air pollution station to obtain the air pollutant levels. However, this study used a sensor to directly capture the sample total oxidants in the ambient air once per minute (Chambers et al., 2018). Future research may explore the different sensitivity and specificity of air pollutants measurements between other methods.

We found evidence that mite sensitization modified the association between air pollutants and PEF levels, with stronger associations in those sensitized to mites during the follow-up period. Our analysis found that mite sensitization modified the associations between air pollutants and respiratory function. This finding further proves the hypothesis of our previous report that exposure to PM2.5 and mite allergens had a synergistic effect on the development of asthma (Wang et al., 2016; Wang, 2013)]. In another study in Puerto Rico, the authors observed a non-significant increase trend of asthma in those who lived closest to the major roads and were exposed to mite sensitization (Stevens et al., 2020). This may be because air pollutants and mite sensitization are related to Th2 inflammation, inducing airway inflammation and airway modeling (Bouazza et al., 2018; Hsu et al., 2020).

Regarding season and PEF, we found that seasons may modify the effect of specific air pollutants on PEF. In the single air pollutant models, concentrations of CO were associated with PEF only in children who were sensitized to the mite. In Taiwan, spring is one of the seasons which reflect high pollution concentration. It is linear with findings, and the natural phenomenon of frequent sand and dust storms transfer from East Asia during the Spring season [28]. It resulted in the additional accumulation of air pollutants and exaggerated PEF in children.

There are some limitations to this study. Firstly, we did not adjust some potential confounding factors, such as seasonality and personal behavior, in our regression model, which may have biased our results. However, we have put average temperature and relative humidity in our model. We have a large sample size. Moreover, repeated measures of air pollutants and emergency room visits were assessed using a generalized estimating equations model with a Poisson distribution to increase power and decrease in this longitudinal study.

The strength of this study is that mobile phone apps are able to collect real-time and precise air pollutant levels on a population scale which allows for capturing the day-to-day dynamics change of these particles. In addition, it easily records the real-time asthma condition by peak flow measurement from smartphone apps. This immediate collection of particles using a smartphone app and its effects on asthmatic patients is also a cost-effective method for collecting data. It overcomes the potential data collection barrier during a pandemic such as COVID-19. By smartphone app internet of Thing platform, data from sensor devices can be shared. This can help physicians to deliver notifications to patients and families about their health condition and to produce health statistics and predictive analysis.

5. Conclusion

In conclusion, smartphone apps may help to collect personal ambient air pollution data. Using our developed smartphone apps, we found that NO_2, CO, and PM10 were higher during the pre-and post-COVID-19 lockdowns than during the lockdown. Exposure to air pollution increased PEF variability in asthma subjects during the monitoring period. Lastly, the association was stronger in children who were sensitized to mites. It is essential to inform asthmatic children about the risk of exposure to high air pollution levels. Ongoing monitoring of air pollution levels is essential for children's health, and sending in time reminders of avoidance of unhealthy air quality may be performed by smartphones to protect children's lung health and acute asthma attacks. The digitally controlled air monitoring and management system and peak flow monitoring may help fight against asthma during and even beyond the pandemic. This smart healthcare model can be the new normal for asthma hybrid care.

Author contributions

I-J.W. designed the studies; **P-Y. L**. performed the analysis; **B-F H** analyzed the data; **P-Y. L. and W.-I. J.** wrote the article. **J-Y W and RP** reviewed and edited, **I.-J. W**. supervision, funding acquisition, and project administration. All authors have read and agreed to the published version of the manuscript.

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Appendix A. Supplementary data

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"Residential greenness, gestational diabetes mellitus (GDM) and microbiome diversity during pregnancy"

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ABSTRACT

Background: Gestational diabetes mellitus (GDM) is associated with reduced gut microbiota richness that was also reported to differ significantly between those living in rural compared to urban environments. Therefore, our aim was to examine the associations between greenness and maternal blood glucose levels and GDM, with microbiome diversity as a possible mediator in these associations.

Methods: Pregnant women were recruited between January 2016 and October 2017. Residential greenness was evaluated as mean Normalized Difference Vegetation Index (NDVI) within 100, 300 and 500 m buffers surrounding each maternal residential address. Maternal glucose levels were measured at 24–28 weeks of gestation and GDM was diagnosed. We estimated the associations between greenness and glucose levels and GDM using generalized linear models, adjusting for socioeconomic status and season at last menstrual period. Using causal mediation analysis, the mediation effects of four different indices of microbiome alpha diversity in first trimester stool and saliva samples were assessed.

Results: Of 269 pregnant women, 27 participants (10.04%) were diagnosed with GDM. Although not statistically significant, adjusted exposure to medium tertile levels of mean NDVI at 300 m buffer had lower odds of GDM (OR = 0.45, 95% CI: 0.16, 1.26, p = 0.13) and decreased change in mean glucose levels ($\beta = -6.28$, 95% CI: 14.91, 2.24, p = 0.15) compared to the lowest tertile levels of mean NDVI. Mixed results were observed at 100 and 500 m buffers, and when comparing highest tertile levels to lowest. No mediation effect of first trimester microbiome on the association between residential greenness and GDM was observed, and a small, possibly incidental, mediation effect on glucose levels was observed.

Conclusion: Our study suggests possible associations between residential greenness and glucose intolerance and risk of GDM, though without sufficient evidence. Microbiome in the first trimester, while involved in GDM etiology, is not a mediator in these associations. Future studies in larger populations should further examine these associations.

Abbreviations: Directed Acyclic Graph, (DAG); Gestational Diabetes Mellitus, (GDM); Glucose Challenge Test, (GCT); Last Menstrual Period, (LMP); Normalized Difference Vegetation Index, (NDVI); Socioeconomic Status, (SES).

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1. Introduction

As urbanization advances, people live in less green environments. Modern urban life is linked to chronic stress, lack of physical activity and exposure to environmental risk factors. In 2016, the World Health Organization published a summary of dozens of epidemiological studies supporting the findings that living near urban green spaces, such as parks, playgrounds and natural vegetation, promotes mental and physical health and reduces morbidity and mortality among city dwellers (World Health Organization, 2016).

Multiple pathways may mediate the associations between green spaces and health, and several have been suggested. The relatively new biodiversity hypothesis links the reduction in green spaces to reduced host microbial biodiversity, which in turn results in adverse effects on health (Haahtela et al., 2013; Hough, 2014). A recent double blinded placebo-controlled study amongst 26 urban-living children, demonstrated that continuous exposure to microbially rich environments can shift host microbiome composition and regulatory immune responses, supporting the biodiversity hypothesis (Roslund et al., 2022).

Gestational diabetes mellitus (GDM) is defined as any glucose intolerance with the first onset or diagnosis during pregnancy. Although previous studies demonstrated associations between greenness and decreased incidence of type 2 diabetes mellitus, as reported and summarized in a recent meta-analysis (Twohig-Bennett and Jones, 2018), we could only identify a handful of studies regarding GDM incidence and greenness. These studies demonstrated reduced risk of GDM incidence with exposure to higher Normalized Difference Vegetation Index (NDVI) values, a measure of vegetation density and greenness (Choe et al., 2018; Liao et al., 2019; Qu et al., 2020; Yu et al., 2023).

GDM prevalence is affected by a variety of risk factors, such as overweight or obesity, ethnicity, socioeconomic status (SES), diet, genetic polymorphisms, advanced maternal age, and family or personal history of insulin resistance diseases (Zhu and Zhang, 2016). Another factor suggested to affect GDM is the gut microbiome, with reduced richness in GDM pregnancies and changes in relative abundance of certain species compared to healthy pregnant women (Calatayud et al., 2019; Pinto et al., 2023; Turjeman et al., 2021). Gut microbiota also differs significantly in abundance and richness between hosts living in rural and urban environments (Bowyer et al., 2022). Thus, it is possible that GDM occurrence is affected by greenness and biodiversity, through their effect on the gut microbiota richness.

Our hypothesis is that residential greenness is inversely associated with GDM incidence in pregnant women, possibly by affecting gut microbiome richness. Our study aimed to evaluate the association between greenness and GDM risk. The results of this research could have implications for public health by identifying beneficial associations that can be the first step towards developing specific recommendations for greenness exposure standards during pregnancy.

2. Methods

2.1. Study population

Data on the gut microbiome and GDM status was obtained from an Israeli pregnancy cohort, as described by Pinto et al. (2023). Briefly, pregnant women (N = 400) were recruited at three Israeli health care clinics between January 2016 and October 2017 and were followed throughout pregnancy and delivery. Inclusion criteria included healthy pregnant women, aged 18–40, at 11–13 weeks gestational age. Exclusion criteria included non-spontaneous pregnancies, diagnosis of type 1 or type 2 diabetes mellitus before pregnancy, antibiotics usage 3 months prior to recruitment and multiple gestation pregnancies. For our main analysis, we focused on women living in urban areas as defined by the Israeli Ministry of Interior, who met the inclusion criteria and had available data on greenness exposure using the high resolution RapidEye satellite system (N = 269) (Supplemental Fig. 1).

2.2. Residential greenness

Participants' residential addresses (N = 269) during first trimester (provided at recruitment) were geocoded using the Google geocoding server, at the home level when possible (N = 264, 98.14%) or at the settlement level (N = 5, 1.86%), as previously described (Agay-Shay et al., 2013a).

To determine the exposure to residential greenness, we used NDVI derived from RapidEye satellite data, at 5 m × 5 m resolution (Planet, 2014). NDVI is a greenness indicator based on land surface reflectance of visible (red) and near infra-red parts of the spectrum (Weier and Herring, 2000), ranging between −1 and 1, with higher values indicating more greenness and negative values indicating non-biomass (water, cloud, snow). Residential greenness was calculated as the mean NDVI at 100, 300 and 500 m buffers around each maternal residential address (N = 269). NDVI values were generated from RapidEye data by using images obtained on October 24th 2014, after the dry season, that were available for our study region. During the dry season only the evergreen canopies of woodland trees, shrubs and irrigated vegetation contribute to NDVI (Helman, 2018). We selected this month (driest, after the summer) to maximize NDVI contrast and to evaluate effects from exposures to evergreen vegetation including irrigated vegetation. Negative values were converted to zero as previously recommended (Martín-Sotoca et al., 2018; Weier and Herring, 2000). Mean NDVI values were categorized into tertiles of exposure defined as low, medium and high levels of NDVI for each buffer.

2.3. Glucose levels and gestational diabetes mellitus

The primary outcome, GDM, was generally assessed for every participant at 24–28 weeks gestational age using a 50 g Glucose Challenge Test (GCT), regularly performed as a screening test in Israel. Blood glucose levels were measured 1 h after glucose administration, and GDM was defined when glucose levels were above 200 mg/dL (Yogev et al., 2009). Participants demonstrating glucose levels between 140 and 200 mg/dL were required to perform a 100 g oral glucose tolerance test (Committee on Practice Bulletins—Obstetrics, 2018). Participants who presented risk factors for GDM such as large for gestational age (infants larger than 10th percentile for gestational age and sex), GDM in previous pregnancies or polyhydramnios, performed the oral glucose tolerance test instead of GCT (N = 28). During the oral glucose tolerance test, blood glucose levels were measured just before glucose administration whilst fasting, and 1 h, 2 h and 3 h after glucose administration. GDM was defined if any of the following conditions existed: 1) fasting glucose level of 95 mg/dL or above; 2) 1 h blood glucose level of 180 mg/dL or above; 3) 2 h blood glucose level of 155 mg/dL or above; and 4) 3 h blood glucose level of 140 mg/dL or above (Committee on Practice Bulletins—Obstetrics, 2018). Diagnosis using one abnormal value instead of two abnormal values is based on Yogev et al. (2009), showing adverse pregnancy outcomes in pregnant women with one abnormal value, and according to the American College of Obstetricians and Gynecologists' recommendations (Committee on Practice Bulletins—Obstetrics, 2018). Glucose levels measured during GCT were used as a continuous outcome (mg/dL) for 242 participants with available data.

2.4. Microbiome diversity

Microbiome diversity indices were considered as possible mediators in the associations between residential greenness and GDM and glucose levels at GCT. First trimester microbiome diversity was assessed using stool and saliva samples that were collected during recruitment as described previously (Pinto et al., 2023). Briefly, sample collection and processing were in accordance with the National Institutes of Health (NIH) Human Microbiome Project standards (Human Microbiome Project Consortium, 2012). Samples were analyzed using 16S rRNA gene

sequencing to identify and quantify bacterial species composition of the gut microbiome. Bacterial DNA was extracted, amplified and sequenced using Illumina adapter sequences which targeted the highly conserved V4 region of the 16S gene (Goodrich et al., 2014). Alpha (within sample) diversity was calculated using four different indices, and values were considered as mediators: 1) Observed species which measures number of the species (amplicon sequence variants in this case) and represents richness (Wagner et al., 2018); 2) Shannon's diversity index which measures richness and evenness (overall diversity) (Wagner et al., 2018); 3) Faith's phylogenetic diversity which measures richness considering phylogenetic distance (Chao et al., 2016); and 4) Pielou's evenness index which measures equity in species abundance (Finotello et al., 2018).

2.5. Statistical analysis

Linearity of the associations between greenness (exposure) and outcomes was assessed using generalized additive models. Because nonlinear associations were observed (Figs. 1–2), we analyzed exposure variables in categories defined by tertiles of exposure, which allow interpretation relative to 'low', 'medium' and 'high' values.

Directed Acyclic Graphs (DAGs) were drawn (Supplemental Figs. 2 and 3), and were analyzed using DAGitty (Shrier and Platt, 2008; Textor et al., 2016). In the DAGs, we included known covariates that could potentially confound the associations between exposure-outcome, mediator-outcome and exposure-mediator.

The covariates evaluated were: maternal age, gravidity, parity, body mass index, family medical history, comorbidities, chronic medication treatment, smoking, GDM in previous pregnancies, education and ethnicity. Additional factors such as sleeping habits, stress levels, physical activity, diet type and eating habits (calorie and carbohydrate consumption) were also evaluated. Data were collected using self-report questionnaires (Pinto et al., 2023), validated stress questionnaires (Cohen et al., 1983), food diaries, and dietitian interviews. Fasting plasma glucose levels, insulin levels, leptin levels and inflammatory markers levels such as several interleukins, granulocyte-macrophage colony-stimulating factor, interferon gamma and tumor necrosis factor alpha were measured using blood samples collected during recruitment and were additionally considered as confounders or mediators. SES, a possible confounder in the association between residential greenness and GDM (Qu et al., 2020), was evaluated based on the residential address provided at recruitment and the Israel Central Bureau of Statistics' 2017th database (Israel Central Bureau of Statistics, 2017). In addition, season during early pregnancy, which is an important possible confounder (Preston et al., 2020), was defined based on the last menstrual period (LMP) date reported by the participants at recruitment, as hot season (LMP dates between March 31st through September 22nd) and cold season (LMP dates between September 23rd through March 30th) (Agay-Shay et al., 2013b; Alpert et al., 2004).

Based on the constructed DAGs, we selected one minimal set of variables needed to block all biasing paths for all models, and we adjusted the models for the following covariates: SES and seasonality at LMP (Supplemental Figs. 2 and 3).

Mean and standard deviation were calculated for continuous variables and percentages of every category were calculated for categorical variables. Descriptive analyses were performed between the covariates and GDM categories and NDVI tertiles. According to the distribution type, Student's T-tests and Mann-Whitney tests were performed for continuous variables for GDM categories, and analysis of variance tests and Kruskal-Wallis tests were performed for the three NDVI tertiles. Chi-squared tests were performed for categorical variables. Univariate analysis was performed in order to examine the association between exposure-outcome (NDVI-GDM, and NDVI-glucose levels).

Generalized linear models with normal distribution and identity link were used to evaluate associations between greenness and changes in mean glucose levels (beta coefficients with 95% confidence intervals). Generalized linear models with binary family log link function were used to evaluate the changes in odds ratios and 95% confidence intervals of GDM. Crude and adjusted models were evaluated.

Mediation analysis was conducted using four alpha diversity indices of first trimester microbiome to evaluate the percentage of the association between residential greenness at 300 m buffer and GDM and glucose levels at GCT, explained by each of the mediators (and 95% CI), estimated via the non-parametric bootstrap method with 500 simulations (Lapointe-Shaw et al., 2018). Causal mediation analysis was based on the following assumptions: 1) no unmeasured confounding of the exposure-outcome effect; 2) no unmeasured confounding of the mediator-outcome effect; 3) no unmeasured confounding of the

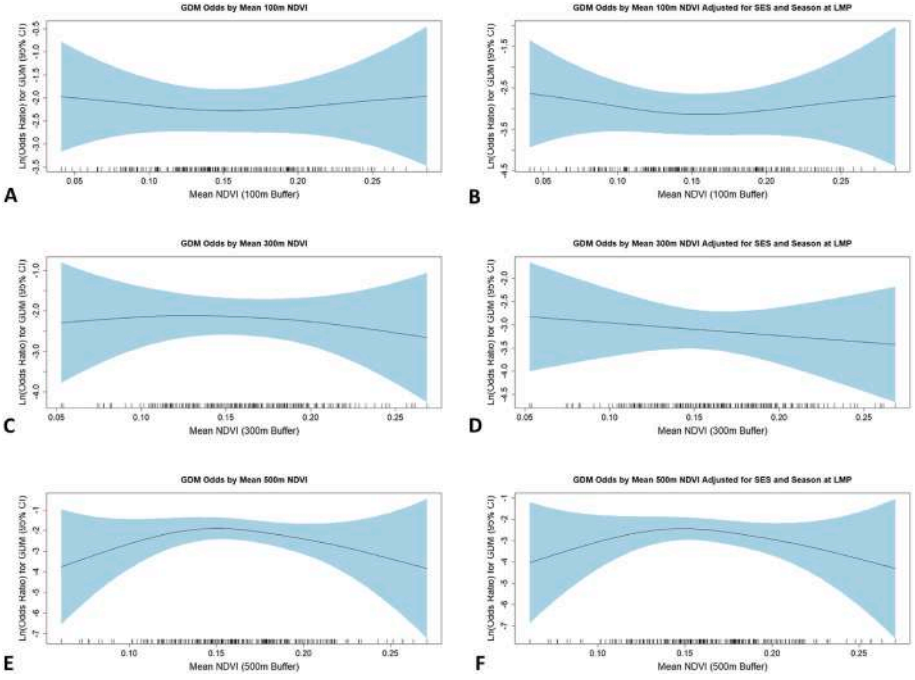

Fig. 1. Unadjusted and adjusted associations between GDM odds and mean NDVI at 100, 300 and 500 m buffers derived from RapidEye data for the urban population (N = 269). A and B present the 100 m NDVI buffer, C and D the 300 m buffer, and E and F the 500 m buffer. Crude models for the different buffers are presented in A, C and E, and adjusted models are in B, D and F. Generalized additive models were used. Models were adjusted for SES and season at LMP. Component smooths are shown with 95% confidence intervals that include the uncertainty about the overall mean (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

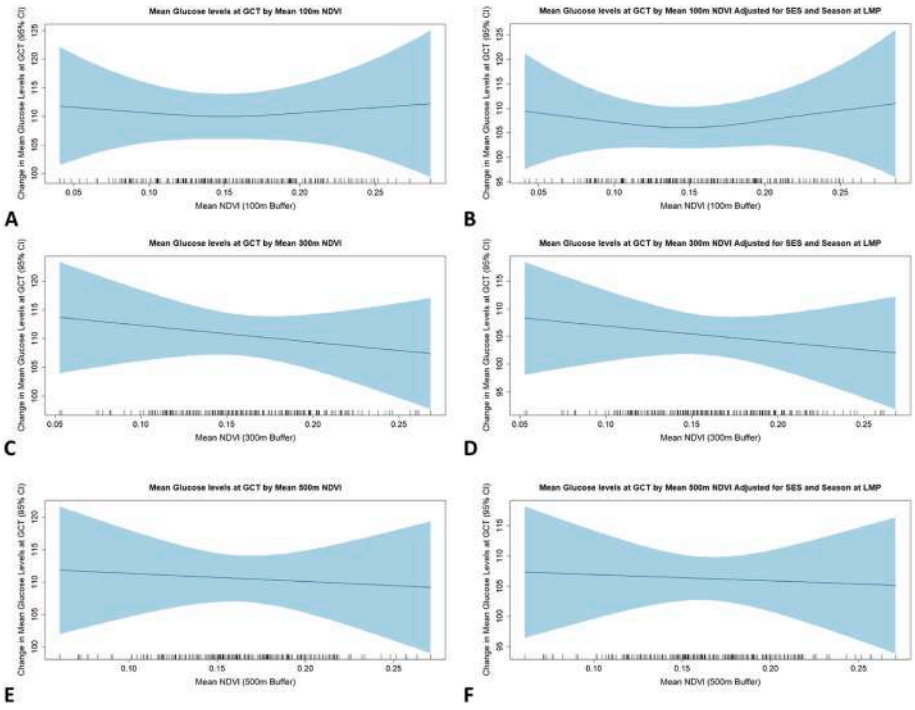

Fig. 2. Unadjusted and adjusted associations between changes in mean glucose levels at GCT and mean NDVI at 100, 300 and 500 m buffers derived from RapidEye data for urban population (N = 242). A and B present the 100 m NDVI buffer, C and D the 300 m buffer, and E and F the 500 m buffer. Crude models for the different buffers are presented are in A, C and E, and adjusted models in B, D and F. Generalized additive models were used. Models were adjusted for SES and season at LMP. Component smooths are shown with 95% confidence intervals that include the uncertainty about the overall mean (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

exposure-mediator effect; and 4) no confounder of the mediator-outcome effect that are affected by the exposure (Rijnhart et al., 2021).

When assumptions of the mediation analysis are met, the proportion mediated describes the proportion of the effect of the exposure (NDVI tertile) on the outcome (GDM/glucose levels at GCT) that goes through the mediator (first trimester microbiome diversity) (Lee et al., 2021). It is calculated by dividing the average causal mediation effect by the total effect, multiplied by 100 to generate percentages. The proportion mediated could be negative if the direct and indirect effects have opposite signs. It is calculated as the sum of the proportion mediated for each mediator at a time. Proportion mediated higher than 1 (larger than 100%) indicates one of the following: 1) there are other mediators with a negative proportion mediated; 2) the mediators affect one another; 3) there are interactions between the effects of the mediators on the outcome (VanderWeele and Vansteelandt, 2014).

A priori statistical significance was defined as $p < 0.05$. R (version 4.1.3; R Development Core Team), RStudio (version 1.3.1073; RStudio Team) and SPSS 25 (version 25.0; IBM SPSS Statistics for Windows) statistical softwares were used for all analyses described above.

2.6. Further analyses

Several sensitivity analyses were performed using Sentinel-2 satellite data and the entire population including non-urban living participants (N = 354). Methods are presented in supplements (Supplemental Methods 1).

2.7. Ethical approval

The study protocol was approved by the Helsinki committee of Rabin Medical Center, Beilinson Campus (No. 0263-15-RMC). Individual written informed consents were provided from all participants at recruitment, in accordance with Clalit's institutional review board approval (No. 0135-15-COM).

3. Results

3.1. Descriptive statistics

Selected maternal sociodemographic and lifestyle characteristics, as well as exposure variables, are presented in Table 1. Out of the study population, 27 participants (10.04%) were diagnosed with GDM. Participants in the GDM group were more likely to be multiparous, had a pre-pregnancy body mass index categorized as overweight or obese, had more GDM-related family history and had more GDM diagnoses in past pregnancies. Moreover, participants in the GDM group presented higher fasting glucose levels during early pregnancy (6–8 weeks of gestation) and were more likely to be smokers (all p values < 0.05). However, assessment of the GDM group compared to the non-GDM group revealed no statistically significant differences in other characteristics examined, including mean NDVI values, seasonality at LMP, SES, medication treatment (although medication types were significantly different), education or ethnicity. These results suggest that, excluding a few variables that were previously known as risk factors for GDM (such as multiparity, weight, and past GDM diagnosis), the study groups are very similar and thus comparable.

Supplemental Table 1 presents the same sociodemographic and lifestyle characteristics, characterized by mean NDVI exposure tertiles at 300 m buffer. There is a statistically significant difference between SES in the different exposure tertiles, with higher mean NDVI values corresponding to higher SES.

3.1.1. GDM diagnosis and mediators

Supplemental Table 2 presents first trimester covariates that were considered as possible mediators in urban study population (N = 269), characterized by GDM incidence. GDM diagnosis-related variables are also presented. As expected, significant differences were observed in first trimester body mass index, GCT and oral glucose tolerance test values, with higher values in the GDM group. We did not observe significant differences in stress levels, sleeping habits, calories and carbohydrates consumption, insulin and leptin levels, alpha diversity indices in saliva samples and most of the indices in stool samples. There was only a statistically significant difference in stool's observed species

Table 1
Study population (urban) characteristics of GDM diagnosed participants (n = 27) compared to healthy non-GDM participants (n = 242). Data reported as mean (SD) or as count (%).

	GDM group (N = 27)	Non-GDM group (N = 242)	Total population (N = 269)	*p* values[a]
Age (Years), Mean (SD)	32.82 (3.93)	31.23 (4.30)	31.39 (4.28)	0.06[b]
Age Category (Years)				0.19[c]
under 25	0 (0.0%)	15 (6.2%)	15 (5.6%)	
25-34	17 (63.0%)	168 (69.4%)	185 (68.8%)	
35+	10 (37.0%)	59 (24.4%)	69 (25.7%)	
Maternal Gravidity, Mean (SD)	2.59 (1.22)	2.33 (1.36)	2.36 (1.35)	0.15[d]
Maternal Gravidity Category				0.05[c]
1	8 (29.6%)	78 (32.2%)	86 (32.0%)	
2	2 (7.4%)	70 (28.9%)	72 (26.8%)	
3	11 (40.7%)	58 (24.0%)	69 (25.7%)	
4+	6 (22.2%)	36 (14.9%)	42 (15.6%)	
Maternal Parity, Mean (SD)	1.15 (1.03)	0.90 (0.91)	0.92 (0.92)	0.20[d]
Parity Category				**0.03**[c]
Nulliparous	10 (37.0%)	95 (39.3%)	105 (39.0%)	
Primiparous	5 (18.5%)	92 (38.0%)	97 (36.1%)	
Multiparous +	12 (44.4%)	55 (22.7%)	67 (24.9%)	
Season At LMP [e]				0.38[c]
Hot Season	9 (33.3%)	102 (42.1%)	111 (41.3%)	
Cold Season	18 (66.7%)	140 (57.9%)	158 (58.7%)	
Height (m), Mean (SD)	1.63 (0.07)	1.64 (0.06)	1.64 (0.06)	0.66[b]
Missing	0	20	20	
Pre-Pregnancy BMI (kg/m^2), Mean (SD)	28.03 (7.44)	22.30 (3.79)	22.92 (4.67)	< **0.001**[d]
Pre-Pregnancy BMI Category [f]				< **0.001**[c]
Missing	0	20 (8.3%)	20 (7.4%)	
Underweight	2 (7.4%)	32 (13.2%)	34 (12.6%)	
Normal	8 (29.6%)	147 (60.7%)	155 (57.6%)	
Overweight	8 (29.6%)	32 (13.2%)	40 (14.9%)	
Obesity	9 (33.3%)	11 (4.5%)	20 (7.4%)	
GDM Related Family History				**0.046**[c]
Missing	4 (14.8%)	81 (33.5%)	85 (31.6%)	
GDM	0 (0.0%)	1 (0.4%)	1 (0.4%)	
Diabetes	7 (25.9%)	20 (8.3%)	27 (10.0%)	
Hypertension	0 (0.0%)	15 (6.2%)	15 (5.6%)	
Diabetes and Hypertension	2 (7.4%)	4 (1.7%)	6 (2.2%)	
No	14 (51.9%)	121 (50.0%)	135 (50.2%)	
Previous GDM Category				**0.01**[c]
Yes	2 (7.4%)	2 (0.8%)	4 (1.5%)	
No	25 (92.6%)	240 (99.2%)	265 (98.5%)	
GDM Related Comorbidities [g]				< **0.001**[c]
Missing	0 (0.0%)	6 (2.5%)	6 (2.2%)	
Yes	9 (33.3%)	14 (5.8%)	23 (8.6%)	
Multiple GDM Related Comorbidities	0 (0.0%)	1 (0.4%)	1 (0.4%)	
Other Comorbidities	5 (18.5%)	74 (30.6%)	79 (29.4%)	
No Comorbidities	13 (48.1%)	147 (60.7%)	160 (59.5%)	
Medication Treatment				0.14[c]
Missing	0 (0.0%)	7 (2.9%)	7 (2.6%)	
Yes	9 (33.3%)	49 (20.3%)	58 (21.6%)	
No	18 (66.7%)	186 (76.9%)	204 (75.8%)	
Antibiotics Treatment During Pregnancy				0.28[c]
Missing	0 (0.0%)	1 (0.4%)	1 (0.4%)	
Yes	0 (0.0%)	10 (4.1%)	10 (3.7%)	
No	27 (100.0%)	231 (95.5%)	258 (95.9%)	
Antibiotics Treatment During First Trimester				0.89[c]
Missing	0 (0.0%)	1 (0.4%)	1 (0.4%)	
Yes	0 (0.0%)	1 (0.4%)	1 (0.4%)	
No	27 (100.0%)	239 (98.8%)	266 (98.9%)	
Possible	0 (0.0%)	1 (0.4%)	1 (0.4%)	
Early Pregnancy Fasting Glucose (mg/dL), Mean (SD)	89.19 (12.05)	83.21 (7.22)	83.85 (8.06)	**0.02**[d]
Fasting Glucose Level Category [h]				**0.01**[c]
Missing	0 (0.0%)	7 (2.9%)	7 (2.6%)	
Not Tested	0 (0.0%)	8 (3.2%)	8 (3.0%)	
Normal	21 (77.8%)	212 (87.6%)	233 (86.6%)	
High and Very High	6 (22.2%)	15 (6.2%)	21 (7.8%)	
Smoking Status				**0.02**[c]
Missing	1 (3.7%)	16 (6.6%)	17 (6.3%)	
Yes	3 (11.1%)	21 (8.7%)	24 (8.9%)	
No	18 (66.7%)	194 (80.2%)	212 (78.8%)	
Past Smoking	5 (18.5%)	11 (4.5%)	16 (6.0%)	
Education (Years), Mean (SD)	14.6 (2.4)	15.3 (2.4)	15.2 (2.4)	0.22[d]
Education Category				0.44[c]
Missing	7 (25.9%)	71 (29.3%)	78 (28/9%)	
High School (Up to 12 Years)	7 (25.9%)	42 (17.4%)	49 (18.2%)	
First Degree (13–16 Years)	9 (33.3%)	74 (30.6%)	83 (30.9%)	
Second Degree or Above (17+ Years)	4 (14.8%)	55 (22.7%)	59 (21.9%)	
Ethnicity				0.27[c]
Missing	5 (18.5%)	70 (28.9%)	75 (27.9%)	
Ashkenazi	13 (48.1%)	64 (26.5%)	77 (28.6%)	
Sephardic	5 (18.5%)	57 (23.6%)	62 (23.0%)	
At Risk (Arab, Yemen, Ethiopian)	1 (3.7%)	14 (5.8%)	15 (5.6%)	
Other	3 (11.1%)	37 (15.3%)	40 (14.9%)	
Socioeconomic Status Level, Mean (SD)	7.93 (1.36)	7.76 (1.82)	7.78 (1.78)	0.99[d]
Socioeconomic Status Category				0.91[c]
Low Socioeconomic Status (1–7)	9 (33.3%)	78 (32.2%)	87 (32.3%)	
High Socioeconomic Status (8+)	18 (66.7%)	164 (67.8%)	182 (67.7%)	
Mean NDVI Level At 100m Buffer (RapidEye), Mean (SD)	0.151 (0.057)	0.152 (0.050)	0.152 (0.050)	0.97[b]
Mean NDVI Level Category At 100m Buffer (RapidEye)				0.41[c]
Low Mean NDVI Level	10 (37.0%)	80 (33.1%)	90 (33.5%)	
Medium Mean NDVI Level	6 (22.2%)	84 (34.7%)	90 (33.5%)	
High Mean NDVI Level	11 (40.7%)	78 (32.2%)	89 (33.1%)	
Mean NDVI Level At 300m Buffer (RapidEye), Mean (SD)	0.16 (0.04)	0.16 (0.04)	0.16 (0.042)	0.70[b]
Mean NDVI Level Category At 300m Buffer (RapidEye)				0.33[c]
Low Mean NDVI Level	12 (44.4%)	78 (32.2%)	90 (33.5%)	
Medium Mean NDVI Level	6 (22.2%)	84 (34.7%)	90 (33.5%)	
High Mean NDVI Level	9 (33.3%)	80 (33.1%)	89 (33.1%)	

(*continued on next page*)

Table 1 (*continued*)

	GDM group (N = 27)	Non-GDM group (N = 242)	Total population (N = 269)	*p* values[a]
Mean NDVI Level At 500m Buffer (RapidEye), Mean (SD)	0.16 (0.03)	0.16 (0.040)	0.16 (0.04)	0.73[b]
Mean NDVI Level Category At 500m Buffer (RapidEye)				0.34[c]
Low Mean NDVI Level	9 (33.3%)	81 (33.5%)	90 (33.5%)	
Medium Mean NDVI Level	12 (44.4%)	78 (32.2%)	90 (33.5%)	
High Mean NDVI Level	6 (22.2%)	83 (34.3%)	89 (33.1%)	

BMI- Body Mass Index; GDM- Gestational Diabetes Mellitus; LMP- Last Menstrual Period; NDVI- Normalized Difference Vegetation Index; SD- Standard Deviation; Categorical variables are presented as count (%) and continuous variables as mean (SD).
Significant differences (*p* value < 0.05) are marked in bold. Missing reported as count (%).
[a] *p* values were calculated according to variables' type and distribution.
[b] Student's T test for normal distributed continuous variables.
[c] Chi-squared Test for categorical variables.
[d] Mann-Whitney Test for non-normal distributed continuous variables.
[e] Hot season between March 31st through September 22nd, cold season between September 23rd through March 30th.
[f] BMI category- underweight <18.5 kg/m^2, normal 18.5–24.9 kg/m^2, overweight 25–29.9 kg/m^2, obesity >30 kg/m^2.
[g] GDM related comorbidities such as past or current preeclampsia, obesity, polycystic ovary syndrome, chronic or gestational hypertension and hyperemesis.
[h] Fasting glucose level- normal <95 mg/dL, high 95–126 mg/dL, very high >126 mg/dL.

index (Supplemental Table 2), which was lower in the GDM group. Most of the cytokines measured were also similar between the two groups, except for interleukin-6 and granulocyte-macrophage colony-stimulating factor which were significantly higher in the GDM group.

Supplemental Table 1 also presents the same covariates and outcome assessments, characterized by NDVI tertiles derived from RapidEye at 300 m buffer. Most covariates did not demonstrate significant differences between the tertiles, with the exception of higher body mass index at first trimester and lower levels of interferon gamma at the lowest tertile of NDVI exposure, and lower values of Shannon's diversity index (stool samples) at the highest tertile of exposure (Supplemental Table 1).

Sensitivity analysis using the entire population including non-urban living participants (N = 354) demonstrated similar sociodemographic characteristics (Supplemental Table 3). GDM diagnosis-related variables and possible mediators were vastly the same, with the exception of observed species index that was similar between the groups, and interleukin-8 concentration which was significantly higher in the GDM group compared to non-GDM.

3.2. Associations between residential greenness and GDM

We observed no statistically significant associations between residential greenness and GDM odds. Although statistical analysis demonstrated weak evidences, women who were exposed to medium tertile levels of mean NDVI at 300 m buffer, had lower GDM odds (OR = 0.46, 95% CI: 0.17, 1.29, p = 0.14) compared to women exposed to lowest tertile levels of mean NDVI (Fig. 3, detailed in Supplemental Table 4). Although also not significant, medium tertile levels of mean NDVI demonstrated lower GDM odds at 100 m buffer and higher odds at 500 m buffer, compared to lowest tertile levels of NDVI (*p* values = 0.30 and 0.49, respectively). Comparing the highest tertile levels to lowest tertile demonstrated higher GDM odds at 100 m buffer and lower odds at 500 m buffer, also without statistical significance (*p* values = 0.80 and 0.43, respectively).

A similar pattern of association was observed after adjusting for SES and season at LMP (OR = 0.45, 95% CI: 0.16, 1.26, p = 0.13) with lower GDM odds in the medium NDVI tertile levels at 300 m buffer compared to the lowest tertile (Fig. 3, detailed in Supplemental Table 4). Adjusted models for 100 and 500 m buffers also presented very similar results, yet again without statistically significant values (*p* values > 0.30).

Sensitivity analyses performed using Sentinel-2 data for urban population (Supplemental Table 4) and using both satellites for the entire population including non-urban living participants (Supplemental Table 5) demonstrated similar results.

3.3. Associations between residential greenness and glucose levels at GCT

For women with GCT measurements (N = 242) some beneficial associations were also observed, yet again without sufficient statistical significance (Fig. 4, detailed in Supplemental Table 6). The strongest effect estimate was observed for women exposed to medium tertile levels of mean NDVI at 300 m buffer, who demonstrated lower (though non-significant) mean glucose levels at GCT compared to the lowest tertile levels of mean NDVI (β = −5.82, 95% CI: 14.43, 2.79, p = 0.19). A stronger, yet still non-significant, effect estimate was observed when adjusting for season at LMP and SES (β = −6.28, 95% CI: 14.91, 2.34, p = 0.15). For the 100 m and 500 m buffers, no significant associations were observed, as indicated by the high *p* values.

Similar results were observed in the sensitivity analyses using Sentinal-2 data for the urban population (Supplemental Table 6), and using both satellites for the entire population including non-urban living participants (Supplemental Table 7).

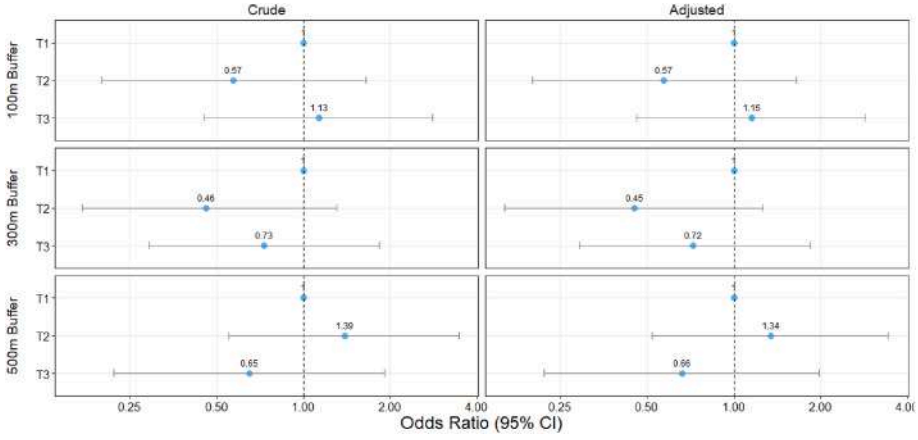

Fig. 3. Generalized Linear Models with binary family log link function evaluating the association between mean NDVI, abstracted from RapidEye data, and Odds Ratio for GDM in urban population (N = 269). Data is represented as crude and adjusted for SES and season at LMP at 100, 300 and 500 m buffers. Medium and highest tertiles were compared to lowest tertile of exposure. CI- Confidence interval; GDM- Gestational Diabetes Mellitus; NDVI- Normalized Difference Vegetation Index; T- tertiles of exposure; T1- reference tertile, low mean NDVI tertile; T2- medium mean NDVI tertile; T3- highest mean NDVI tertile; Values are detailed at Supplemental Table 4.

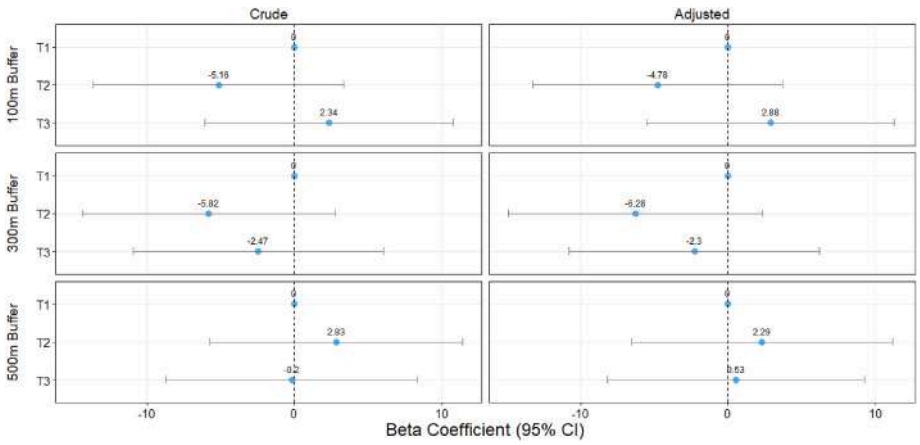

Fig. 4. Generalized linear models with normal distribution and identity link evaluating the association between mean NDVI, abstracted from RapidEye data, and change in mean glucose at GCT in urban population (N = 242). Data is represented as crude and adjusted for SES and season at LMP at 100, 300 and 500 m buffers. Medium and highest tertiles were compared to lowest tertile of exposure. CI- Confidence interval; GCT- Glucose Challenge Test; NDVI- Normalized Difference Vegetation Index; T- tertiles of exposure; T1- reference tertile, low mean NDVI tertile; T2- medium mean NDVI tertile; T3- highest mean NDVI tertile; Values are detailed at Supplemental Table 6.

3.4. Microbiome as a mediator

The results of the mediation analysis are reported in Supplemental Tables 8–11. The total effects of the mean NDVI tertiles at 300 m buffer on GDM (Supplemental Tables 8–9) and glucose levels at GCT (Supplemental Tables 10–11) are generally similar to the results reported in Supplemental Tables 4 and 6

3.4.1. Mediation on GDM

For those with microbiome data (N = 107 with saliva samples, N = 122 with stool samples), analyzing the possible mediation of microbiome on the crude association between NDVI and GDM odds did not reveal statistically significant change of the total effects in the highest and medium tertiles of mean NDVI at 300 m buffer compared to the lowest tertile (Supplemental Table 8). In each of the mediators inspected for stool and saliva samples, the direct effects were nearly the same as the total effects, hence there was practically no mediation effect of first trimester microbiome on the association between residential greenness and GDM. Similar patterns were observed in the adjusted models (Supplemental Table 9).

3.4.2. Mediation on glucose levels at GCT

Generally, for those with both GCT and microbiome data (N = 95 and 109 with saliva and stool samples in the medium tertile levels, and N = 98 and 113 with saliva and stool samples in the highest tertile levels, respectively), mediation of first trimester microbiome in the association between NDVI and glucose levels at GCT was not observed. However, although non-significant, in the crude model of the highest versus lowest tertile of mean NDVI at 300 m buffer for Faith's phylogenetic diversity index (stool sample), the direct effect of NDVI on the change in mean glucose levels had beta coefficient of −7.20 (95% CI: 17.39, 3.46, p = 0.23), whereas the total effect comprising the mediation effect had beta coefficient of −8.32 (95% CI: 19.31, 2.29, p = 0.12), with proportion mediated of 13.5% (95% CI: 42.31%,210.60%, p = 0.27) as presented in Supplemental Table 10. A similar pattern for the same index was observed in the adjusted model for SES and season at LMP (Supplemental Table 11).

4. Discussion

Although the associations we found were not statistically significant, lower odds of GDM and lower mean glucose levels were observed in women exposed to medium levels of residential greenness compared to low levels (mean NDVI at 300 m buffer). This pattern of association was maintained (though still not significant) after adjusting for SES and seasonality at LMP. To the best of our knowledge, this is the first study that evaluated mediation through first trimester microbiome, using four alpha diversity indices in stool and saliva samples. The mediation analysis did not reveal significant evidence of mediation of first trimester microbiome in the associations between residential greenness and GDM and glucose levels at GCT.

Our results are consistent with current equivocal literature, with several studies that could not identify significant associations between residential greenness, glucose intolerance and risk of GDM such as Rammah et al. performed in Spain amongst 2263 participants, and Lin et al. performed in China amongst 587 participants (Lin et al., 2021; Rammah et al., 2021). On the contrary, larger studies did find significant associations (Liao et al., 2019; Qu et al., 2020; Yu et al., 2023). Liao et al. reported reduced incidence of impaired glucose tolerance and GDM amongst 6807 participants in China. In their study, impaired glucose tolerance was defined as participants demonstrating some level of abnormal glucose tolerance, though not fully meeting the GDM diagnosis criteria (Liao et al., 2019). Qu et al. reported lower risk for GDM with higher NDVI exposure amongst 5327 participants in China (Qu et al., 2020). Yu et al. reported similar results during second trimester amongst 46,665 participants in China, with no protective effect of first trimester NDVI exposure on GDM risk (Yu et al., 2023). Both Qu et al. and Yu et al. used different exposure buffers than those used in our study. Furthermore, most of the previous studies reported linear associations between NDVI and blood glucose levels and GDM risk, with higher values of NDVI associated with the strongest beneficial effects (Liao et al., 2019; Lin et al., 2021; Yu et al., 2023). In our study, although non-significant, the patterns of associations between greenness and the outcomes were non-linear. It is rather hard to compare these finding to our results, since both the outcome and exposure were evaluated differently.

Our study population was much smaller compared to the studies described above. In addition, unlike our study, Qu et al., Yu et al. and Liao et al. all used GDM diagnosis using a one-step diagnosis approach with 75 g oral glucose tolerance test, which presumably identifies less women as GDM-positive compared to the 100 g oral glucose tolerance test performed in our study (Yogev et al., 2009). Liao et al. also used an impaired glucose tolerance diagnosis for participants who presented moderate glucose intolerance. Therefore, these studies included the more explicit GDM diagnosed participants (Liao et al., 2019; Qu et al., 2020; Yu et al., 2023). It is possible that stratifying the GDM-positive group to different glucose intolerance levels, might have made the differences in greenness exposure levels more distinguishable. These studies also used a lower resolution satellite (500 m resolution by Qu et al. and 250 m resolution by Yu et al. and Liao et al.) compared to the high resolution RapidEye (5 m × 5 m) and Sentinel-2 (10 m × 10 m) satellites that were used in our study. Using lower resolution satellite may cause exposure misclassification. However, this exposure misclassification is presumably random and therefore through the null and thus it cannot explain these gaps. Although we did not find statistically significant results in our study, in future studies, it might be beneficial to

use high resolution satellite data as in our study.

Although the possible interactions between the host, the environment, and the microbiome were suggested to affect human health (Panthee et al., 2022), our study did not find a role when considering GDM. Recent studies reported associations between microbiome diversity and greenness, changes in microbial compositions and specific species domination at different levels of NDVI exposure, and higher soil bacterial diversity in green spaces with higher plant diversity (Baruch et al., 2021; Gacesa et al., 2022; Wu et al., 2022). On the contrary, a previous study performed by Bowyer et al. amongst 2443 non-pregnant twin participants in the United Kingdom, could only find small compositional differences in stool samples across greenness quartiles at 3000 m buffers, whilst differences in abundancies of specific genera were observed for all measures according to urban-rural status (Bowyer et al., 2022).

To the best of our knowledge, our study is the first that aimed to evaluate mediation of microbiome on the possible beneficial association between greenness and GDM amongst pregnant women. We could not demonstrate sufficient evidence regarding the mediating effect of first trimester microbiome in the associations between residential greenness and GDM and glucose levels at GCT. First trimester microbiome diversity was vastly similar between GDM and healthy pregnant women in the urban population, except a significantly lower number of species in the GDM group, as represented by the observed species index in stool samples. It is rather possible that evaluating the more complex microbial composition (abundance of specific species comprising the microbiome) in future studies is needed, using samples from the pre-pregnancy period and from each trimester during pregnancy. This might be supported by Crusell et al., demonstrating only minor aberration in salivary microbiome during late pregnancy amongst 213 GDM participants in Denmark (Crusell et al., 2020), and by Pinto et al., who demonstrated a change in specific bacterial species amongst GDM and non-GDM participants, using the same database that was used in our study (Pinto et al., 2023). Nevertheless, inconsistent results regarding species abundancies amongst GDM-diagnosed participants were demonstrated in recent reviews, and therefore further studies should be performed (Ionescu et al., 2022; Rold et al., 2022).

Our study has a few limitations that should be mentioned. Evaluation of exposure to residential greenness was based on surrounding NDVI. NDVI is measured by satellite and it is a general measure of greenness, disregarding plant richness types, quality and use of green spaces. The residential address used for geocoding, seasonality and microbiome were all evaluated during first trimester of pregnancy. Hence, we did not have any knowledge about residential address or microbiome diversity prior to pregnancy. It is fairly possible that evaluating these variables before pregnancy and during the first and second trimesters, might have revealed a more comprehensive picture of the association. Furthermore, we did not have data on whether the residential address has changed for participants during later stages of pregnancy in which GDM diagnosis is made, which might have tipped the scales regarding exposure.

The study population was not sufficiently diverse. The majority of the participants are educated, from higher SES, living in cities in central Israel, without sufficient representation of minorities and periphery-living participants. Furthermore, the questionnaires given during recruitment were not translated into different languages such as Russian, Arabic, and Amharic. Since no compliance percentages were calculated during cohort recruitment, there may be a compliance bias that affects our external validity. A larger population size would have probably been helpful in determining significant associations. Another significant selection bias that was unintentionally present in our study was that participants with higher risk for GDM such as previous GDM diagnosis or polyhydramnios (N = 28), did not perform the GCT but only oral glucose tolerance test and therefore were excluded from the analysis of association between residential greenness and glucose levels at GCT. We can only assume that including these participants, with presumably higher risk for higher glucose levels at GCT, might have increased the strength of the associations observed.

In spite of the limitations present in our study, to the best of our knowledge, this is the first study that examined the associations between residential greenness and GDM and glucose levels at GCT with microbiome as a mediator, and the first to ever explore these associations among the Israeli population. Another strength is that microbiome was evaluated using both saliva and stool samples, with data collected prospectively. We had information on many personal, behavioral and biological variables that were considered in detailed DAGs and helped determine the minimal set of confounders for our models. In addition, we performed several sensitivity analyses. Albeit not statistically significant, our results suggest a non-linear association between residential greenness and risk for GDM, but did not demonstrate sufficient evidence for mediation by microbiome. This study provides a novel approach regarding the well investigated association between residential greenness and health benefits, and GDM in particular, that should be further evaluated in future studies.

5. Conclusions

Although not statistically significant, our study suggests possible associations between residential greenness and glucose intolerance and risk of GDM during pregnancy, with no sufficient evidence for mediation thorough microbiome. Our study does not provide sufficient evidence to support the hypothesis that the beneficial effect of residential greenness is mediated through microbiome, and further studies should examine this possible association, with emphasis on population size and diversity, and microbiome composition.

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CRediT authorship contribution statement

Ofir Avizemel: Data Curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - Original Draft, Writing - Review & Editing. **Sigal Frishman:** Formal analysis, Investigation, Resources. **Yishay Pinto:** Formal analysis, Investigation, Resources. **Yaron Michael:** Methodology, Data Curation. **Sondra Turjeman:** Formal analysis, Writing - Review & Editing. **Kinneret Tenenbaum-Gavish:** Resources. **Or Yariv:** Resources. **Yoav Peled:** Resources. **Eran Poran:** Resources. **Joseph Pardo:** Resources. **Rony Chen:** Resources. **Moshe Hod:** Resources. **Betty Schwartz:** Supervision. **Eran Hadar:** Resources, Supervision. **Omry Koren:** Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition. **Keren Agay-Shay:** Conceptualization, Methodology, Data Curation, Software, Formal analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare they have no actual or potential competing financial interest or any other conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114191.

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Sex-specific effect of perfluoroalkyl substances exposure on liver and thyroid function biomarkers: A mixture approach

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ABSTRACT

Although studies have investigated the effects of perfluoroalkyl substances (PFASs) on liver and thyroid function, little is known about its combined and sex-specific effect. A total of 688 participants were interviewed and serum PFASs concentration was measured using liquid chromatography/mass spectrometry. Five biomarkers of liver and thyroid function (ALT, GGT, TSH, FT3 and FT4) were chosen as outcomes. A restriction cubic spline function was applied to capture the dose-response relationship between PFASs and liver enzymes and thyroid hormones. Multivariable regression and Bayesian kernel machine regression (BKMR) models were performed to assess the single and overall associations of PFASs with targeted biomarkers. Single-pollutant analyses indicated that increased PFASs concentrations were associated with elevated ALT and GGT levels. BKMR models suggested positive dose-response relationships between PFASs mixtures and ALT and GGT levels. Significant associations were only detected between several PFASs and thyroid hormones, and joint effect of PFASs mixtures on FT3 levels was found at higher concentrations. Meanwhile, sex differences were found in the associations of PFASs with ALT and GGT levels, with significant results only in males. Our findings provide epidemiological evidence for combined and sex-specific effects of PFASs on ALT and GGT levels.

1. Introduction

Perfluoroalkyl substances (PFASs) are a group of man-made fluorinated organic compounds that are widely used in industrial and consumer products due to the thermal stability, and hydrophobic and oleophobic properties (Lindstrom et al., 2011). Nevertheless, perfluorooctane-sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), the two most widely used PFASs, have been listed as persistent organic pollutants since they were found to be highly toxic, bioaccumulative, long-distance migratory and environmentally persistent (Liu et al., 2022a). Humans are widely exposed to PFASs through contaminated food, drinking water, dust, and consumer products (Haug et al., 2011; Banzhaf et al., 2017; Akhbarizadeh et al., 2020), which results in a high detection rate of PFASs in biological samples.

Accumulating evidence has suggested that exposure to PFASs is associated with a range of adverse health conditions, and that the liver and thyroid are the target organs for PFASs (Coperchini et al., 2017; Costello et al., 2022). Rodent studies showed consistent evidence for PFASs hepatotoxicity, including increased liver weight and histopathological alterations (Costello et al., 2022). On the other hand, laboratory data suggested that PFASs may be thyroid toxicants that cause hypothalamic-pituitary-thyroid axis disorders by regulating the

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biosynthesis and transport of thyroid hormones and by interfering with thyroid receptors (Weiss et al., 2009; Ren et al., 2016; Coperchini et al., 2017). There are, of course, numerous epidemiological studies that have attempted to explore the effects of PFASs exposure on human liver and thyroid function, but the results are controversial (Olsen et al., 2003; Khalil et al., 2018; Mora et al., 2018; Salihovic et al., 2018; Nian et al., 2019; Xiao et al., 2020; Li et al., 2021; Derakhshan et al., 2022; Jensen et al., 2022; Li et al., 2022; Liu et al., 2022a). In addition to differences in study design, population characteristics and sample size, potential interactions between PFASs (additive or antagonistic) may account for the inconsistent results. As the study on mixtures in the field of environmental epidemiology continues to evolve, researchers are committed to quantify the risk of disease caused by environmental chemical mixtures, which may be helpful to identify modifiable exposures that are amenable for public health interventions (Braun et al., 2016).

To our knowledge, three studies reported the combined effects of mixed PFASs on liver enzymes with a positive association in general population of Chinese and Canadian adults (Borghese et al., 2022; Liu et al., 2022a) and a nonsignificant association in the European birth cohort (Stratakis et al., 2020). Furthermore, five population-based studies evaluated the overall association between PFASs exposure and thyroid hormones, including one study in the general adult population in China (Li et al., 2022) and four studies in pregnant women or newborns in China, Boston, and the United States (Aimuzi et al., 2020; Lebeaux et al., 2020; Preston et al., 2020; Guo et al., 2021). Thus, the epidemiological evidence for joint effects of PFASs mixtures on liver and thyroid function remains insufficient, especially for adults.

Sex differences in the rate of elimination of PFASs have been reported. Breastfeeding and menstruation were one of the pathways of PFASs excretion (Thomsen et al., 2010; Mondal et al., 2014; Wong et al., 2014), which may lead to rapid elimination of PFASs in young women (Attanasio, 2019). Moreover, the liver was considered a sexually dimorphic organ, since it could express sex hormone receptors (Attanasio, 2019). Thyroid-related disorders showed a sex-specific prevalence, with a 5-20-fold higher susceptibility in women than in men (Tunbridge et al., 1977; Vanderpump et al., 1995; Gietka-Czernel, 2017), for which the sex steroid environment may be a key determinant (Baksi and Pradhan, 2021). Therefore, the sex-specific effects of PFASs exposure on liver and thyroid function deserve profound consideration.

The purpose of this study was to assess single and overall associations of PFASs with biomarkers of liver and thyroid function and to explore potential modified effect of sex in a Chinese adult population.

2. Methods

2.1. Study population

The study was originally initiated to explore whether PFASs exposure was associated with risk of type 2 diabetes (T2DM), applying a case-control study design. T2DM cases were patients from the endocrinology department who donated the blood sample. We excluded patients with severe respiratory and cardiac failure, metabolic disease crises (e.g., diabetes and thyroid), type 1 diabetes, infection, surgery, or bed rest. Diabetes cases were matched (1:1) according to sex and age (±2) with controls that recruited from Medical Examination Center. A total of 688 participants recruited at two hospitals in Tianjin, China, were interviewed from April 2021 to March 2022. In current analyses, participants with missing data on hepatic enzymes (n = 24) and thyroid hormone (n = 94) were excluded. Thus, a total of 664 and 594 individuals were ultimately used to investigate the association between PFASs and liver and thyroid function biomarkers, respectively. All participants provided informed consent, and the study protocol was reviewed and approved.

2.2. Serum PFASs measurement

A total of 24 PFASs were identified and quantified in serum samples using a liquid chromatography-mass spectrometry (Shimadzu 8050, Japan). The limit of detection (LOD) and limit of quantification (LOQ) were considered, as the peak of compounds reached the signal-to-noise ratio of 3 and 10, respectively. In present study, 8 PFASs with detection rates higher than 70% were used for statistical analysis, including potassium perfluoro-1-octanesulfonate (PFOS), perfluoro-n-octanoic acid (PFOA), potassium perfluoro-1-hexanesulfonate (PFHxS), perfluoro-n-nonanoic acid (PFNA), perfluoro-n-decanoic acid (PFDA), perfluoro-n-undecanoic acid (PFUnDA), sodium 1H,1H,2H,2H-perfluoro-1-octanesulfonate (6:2 FTS), sodium perfluoro-1-heptanesulfonate (PFHpS), and serum concentrations of compounds below the LOD were replaced by LOD/$\sqrt{2}$. Detailed information was given in the Supplement, as reported in previous study (Liu et al., 2022b).

2.3. Outcome assessment

Serum alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) levels were measured by enzymatic methods. Concentrations of thyroid stimulating hormone (TSH), free triiodothyronine (FT3), and free thyroxine (FT4) were measured by a chemiluminescent immunoassay. When the liver enzyme level exceeded its upper limit of normal, the biomarkers of liver function were further classified into dichotomous outcomes, i.e., abnormal ALT and abnormal GGT. The clinical reference values of ALT and GGT in this study were ≤41 U/L in men and ≤31 U/L in women, and ≤60 U/L in men and ≤40 U/L in women, respectively (Liu et al., 2022a). Similarly, the thyroid functional status of the study participants was classified according to the thyroid hormone levels (Cappola et al., 2006), but further analysis was not performed because more than 90% of the participants had normal thyroid function.

2.4. Covariates

All participants were interviewed by two well-trained researchers and information on covariates was collected using a structured questionnaire. Confounders adjusted for in this study were as follows: age (year), sex (male or female), BMI (kg/m^2), drinking (yes or no), smoking (yes or no), diabetes (yes or no), hypertension (yes or no), and dyslipidemia (yes or no). Drinking was defined as consuming alcohol at least once a week for one year. Participants who self-reported smoking ≥1 cigarette per day for at least 6 months were considered to be smokers. Diabetes was considered if fasting glucose was ≥7.0 mmol/L, or self-reported on glucose-lowering therapy. Hypertension was defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg, or taking anti-hypertensive medication. Dyslipidemia was defined as having one or more of the following conditions: total cholesterol ≥6.2 mmol/L (hypercholesterolemia), triglyceride ≥2.3 mmol/L (hypertriglyceridemia), low-density lipoprotein cholesterol ≥4.1 mmol/L (hyperbetalipoproteinemia), and high-density lipoprotein cholesterol <1.0 mmol/L (hypoalphalipoproteinemia), consistent with the Chinese national guidelines for the prevention and treatment of adult dyslipidemia (Kong et al., 2022).

2.5. Statistical analysis

The normality of the data was examined using Q-Q plots. The subject characteristics were presented as mean (standard deviations, SDs) for normally distributed variables, median (interquartile range, IQR) for skewed variables, and as number (percentage) for categorical variables, respectively. Student t-test/Wilcoxon rank-sum test and chi-square test were used to compare sex differences in participant characteristics. Q-Q plots showed skewed distributions of PFASs and biomarkers of liver and thyroid function; therefore, natural log-transformation was performed for the above continuous variables.

Restricted cubic spline (RCS) regression was developed to assess the dose-response curves of PFASs with liver and thyroid biomarkers. 3

knots located at the 10th, 50th and 90th percentiles were selected for the RCS function according to the lowest Akaike Information Criterion. Multivariable linear regression models were established to assess the difference percentage of liver and thyroid biomarkers associated with ln-unit increment in PFASs, since most of the relationships between liver and thyroid biomarkers and PFASs concentration were linear (Fig. S1 to Fig. S5). Regression coefficients (β) and standard errors (SE) were used to calculate the percentage differences in the dependent variables and corresponding 95% confidence intervals (95% CI) based on the following two formulas: [exp (β)-1] × 100% and [exp (β ± 1.96 × SE)-1] × 100% (Nian et al., 2019). Multivariable logistic regression models were additionally applied to explore the associations between PFASs and abnormal liver functions. Furthermore, the mixed effect of PFASs on biomarkers of liver and thyroid function was evaluated using BKMR models with 10,000 iterations by a Markov chain Monte Carlo algorithm. The overall effect was evaluated when all PFASs mixtures were fixed at a certain percentile concentration (in the range of 25th to 75th percentile, with 5% increments), and compared to the mixture concentration that remained at the median. The posterior inclusion probabilities (PIPs) were calculated to identify the main components contributing to the effect, using 0.5 as the threshold.

A sex-stratified analysis was conducted to evaluate whether sex modified the relationship of PFASs exposure with biomarkers of liver and thyroid function. In addition, Bayesian kernel machine regression (BMKR) models were further developed in men and women, respectively, to assess the combined effects of PFASs mixtures on targeted biomarkers. Several sensitivity analyses were additionally performed to assess the robustness of the main models. (1) Considering that renal failure may confuse the associations between PFASs and liver enzymes and thyroid hormones (Jain and Ducatman, 2019b), the participants with chronic kidney disease (CKD), defined as an estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m^2, were excluded. In current study, the Japanese coefficient-modified Chronic Kidney Disease Epidemiology Collaboration equation was used to calculate eGFR (Horio et al., 2010). (2) Given the bias stemmed from missing data of thyroid function, multiple imputation by chained equations was applied to interpolate the missing thyroid hormone values. (3) Additional adjustment was performed in the sensitivity analyses for medication of diabetes and hypertension.

Data analyses were carried out by R software (version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria). $P < 0.05$ in the main analyses and $P < 0.1$ in the subgroup analyses were considered statistically significant (both two-tailed) (Tian et al., 2019; Webster et al., 2016; Zeng et al., 2019).

3. Results

3.1. Population characteristics

Table 1 presents the general characteristics of the 688 subjects, with 325 of them being male. The average age of all participants was 57.58 ± 12.57 years. Among the 8 PFASs with a detection rate higher than 70%, the highest serum concentration of PFASs was PFOS (median: 16.79 ng/ml), followed by PFOA (median: 9.40 ng/ml), PFNA (median: 1.86 ng/ml), PFHxS (median: 1.63 ng/ml), PFDA (median: 1.33 ng/ml), PFUnDA (median: 0.75 ng/ml), PFHpS (median: 0.43 ng/ml), and 6:2 FTS (median: 0.42 ng/ml).

3.2. Associations of single-PFASs exposure with liver and thyroid function

Table 2 presents the individual associations of PFASs with estimated changes of liver enzymes and thyroid hormones. It was observed that serum PFASs was positively associated with ALT and GGT. For example, per ln-unit increment in PFOA and PFOS exposure was associated with 5.98% (95% CI: 2.10%, 10.01%) and 6.16% (95% CI: 0.44%, 12.22%) higher GGT levels in serum, respectively. Likewise, as illustrated in

Table 1
Characteristics of the study population (n = 688).

	Total	Males	Females	P value
PFOA, ng/ml (M, IQR)	9.40 (5.32, 15.10)	9.42 (5.61, 15.46)	9.33 (5.06, 14.77)	0.431
PFHxS, ng/ml (M, IQR)	1.63 (1.21, 2.20)	1.78 (1.31, 2.33)	1.53 (1.07, 2.03)	< 0.001
PFOS, ng/ml (M, IQR)	16.79 (10.00, 28.67)	18.80 (11.73, 33.71)	14.80 (9.15, 24.94)	< 0.001
PFNA, ng/ml (M, IQR)	1.86 (1.15, 2.73)	1.93 (1.24, 2.83)	1.72 (1.10, 2.57)	0.025
PFDA, ng/ml (M, IQR)	1.33 (0.72, 2.94)	1.52 (0.79, 3.47)	1.21 (0.70, 2.41)	0.001
PFUnDA, ng/ml (M, IQR)	0.75 (0.46, 1.23)	0.82 (0.45, 1.36)	0.71 (0.47, 1.10)	0.056
6:2 FTS, ng/ml (M, IQR)[a]	0.42 (0.16, 0.64)	0.48 (0.29, 0.67)	0.34 (0.16, 0.59)	< 0.001
PFHpS, ng/ml (M, IQR)	0.43 (0.22, 0.71)	0.48 (0.29, 0.74)	0.36 (0.17, 0.68)	< 0.001
Age, years	57.58 ± 12.57	55.36 ± 12.36	59.58 ± 12.44	< 0.001
BMI, kg/m^2	25.79 ± 3.86	26.20 ± 3.45	25.41 ± 4.16	0.009
Smoking, n (%)	241 (35.0%)	195 (60.0%)	46 (12.7%)	< 0.001
Drinking, n (%)	199 (28.9%)	167 (51.4%)	32 (8.8%)	< 0.001
Hypertension, n (%)	424 (61.6%)	207 (63.7%)	217 (59.9%)	0.289
Diabetes, n (%)	344 (50.0%)	159 (48.9%)	185 (51.1%)	0.621
Hyperlipidemia, n (%)	335 (48.7%)	185 (56.9%)	150 (41.4%)	< 0.001
ALT, U/L (M, IQR)[b]	19.10 (14.10, 27.63)	21.90 (15.75, 31.35)	16.85 (13.00, 24.42)	< 0.001
GGT, U/L (M, IQR)[b]	21.95 (15.97, 34.00)	25.60 (19.10, 40.40)	19.00 (14.07, 28.40)	< 0.001
TSH, mIU/L (M, IQR)[c]	1.85 (1.21, 2.76)	1.69 (1.15, 2.45)	2.02 (1.35, 3.10)	< 0.001
FT3, pmol/L (M, IQR)[c]	3.57 (1.04, 4.68)	2.07 (1.04, 4.68)	3.88 (1.04, 4.68)	0.617
FT4, pmol/L (M, IQR)[c]	10.55 (8.47, 13.66)	10.50 (8.46, 13.74)	10.66 (8.57, 13.51)	0.650

[a] The concentrations below the LOD were replaced by LOD/$\sqrt{2}$.
[b] 664 individuals were used for analysis.
[c] 594 individuals were used for analysis.

Table S4, significant associations of PFOA (OR = 1.23, 95% CI: 1.00, 1.56), PFNA (OR = 1.70, 95% CI: 1.18, 2.48), PFDA (OR = 1.51, 95% CI: 1.17, 1.98), and PFUnDA (OR = 1.66, 95% CI: 1.18, 2.39) with greater odds of abnormal GGT were also detected in multivariable logistic regression models. In addition, several PFASs were found to be associated with decreased TSH and FT4 levels and increased FT3 levels. A ln-unit increase in PFHxS was associated with decreasing of 12.74% (95% CI: 3.23%, 21.31%) TSH level.

The results of subgroup analyses stratified by sex are described in Table 3. Sex-specific associations of serum PFASs concentrations with liver function biomarkers were found in present study. For instance, exposure to PFOA, PFHxS and PFOS significantly increased GGT concentrations in males (PFOA: 9.43%, 95% CI: 4.54%–14.55%; PFH_XS: 18.38%, 95% CI: 5.91%–32.32%; PFOS: 8.74%, 95% CI: 0.82%–17.27%), but not in females (PFOA: -1.07%, 95% CI: -6.62%–4.80%; PFHxS: 1.10%, 95% CI: -10.63%–14.37%; PFOS: 1.83%, 95% CI: -5.80%–10.08%) (all P for interaction <0.1). However, no modified effect of sex on the associations of PFASs concentrations with thyroid hormones levels was seen in our results.

3.3. Associations of PFASs mixtures with liver and thyroid function

The PIPs identified PFNA as a significant chemical associated with liver function, while PFNA and PFHxS were recognized as important chemicals linked to thyroid function (Table S5). Fig. 1 depicts the overall effect of an increase in PFASs mixtures on ALT and GGT levels when the

Table 2
The estimated percentage difference (95% CI) for liver and thyroid function biomarkers associated with per ln-PFASs increment in multivariable linear regression models.

Ln-PFASs (ng/ml)	ALT[a]	GGT[b]	TSH[c]	FT3[d]	FT4[e]
PFOA	2.99 (−0.55, 6.64)	**5.98 (2.10, 10.01)**	−2.78 (−7.13, 1.78)	**2.65 (0.77, 4.56)**	**−2.63 (-4.31, -0.92)**
PFHxS	**10.09 (1.78, 19.07)**	**11.44 (2.44, 21.24)**	**−12.74 (-21.31, -3.23)**	0.27 (−3.87, 4.58)	−2.27 (−6.07, 1.68)
PFOS	4.62 (−0.65, 10.17)	**6.16 (0.44, 12.22)**	−5.33 (−11.49, 1.25)	1.34 (−1.39, 4.15)	**−2.93 (-5.38, -0.41)**
PFNA	**10.58 (3.44, 18.20)**	**14.33 (6.45, 22.79)**	**−10.64 (-18.12, -2.47)**	**4.96 (1.31, 8.74)**	−2.14 (−5.37, 1.20)
PFDA	**6.31 (1.39, 11.46)**	**8.84 (3.46, 14.50)**	**−6.92 (-12.68, -0.79)**	−1.47 (−4.00, 1.11)	−1.57 (−3.94, 0.87)
PFUnDA	**7.95 (1.65, 14.63)**	**11.00 (4.08, 18.38)**	**−8.56 (-15.58, -0.95)**	−0.43 (−3.62, 2.86)	−1.17 (−4.15, 1.91)
6:2 FTS	**9.95 (2.19, 18.31)**	**9.42 (1.13, 18.39)**	−4.11 (−13.27, 6.02)	1.79 (−2.27, 6.01)	−1.61 (−5.31, 2.24)
PFHpS	**7.02 (0.88, 13.53)**	0.57 (−5.65, 7.19)	−5.16 (−12.53, 2.84)	2.23 (−1.07, 5.64)	**−3.17 (-6.11, -0.14)**

Adjusted for age, sex, BMI, smoking, drinking, diabetes, hypertension, and hyperlipidemia.
Bold indicates $P < 0.05$.
[a] Ln-ALT (U/L).
[b] Ln-GGT (U/L).
[c] Ln-TSH (mIU/L).
[d] Ln-FT3 (pmol/L).
[e] Ln-FT4 (pmol/L).

8 PFASs mixtures are fixed at the 50th percentile. The BKMR models indicated positive dose-response relationships between PFASs mixtures and ALT and GGT in the total population. Further analyses of BKMR models run separately in males and females revealed that significant joint effect of PFASs mixtures on liver function biomarkers was only found in males, consistent with the results of multivariable linear regression models. When holding all PFASs at the higher levels (65th, 70th and 75th percentile) compared to their median concentrations, PFASs mixtures were negatively associated with the FT3 levels (Fig. S6). However, the joint effect of PFASs on FT3 levels was nonsignificant in both men and women (Fig. S6). Furthermore, the overall associations of PFASs mixtures with TSH and FT4 levels were not significant across population subgroups (Fig. S6). As illustrated in Fig. S7, it was found that an IQR increase in the ln-transformed concentration of PFDA and PFHxS was negatively associated with ln-FT3 level when other chemicals were set at the 25th, 50th and 75th percentile, respectively. However, for PFNA, the results were in the opposite direction (Fig. S7). In addition, the bivariate exposure-response function for PFASs did not exhibit interactions, as evidenced by the parallel curves with equal slopes at the 10th, 50th, and 90th percentiles (Figs. S8–S12).

3.4. Sensitivity analyses

Table S6 shows the associations of single PFASs exposure with interested biomarkers estimated using multivariate linear regression models after excluding participants with CKD. Overall, positive associations between PFASs and ALT and GGT were still detected and several PFASs were positively or negatively associated with FT3, TSH and FT4, similar to the main analyses. Consistent results were also found in the BKMR models (Figs. S13–S14). Because missing data on thyroid hormones in this study may bias the association of PFASs with thyroid

Table 3
The estimated percentage difference (95% CI) for liver and thyroid function biomarkers associated with per ln-PFASs increment in multivariable linear regression models, stratified by sex.

Biomarkers	Ln-PFASs (ng/ml)	Males	Females	P inter
ALT[a]	PFOA	**4.95 (0.39, 9.72)**	−0.65 (−5.97, 4.97)	**0.019**
	PFHxS	**12.82 (1.38, 25.55)**	5.96 (−5.79, 19.18)	0.241
	PFOS	5.81 (−1.58, 13.76)	2.67 (−4.69, 10.59)	0.295
	PFNA	**13.37 (3.16, 24.60)**	7.72 (−2.00, 18.40)	0.432
	PFDA	**6.75 (0.06, 13.89)**	4.83 (−2.25, 12.42)	0.169
	PFUnDA	7.41 (−0.75, 16.24)	8.42 (−0.99, 18.73)	0.319
	6:2 FTS	**15.27 (4.08, 27.67)**	3.07 (−7.56, 14.92)	**0.080**
	PFHpS	**13.13 (4.25, 22.77)**	1.13 (−7.38, 10.42)	**0.082**
GGT[b]	PFOA	**9.43 (4.54, 14.55)**	−1.07 (−6.62, 4.80)	**0.000**
	PFHxS	**18.38 (5.91, 32.32)**	1.10 (−10.63, 14.37)	**0.054**
	PFOS	**8.74 (0.82, 17.27)**	1.83 (−5.80, 10.08)	**0.081**
	PFNA	**24.47 (12.98, 37.13)**	3.07 (−6.68, 13.84)	**0.003**
	PFDA	**10.66 (3.46, 18.36)**	5.33 (−2.11, 13.34)	**0.018**
	PFUnDA	**12.06 (3.22, 21.65)**	9.85 (−0.11, 20.80)	**0.030**
	6:2 FTS	**18.33 (6.36, 31.64)**	−2.68 (−13.17, 9.07)	**0.013**
	PFHpS	4.09 (−4.57, 13.53)	−4.85 (−13.21, 4.31)	0.334
TSH[c]	PFOA	0.14 (−5.87, 6.54)	−4.63 (−11.03, 2.22)	0.231
	PFHxS	−8.28 (−20.79, 6.20)	**−14.67 (-26.56, -0.84)**	0.105
	PFOS	−7.87 (−16.49, 1.64)	−2.53 (−11.21, 7.00)	0.956
	PFNA	**−12.55 (-23.07, -0.60)**	−6.60 (−17.26, 5.43)	0.778
	PFDA	**−11.39 (-18.87, -3.23)**	−2.79 (−11.34, 6.59)	0.569
	PFUnDA	**−10.87 (-20.07, -0.62)**	−7.43 (−17.66, 4.07)	0.946
	6:2 FTS	−2.82 (−15.66, 11.97)	−1.15 (−14.84, 14.74)	0.292
	PFHpS	−1.41 (−12.01, 10.47)	−5.81 (−16.38, 6.10)	0.134
FT3[d]	PFOA	**2.90 (0.95, 4.90)**	2.84 (−0.55, 6.35)	0.561
	PFHxS	3.99 (−0.68, 8.89)	−2.27 (−9.16, 5.15)	0.422
	PFOS	−0.48 (−3.52, 2.65)	3.27 (−1.27, 8.02)	**0.074**
	PFNA	0.75 (−3.26, 4.92)	**8.44 (2.34, 14.90)**	**0.031**
	PFDA	−2.02 (−4.72, 0.76)	−0.57 (−4.90, 3.96)	0.202
	PFUnDA	−1.41 (−4.75, 2.04)	0.64 (−4.92, 6.52)	0.189
	6:2 FTS	0.79 (−3.60, 5.39)	4.22 (−3.01, 11.99)	0.233
	PFHpS	−0.37 (−3.87, 3.26)	**6.11 (0.21, 12.35)**	**0.070**
FT4[e]	PFOA	**−3.42 (-5.77, -1.00)**	−2.14 (−4.55, 0.34)	0.658
	PFHxS	−3.28 (−8.84, 2.63)	−2.19 (−7.38, 3.29)	0.688
	PFOS	**−4.20 (-7.92, -0.33)**	−2.19 (−5.41, 1.15)	0.498
	PFNA	−3.38 (−8.27, 1.78)	−1.80 (−6.00, 2.58)	0.778

(continued on next page)

Table 3 (*continued*)

Biomarkers	Ln-PFASs (ng/ml)	Males	Females	P inter
	PFDA	−2.02 (−5.48, 1.57)	−1.15 (−4.38, 2.19)	0.831
	PFUnDA	−1.39 (−5.66, 3.08)	−0.71 (−4.83, 3.58)	0.994
	6:2 FTS	−3.59 (−8.94, 2.08)	−0.63 (−5.83, 4.85)	0.355
	PFHpS	−3.80 (−8.10, 0.70)	−3.80 (−7.82, 0.40)	0.788

Adjusted for age, sex, BMI, smoking, drinking, diabetes, hypertension, and hyperlipidemia.
Bold indicates $P < 0.05$ or $P < 0.1$.
[a] Ln-ALT (U/L).
[b] Ln-GGT (U/L).
[c] Ln-TSH (mIU/L).
[d] Ln-FT3 (pmol/L).
[e] Ln-FT4 (pmol/L).

function, single- and multi-pollutant analyses were performed again after multiple interpolation of the missing data, and the results were comparable to those in the main models (Table S7 and Fig. S15). The results found in the sensitivity analyses with additional adjustment for medication were consistent with the main findings (Table S8 and Figs. S16-S17).

4. Discussion

The results of current analyses suggested positive associations of single and joint PFASs exposure with ALT and GGT levels by using multivariable linear regression and BKMR models. Whereas for thyroid function, several PFASs were inversely correlated with TSH and FT4 levels and positively correlated with FT3 levels in the single-pollutant analyses. However, the BKMR models only showed that PFASs mixtures were negatively correlated with FT3 at higher levels. Additionally, the sex-specific effects of PFASs on ALT and GGT were observed, with significant associations only in males. Our findings provide epidemiological evidence for combined and sex-specific effects of PFASs on ALT and GGT levels in Chinese adults.

PFOS (median: 16.79 ng/ml) and PFOA (median: 9.40 ng/ml) were the most contaminated PFASs, which were comparable to levels reported in previous studies conducted in Chinese populations (Li et al., 2022). For example, a cross-sectional investigation of general adult population in Guangzhou, China reported the concentrations of PFOS and PFOA were 14.85 ng/ml and 8.97 ng/ml, respectively. Another case-control study conducted in Tianjin, China found the serum concentrations were 11.64 ng/ml for PFOS and 12.28 ng/ml for PFOA (Duan et al., 2021). However, PFOS and PFOA concentrations in this study were higher than those reported in studies undertaken in Canada (3.3 ng/ml for PFOS; 1.3 ng/ml for PFOA) (Cakmak et al., 2022), the United States (6.3 ng/ml for PFOS; 2.2 ng/ml for PFOA) (Jain and Ducatman, 2019a), Australia (1.8 ng/ml for PFOS; 2.3 ng/ml for PFOA) (Eriksson et al., 2017), and Sweden (0.57 ng/ml for PFOS; 2.53 ng/ml for PFOA) (Salihovic et al., 2018).

Numerous epidemiological studies have investigated the individual associations of PFASs with liver and thyroid function. Overall, positive (Salihovic et al., 2018; Nian et al., 2019; Borghese et al., 2022; Cakmak et al., 2022; Liu et al., 2022a) or null (Mundt et al., 2007; Sakr et al., 2007; Khalil et al., 2018; Nilsson et al., 2022) associations were found for PFASs-ALT and PFASs-GGT analyses in published studies. For instance, Nian et al. found positive associations between PFASs and liver function biomarkers, including ALT and GGT, in the general adult population (Nian et al., 2019). Positive associations were also found for PFASs-ALT and PFASs-GGT in a nationally representative sample of 6768 individuals from the Canadian Health Measures Survey (Cakmak et al., 2022). However, there was no statistically significant relationship between PFASs and ALT levels in Australian firefighters (Nilsson et al., 2022). Similarly, no significant association of PFASs with ALT levels was observed in obese children (Khalil et al., 2018). In addition, epidemiological findings for PFASs-thyroid hormones were inconsistent, and pregnant women and newborns were priority populations of concern. For example, previous evidence showed that PFASs were positively (Webster et al., 2014; Webster et al., 2016; Byrne et al., 2018), negatively (Aimuzi et al., 2019; Liang et al., 2020; Li et al., 2022), or insignificantly (Preston et al., 2018; van Gerwen et al., 2020) correlated with TSH levels.

In current analyses, we also found that PFASs were associated with elevated ALT and GGT levels, which was similar to the documented results. On the other hand, multivariable linear regression models suggested that several PFASs were negatively correlated with TSH and FT4 levels and positively correlated with FT3 levels. The controversial results may be attributed to differences in study population, study design,

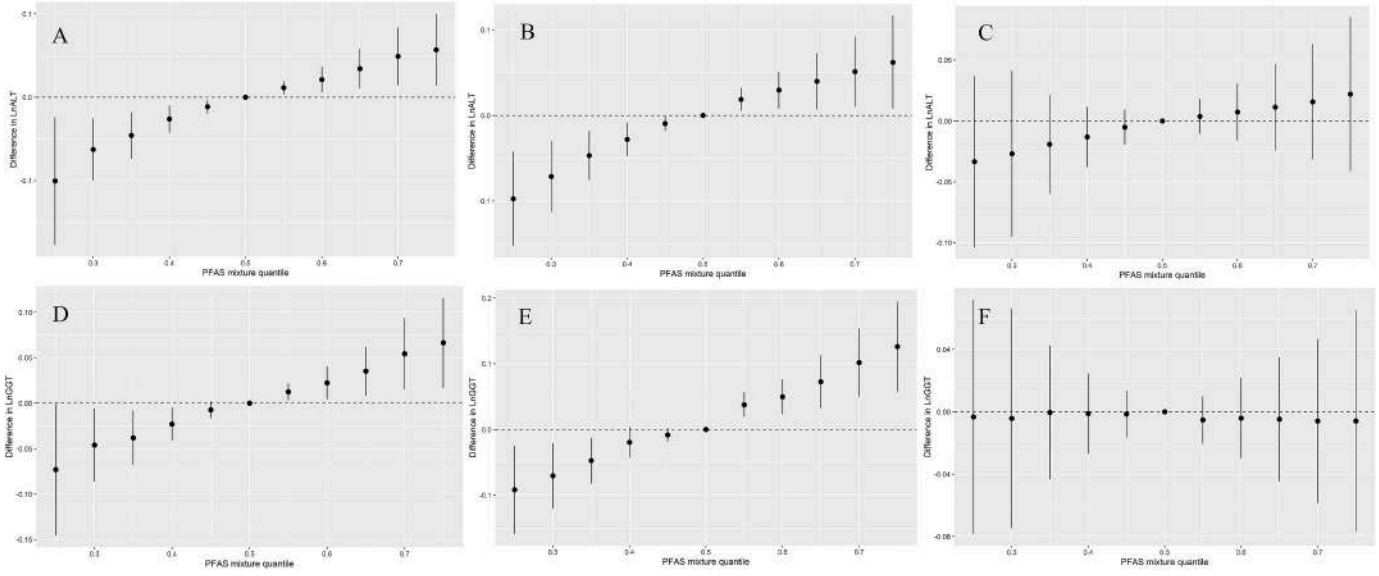

Fig. 1. Overall effects of PFASs mixtures on hepatic enzymes in (A, D) total population, (B, E) males, and (C, F) females using BKMR model. Models were adjusted for age, sex, BMI, smoking, drinking, diabetes, hypertension, and hyperlipidemia.

concentration and composition of PFASs, and genetic background. Exposure assessment of PFASs in environmental media such as soil, river and atmosphere showed a distinct spatial distribution of PFASs (Lin et al., 2020; Lin et al., 2021; Shi et al., 2021; Du et al., 2022; Mattias et al., 2022), which may be related to the fluoridation industry sites and human activities. Meanwhile, accumulating evidence suggested that the health effects of PFASs may be non-monotonic and non-linear (Aimuzi et al., 2020; Skogheim et al., 2021; Li et al., 2022; Liu et al., 2022a), suggesting that differences in PFASs concentrations across studies may lead to complex and inconsistent results (Han et al., 2021).

To date, little is known about joint effects of PFASs mixtures on liver and thyroid function. After an extensive search of previous studies, we found three population studies that assessed the overall associations of combined PFASs exposure with liver enzymes levels (Stratakis et al., 2020; Borghese et al., 2022; Liu et al., 2022a). Liu et al. observed a positive dose-response pattern of PFASs mixtures with ALT and GGT levels by applying BKMR models in a general population of Chinese adults (Liu et al., 2022a). Data from the Canadian Health Measures Survey suggested that one-quartile increment in the PFASs mixtures was positively associated with GGT, as assessed by the quantile g-computation (Borghese et al., 2022). Another study by Stratakis et al. evaluated the overall association of prenatal PFASs exposure with liver function, and the results showed a positive association between PFASs mixtures and liver injury risk, but the combined effects on ALT and GGT levels were almost insignificant (Stratakis et al., 2020). In this study, after fully adjusting for potential confounders in BKMR models, significant associations between PFASs mixtures and elevated ALT and GGT levels were obtained, consistent with prior studies.

In addition, to our knowledge, five studies investigated the combined effects of PFASs mixtures on thyroid hormones (Aimuzi et al., 2020; Lebeaux et al., 2020; Preston et al., 2020; Guo et al., 2021; Li et al., 2022). Li et al. found a nonlinear combined effect of PFASs mixtures on TSH and FT4 levels in the Chinese adult population (Li et al., 2022). Guo et al. evaluated the overall association between cord serum PFASs concentration and neonatal thyroid function using weighted quantile sum (WQS) regression models and results showed that PFASs mixtures were positively associated with FT4 and negatively associated with TSH, but not with FT3 (Guo et al., 2021). Aimuzi et al. observed a negative overall association of PFASs mixtures with maternal FT4 level, and null associations with TSH and FT3 levels (Aimuzi et al., 2020). Moreover, two studies explored the mixed effects of prenatal PFASs exposure on maternal and neonatal thyroid function. Only maternal free T4 index was inversely associated with PAFSs mixtures in the study by Preston et al. (Preston et al., 2020), while another study showed non-significant associations between PFASs mixtures and biomarkers of thyroid function in both mothers and newborns (Lebeaux et al., 2020). In the current study undertaken in Chinese adult population, BKMR models showed that high concentrations of PFASs mixtures were inversely correlated with FT3 level, but not with either TSH or FT4. The available evidence for the effects of PFASs mixtures on thyroid hormones is contradictory, and well-designed studies are encouraged to address this confusion.

Given the sex differences in PFASs elimination and the sexually dimorphic effect of liver and thyroid, this study further assessed whether the association of PFASs with liver and thyroid biomarkers was sex-specific. First, we established stratified analyses by sex and found that the single associations of PFASs with ALT and GGT levels were stronger in males with significant interaction terms ($P < 0.1$). Further, BKMR models run across sex also demonstrated that the significant joint effects of PFASs mixtures on ALT and GGT levels were observed only in males. However, we did not find the sex-specific association between PFASs and thyroid hormones. Stratified analyses by BMI, smoking, drinking, diabetes, hypertension, and dyslipidemia were conducted to explore potential modifiers of the association between PFASs and thyroid function biomarkers, because growing evidence has shown that smoking, obesity, hyperglycemia, and pre-existing cardiometabolic diseases can worsen TSH in the circulation. The results showed decreased TSH levels associated with PFASs were only found in overweight individuals and those with dyslipidemia (Table S9). Published studies suggested controversial results for sex differences in the association between PFASs and liver function. Analysis of data from the general adult population in China did not show sex differences in the associations of PFASs with GGT and ALT levels (Liu et al., 2022a). Similarly, a study by Mora et al. investigated the relationship between prenatal and mid-childhood PFASs concentrations and mid-childhood ALT levels stratified by child sex and showed a non-significant interaction term between PFASs and child sex (Mora et al., 2018). Using data from NHANES 2013–2016, another study was conducted to explore the sex differences in the association of PFASs and liver function in adolescents aged 12–19 years (Attanasio, 2019). The authors reported the sex differences in the association of PFASs and liver function, such that PFOA was correlated with increased ALT level and a higher risk of elevated ALT in females, while the reversed results were found in males.

The underlying mechanisms of the hepatotoxicity associated with PFASs exposure are unclear. In vitro experiments showed that PFOA treatment could induce endoplasmic reticulum stress and mitochondrial-mediated apoptosis in human liver L02 cells via endoplasmic reticulum-mitochondria communication (Wang et al., 2022b). Animal models suggested that PFOS could form an ion pair with choline, which in turn caused hepatic steatosis and oxidative stress (Zhang et al., 2016). Zebrafish liver cells exhibited inhibition of cell viability and massive accumulation of autophagic vacuoles after PFOA exposure, and further transcriptome analysis revealed significant changes in autophagy, signal transduction, tight junctions, endocrine system, immune system, and metabolism-related pathways (Wu et al., 2022). Lin et al. reported that exposure to environmentally relevant concentrations of PFOS and PFOA could result in liver damage and oxidative stress in male black-spotted frogs, which might be associated with dysregulation of the gut microbiota (Lin et al., 2022b). In addition, it has been shown that the PFASs-induced abnormalities in hepatic lipid metabolism are mediated in part through the peroxisome proliferator-activated receptor (Jiang et al., 2021; Lin et al., 2022a; Wang et al., 2022a; Wang et al., 2022c). Sex differences in tissue distribution and pharmacokinetics of PFASs may explain the sex-specific effect of PFASs on liver function. Research conducted by Kim et al. found that the tissue-plasma partition coefficient (Kp) values of PFOS and PFOA in the liver showed significant differences between male and female rats (Kim et al., 2016). Specifically, the study reported that male rats had Kp values for PFOS and PFOA that were approximately 1.9 and 2.9 times higher, respectively, than those in female rat. Additionally, a review of literature revealed that female rats exhibited higher urinary excretion of PFASs compared to males, which could potentially account for the observed sex differences in the distribution of specific PFASs, such as PFHxS and PFOA, in rats (Pizzurro et al., 2019).

Several limitations in this study must be acknowledged. First, non-longitudinal data cannot make causal inferences about PFASs exposure and targeted biomarkers. Second, while adjusting for several important covariates, the confounding effects of other factors, such as medications, diet, and unmeasured environmental contaminants, could not be completely eliminated, similar to published studies (Attanasio, 2019; Cakmak et al., 2022; Liu et al., 2022a). In present study, additional adjustment for medication of hypertension and diabetes was performed in the sensitivity analyses, and the results were similar to the main analyses. Third, the missing data of thyroid hormones may cause bias in the results. However, sensitivity analysis of multiple interpolations of thyroid hormones showed comparable results to those obtained by the main models. Fourth, participants were from a case-control study assessing the association of PFASs with risk of T2DM, which may lead to the sample size of the current study is inadequate to provide sufficient statistical power for certain analyses and limit the generalization of the findings. Fifth, whether differences in PFASs concentrations across sex are responsible for the sex-specific effects of PFASs on ALT and GGT warrants further investigation.

5. Conclusion

In conclusion, we found positive associations between single and joint exposure to PFASs and ALT and GGT levels in Chinese adults, and the significant associations were found only in males. Our findings provide population-based evidence for the hepatotoxicity of combined PFASs exposure and the sex-specific adverse effects of PFASs on liver function.

Author contribution

Ze Yang: Conceptualization, Methodology, Formal analysis, Data Curation, Writing-Original Draft; **Ruifang Liu:** Formal analysis, data Curation, Writing-Original Draft; **Hongbo Liu**: Methodology, Formal analysis; **Jiemin Wei**: Conceptualization, Validation; **Xiaohui Lin**: Methodology; **Mingyue Zhang**: Methodology; **Yu Chen**: Conceptualization; **Jingyun Zhang**: Data Curation; **Meiqing Sun**: Methodology; **Zhe Feng**: Methodology; **Jian Liu**: Methodology; **Xiangyang Liu**: Methodology; **Xiaoxu Huo**: Data Curation, **Kun Men**: Resources; **Qiaoyun Yang**: Methodology, Data Curation, **Xi Chen**: Data Curation; **Nai-jun Tang**: Writing-Review & Editing, Supervision, Funding acquisition.

Declaration of competing interest

Authors declare no actual or potential competing financial interests.

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Appendix A. Supplementary data

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Urinary neonicotinoid insecticides and adiposity measures among 7-year--old children in northern China: A cross-sectional study

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ABSTRACT

Background: Neonicotinoid insecticides (NEOs) are emerging synthetic insecticides used in various pest management regimens worldwide. Toxicology studies have indicated the obesogenic potential of NEOs, but their associations with adiposity measures are largely unknown.
Objectives: We aimed to assess urinary levels of NEOs/metabolites and their associations with children's adiposity measures, and to further investigate the potential role of oxidative stress.
Methods: This study included 380 children who participated in the 7th year's follow-up of the Laizhou Wan Birth Cohort in northern China. Urinary levels of seven NEOs and two metabolites and a biomarker of lipid peroxidation named 8-iso-prostaglandin-F2α (8-iso-PGF2α) were detected. A total of nine indicators of adiposity were measured. Body mass index (BMI) z-score ≥85th percentile was defined as overweight/obesity, and waist-to-height ratio (WHtR) ≥0.5 was considered as abdominal obesity. Multiple linear regression, binary logistic regression and mediation analysis were performed.
Results: Six NEOs [imidacloprid (IMI, 99.7%), clothianidin (CLO, 98.9%), dinotefuran (DIN, 97.6%), thiamethoxam (THM, 95.5%), acetamiprid (ACE, 82.9%), thiacloprid (THD, 77.6%)] and two metabolites [N-desmethyl-acetamiprid (N-DMA, 100.0%), 6-chloronicotinic acid (6-CINA, 97.9%)] exhibited high detection rates. Multiple linear regressions showed positive associations of waist circumference with urinary levels of IMI and THM, of WHtR with IMI and THM levels, and of body fat percentage with 6-CINA levels. In contrast, exposure to N-DMA was negatively associated with body fat percentage and fat mass index. Binary logistic regressions further revealed that higher IMI levels were associated with overweight/obesity (OR = 1.556, 95% CI: 1.100, 2.201) and abdominal obesity (OR = 1.478, 95% CI: 1.078, 2.026) in children. 8-iso-PGF2α demonstrated 27.92%, 69.52% and 35.37% mediating effects in the positive associations of IMI, THD and THM with WHtR, respectively. Sex modified the associations of DIN with body fat mass (p_{int} = 0.032), body fat percentage (p_{int} = 0.009), fat mass index (p_{int} = 0.037) and the overweight/obesity rate (p_{int} = 0.046), with negative associations in girls and nonsignificant positive associations in boys.
Conclusions: School-age children in northern China were widely exposed to NEOs/metabolites. Urinary levels of NEOs/metabolites were associated with adiposity measures through the mediating role of 8-iso-PGF2α. These associations were mixed, and a sex-specific effect might exist.

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1. Introduction

Neonicotinoid insecticides (NEOs) are emerging synthetic insecticides serving as a replacement for organophosphorus and pyrethroid insecticides (Lu et al., 2018; Oya et al., 2021). Due to their high insecticidal activities and low mammalian toxicities, NEOs are widely used in agriculture, garden forestry, and animal husbandry for pest control (Matsuda et al., 2005; Ospina et al., 2019; Wang et al., 2019). The usage of NEOs has dramatically increased worldwide since the early 1990s, and it accounted for more than 25% of the global insecticide market share in 2014 (Bass et al., 2015; Cimino et al., 2017). Until now, NEOs/metabolites have been ubiquitous in ecosystems and they have been broadly detected in various media, such as air, soil and water (Bonmatin et al., 2015, 2021; Casillas et al., 2022). Human exposure to NEOs mainly occurs via the intake of contaminated drinking water and food (Chen et al., 2020; Mahai et al., 2021). China is an important producer and consumer of NEOs (Zhang et al., 2019b). The general population in China have increased exposure to NEOs due to their wide application for pest control for planting and residential purposes (Pan et al., 2022; Song et al., 2020); in particular, the elevated levels of NEOs among Chinese children have raised significant concerns in public health (Wang et al., 2020a).

Childhood obesity is considered to be a global public health problem, and the prevalence of overweight/obesity in children has been increasing worldwide over the past decades (Fan and Zhang, 2020; NCD-RisC, 2017; Torres-González et al., 2020; Wang et al., 2020b). Notably, children with obesity are more likely to become obese in adulthood and develop cardiometabolic complications, metabolic disorders, and cancer later in life (Gurnani et al., 2015; Weihrauch-Blüher et al., 2019). Although the underlying factors are far from fully clear, growing evidence has proposed endocrine disrupting chemicals (EDCs) as emerging risk factors for childhood obesity (Heindel et al., 2015; Muscogiuri et al., 2017; Yang et al., 2018). In recent years, an increasing body of evidence has revealed that NEOs possess endocrine disrupting properties (Caron-Beaudoin et al., 2018; Mesnage et al., 2018; Mikolić and Karačonji, 2018) that may disturb adipose metabolism and thereby increase fat accumulation (Mesnage et al., 2018; Sun et al., 2017). Limited epidemiological studies on the influence of NEOs/metabolites on adiposity measures are available, and more research is needed to investigate these associations.

The underlying mechanisms of NEOs' toxicity have not been clearly elucidated, but *in vivo* and *in vitro* studies have suggested that oxidative stress may play an important role, e.g., the generation of reactive oxygen/nitrogen species (ROS/RNS) (El-Gendy et al., 2010; Sheets et al., 2016). Previous epidemiological studies reported that exposure to NEOs was associated with increased oxidative stress biomarkers of lipid damage (Li et al., 2020; Zhang et al., 2021). Elevated levels of oxidative stress were shown to be correlated with children's adiposity measures (Arogbokun et al., 2021; Loy et al., 2014). Moreover, animal studies have shown that NEOs cause oxidative stress, which may further induce lipid accumulation and obesity (Lu et al., 2021; Yan et al., 2020). However, it remains unclear whether oxidative stress plays a role in the associations between NEOs and adiposity measures in epidemiological studies. Therefore, we explored the mediating effect of oxidative stress in these associations among children.

Based on the information collected from the follow-up survey in the 7th year of the Laizhou Wan Birth Cohort (LWBC) in northern China, we aimed to assess urinary levels of NEOs/metabolites in children, their associations with adiposity measures and the role of 8-iso-PGF2α in these associations. Additionally, we further examined whether sex modified the relationships between NEOs/metabolites and adiposity measures in children.

Abbreviations

NEOs	neonicotinoid insecticides
8- iso-PGF2α	8-iso-prostaglandin F2α
BMI	body mass index
WHtR	waist-to-height ratio
IMI	imidacloprid
CLO	clothianidin
DIN	dinotefuran
THM	thiamethoxam
ACE	acetamiprid
THD	thiacloprid
N- DMA	N-desmethyl-acetamiprid
6- CINA	6-chloronicotinic acid
EDCs	endocrine disrupting chemicals
LWBC	Laizhou Wan Birth Cohort
NIT	nitenpyram
HPLC–MS/MS	high-performance liquid chromatography coupled with triple quadrupole mass spectrometry
RSDs	relative standard deviations
LODs	limits of detection
SD	standard deviation
ORs	odds ratios
CIs	confidence intervals
TE	total effect
IE	ndirect effect
DE	direct effect

2. Materials and methods

2.1. Study population

The Laizhou Wan Birth Cohort was established in 2010–2013 in the southern coastal area of Laizhou Wan (Bay) of the Bohai Sea, Shandong Province, northern China. Detailed information about the cohort was published elsewhere (Ding et al., 2013; Han et al., 2018; Yao et al., 2019). Briefly, the eligibility criteria for recruitment included expectant mothers with aged ≥18 years old, singleton pregnancy, residence in the area for ≥3 years and having no report of assisted reproduction, chronic or pregnancy-associated hypertension and diabetes, HIV or AIDS infection, and illicit drug use. In total, 773 pregnant women with an average gestational week of 39.47 ± 1.39 weeks met the recruitment criteria and participated in the study (baseline population). Of these 773 subjects, a total of 456 children with their mothers participated in the 7th year follow-up survey. We excluded 55 children without sufficient urine samples for NEOs and oxidative stress measures, 10 children with creatinine concentrations <0.1 g/L, which were considered to be too dilute for accurate analysis (Ding et al., 2017; Eskenazi et al., 2004), and 11 children who were missing important confounder variables. Ultimately, 380 children were included in this study. This research was approved by the Medical Ethics Committee of Xinhua Hospital affiliated with Shanghai Jiao Tong University School of Medicine.

2.2. Data collection and adiposity measures

At the baseline survey, pregnant women were interviewed by trained nurses using standardized questionnaires to collect information on social demographics, living habits, and perceived environmental exposures. Medical and obstetric histories were obtained from medical

records. At the 7th year's follow-up survey, main caregivers of the participating 7-year-old children were asked to complete the follow-up questionnaires, including information on children's dietary habits, sleep quality, and disease history. Every participant provided written informed consent prior to participating in this study.

Children's adiposity measures were measured by trained investigators during the 7-year follow-up. Height (cm) was measured while the children were not wearing hats or shoes with their heels against the wall and standing straight; weight (kg) was measured while the children were wearing thin clothes, without shoes, and with an empty bladder; waist circumference (cm) was measured using a plastic measuring tape, which was placed around the abdomen and close to the skin (Hu et al., 2022). Body fat mass (kg), body fat percentage (%), and visceral fat area (cm^2) were estimated using a body composition analyzer (S10, Inbody Co. Ltd) while the children were wearing thin clothes with bare hands and feet, and had fasted or eaten at least 2–3 h previously (Bukowska et al., 2021).

Body mass index (BMI), waist-to-height ratio (WHtR) and fat mass index were calculated using the following formulas: BMI = weight (kg)/height^2 (m^2); WHtR = waist circumference (cm)/height (cm); and fat mass index = body fat mass (kg)/height^2 (m^2). Sex- and age-specific height z-scores, weight z-scores and BMI z-scores were calculated according to the 2007 World Health Organization growth standard (De Onis et al., 2009). Children with BMI z-scores ≥85th percentile were classified as overweight/obesity (Grace et al., 2021), and children with WHtR ≥0.5 were defined as abdominal obesity (Gibson and Ashwell, 2020).

2.3. Urinary NEOs/metabolites measurements

Spot random urine samples were collected under the guidance of the children's main caregivers and then aliquoted and stored in polypropylene (PP) tubes at −80 °C until further analysis. During measurement, analytical standards of acetamiprid (ACE), imidacloprid (IMI), thiamethoxam (THM), thiacloprid (THD), clothianidin (CLO), dinotefuran (DIN), nitenpyram (NIT) (Sigma, USA), N-desmethyl-acetamiprid (N-DMA), and 6-chloronicotinic acid (6-CINA) (Dr. Ehrensorfer, GER) and isotopically labeled internal standards of ACE-d_3, IMI-d_4, THM-d_3, THD-d_4 (Sigma, USA), NIT-d_5, 6-CINA-$^{13}C_6$, and N-DMA-$^{13}C_2$ (Cambridge Isotope Laboratories, USA) were used. NEOs/metabolites were detected based on the modified method reported by Pan et al. (2022) via high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (HPLC−MS/MS, Agilent 1290 infinity, Sciex Triple Quad™4500, USA). Briefly, a 3-mL urine sample was spiked with 10 μL of 100 ng/mL internal standards and 300 μL of β-glucuronidase solution (100 Unit/mL). After incubating at 37 °C for 12 h, the urine was extracted and purified by solid-phase extraction (SPE) and was subsequently concentrated to near dryness. After resuspension in 100 μL acetonitrile, the sample was fully vortexed and centrifuged (13200 rpm, 5 min). A total of 10 μL of sample extract was injected into an Eclipse Plus 95A-C18 column (5 μm, 2.1*150 mm; Agilent, USA) with a flow rate of 0.3 mL/min. The mobile phase consisted of phase A (0.1% formic acid solution) and phase B (acetonitrile solution), starting with 70% and 30%, respectively. In 5 min, phases A and B reached 50%, then dropped to 10% and rose to 90%, and ultimately returned to the initial percentage. Strict quality control was conducted during analysis. At intervals of every 20 samples, two filed blanks and two low-concentration quality control samples (0.5 ng/mL) were measured repeatedly. The recovery of spike samples ranged from 80% to 120%. The intra-day and inter-day relative standard deviations (RSDs) were 2.21–9.40% and 7.98–16.63%, respectively. The limits of detection (LODs) ranged from 0.001 to 0.040 ng/mL in this study. Concentrations below the LODs were replaced with the LOD divided by the square root of 2 (Furukawa et al., 2010).

2.4. Oxidative stress measurements

Among the multiple accepted biomarkers for oxidative stress, 8-iso-PGF2α is considered one of the best due to its availability and stability in biological fluids (Gołąb et al., 2022; Mure et al., 2015). 8-iso-PGF2α is produced by the nonenzymatic peroxidation of arachidonic acid in membrane phospholipids (Basu, 2010), which is closely related to childhood obesity as a valid biomarker of lipid peroxidation (Arogbokun et al., 2021). Hence, we used 8-iso-PGF2α to represent the oxidative stress levels in this study. Urinary concentrations of 8-iso-PGF2α were detected using an 8-iso-PGF2α ELISA Kit (Cayman, USA) according to the manufacturers' instructions. The 8-iso-PGF2α values were calculated based on calibration sigmoid plots (ELISA Calc Software) of the absorbance at 405 nm of a standard at various concentrations. In addition, urinary creatinine concentrations were measured by an automated chemistry analyzer (7100, Hitachi, Japan) using a clinically validated enzymatic method.

2.5. Statistical analysis

Descriptive statistics were calculated for the maternal sociodemographic characteristics and childhood adiposity measures using the mean ± standard deviation (SD) or n (%). Distributions of NEOs/metabolites are described with detection rate, range, and quartiles. Independent t tests and chi-square tests were conducted to compare the differences in the baseline characteristics between the included population and the excluded population. Given the skewed distributions, urinary levels of NEOs/metabolites and 8-iso-PGF2α were ln-transformed as continuous variables before analyses. Statistical analyses were limited to NEOs/metabolites with detection rates above 75%.

The associations of NEOs/metabolites with continuous adiposity measures were analyzed by multiple linear regressions, and binary logistic regressions were conducted to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for overweight/obesity and abdominal obesity. To explore the mediating effects of 8-iso-PGF2α on the relationship between NEOs/metabolites and adiposity measures, a mediation analysis was conducted using the R package 'regmedint' if the following criteria were met (Valeri and Vanderweele, 2013; VanderWeele, 2016): 1) exposure significantly affected the mediator; and 2) the mediator significantly affected the outcome. The total effect (TE) of NEOs/metabolites on adiposity measures was decomposed into the indirect effect (IE; i.e., the effect of NEOs/metabolites on adiposity measures that is mediated by 8-iso-PGF2α) and the direct effect (DE; i.e., the estimated effect after controlling for 8-iso-PGF2α), and the estimated proportion mediated by 8-iso-PGF2α was calculated as the β coefficient of the IE divided by the β coefficient of the TE. Considering the potential sex-specific effects of NEOs (Arslan et al., 2016; Godbole et al., 2022), interaction terms between NEOs/metabolites and sex were included in the previously described models, and stratified analysis by sex was further performed.

Directed acyclic graph (DAG) (Fig. S1) was used to select the potential covariates according to our prior knowledge and published literature (Pan et al., 2022; Zhao et al., 2022), including sex (boy, girl), pediatric age (continuous, years), maternal education level (less than high school, high school, college and above), and household monthly income (<3000 CNY, 3000–5000 CNY, >5000 CNY; the median household monthly income of residents in Shandong, China was about 4000 CNY, as a reference). Pre-pregnancy BMI (continuous, kg/m^2), parity (primiparous, multiparous), and method of delivery (vaginal delivery, caesarean) were also included as confounding factors due to their influences on childhood obesity (Gaillard et al., 2014; Huang et al., 2022; Önnestam et al., 2022). Urinary creatinine levels were adjusted as continuous covariates in the final models to control for urine dilution (Jacobson et al., 2019; Liu et al., 2019). Multicollinearity was not detected, as the variance inflation factor was <2 for all covariates.

Several sensitivity analyses were conducted to confirm the

robustness of our results. First, considering that vegetables and fruits are the main sources of dietary NEOs exposure (Chen et al., 2020), and that sleep duration is strongly associated with childhood obesity (Glasgow et al., 2022), the frequency of food (vegetables, fruits) consumption (<1 time/week, 1–3 times/week, ≥4 times/week) and sleep duration (continuous, hours) were included as additional covariates in the models. Second, children with preterm delivery (<37 weeks of gestation, n = 7) or low birth weight (<2500 g, n = 7) were further excluded to verify our findings, and 366 children were ultimately included in the sensitivity analyses. Moreover, the restricted cubic spline (RCS) model was used to evaluate the nonlinear relationship between NEOs/metabolites and adiposity measures. Statistical evaluations were carried out using SPSS v.22 and R software (version 4.1.3). All statistical tests were two-sided tests and $P < 0.05$ was considered significant.

3. Results

3.1. Population characteristics and adiposity measures

The characteristics and adiposity measures of the study population are shown in Table 1 (Table S1). A total of 68.2% of the mothers were primiparous, 56.5% had a high school education or higher, and 37.9% had a household monthly income of more than 3000 CYN. No significant difference was observed in demographic characteristics between the included population (n = 380) and the excluded population (n = 393) based on the LWBC (Table S1). The average age of the 380 participating children was 7.68 ± 0.62 years old, and 53.2% of them were boys. The mean values (SDs) of various adiposity measures were 128.78 (6.44) cm for height, 28.01 (6.81) kg for weight, 16.73 (3.04) kg/m^2 for BMI, 58.79 (8.23) cm for waist circumference, 0.46 (0.05) for WHtR, and 21.93 (8.51)% for body fat percentage. Of these children, 57 (15.0%) were considered as overweight/obesity, and 67 (17.6%) were considered as abdominal obesity. The adiposity measures were significantly higher in boys than in girls ($P < 0.05$), except for height (Table 1).

Table 1
Adiposity measures of 7-year-old children in this study based on the Laizhou Wan Birth Cohort.

Adiposity measures	Mean ± SD or n (%)			*P* value[a]
	Total (n = 380)	Boys (n = 202)	Girls (n = 178)	(Boys vs Girls)
Height (cm)	128.78 ± 6.44	129.61 ± 6.28	127.84 ± 6.50	0.704
Weight (kg)	28.01 ± 6.81	29.37 ± 7.40	26.47 ± 5.71	0.001
BMI (kg/m^2)	16.73 ± 3.04	17.32 ± 3.35	16.07 ± 2.50	<0.001
Height z-score	0.65 ± 0.96	0.71 ± 0.93	0.59 ± 0.99	0.592
Weight z-score	0.67 ± 1.35	0.92 ± 1.45	0.40 ± 1.18	0.004
BMI z-score	0.37 ± 1.49	0.64 ± 1.65	0.06 ± 1.22	<0.001
Waist circumference (cm)	58.79 ± 8.23	60.45 ± 8.78	56.91 ± 7.13	0.002
WHtR	0.46 ± 0.05	0.47 ± 0.06	0.45 ± 0.05	0.005
Body fat mass (kg)	6.61 ± 4.23	7.05 ± 4.84	6.10 ± 3.35	<0.001
Body fat percentage (%)	21.93 ± 8.51	22.04 ± 9.54	21.82 ± 7.19	<0.001
Fat mass index (kg/m^2)	3.90 ± 2.30	4.10 ± 2.62	3.66 ± 1.85	<0.001
Visceral fat area (cm^2)	30.25 ± 21.58	32.73 ± 25.02	27.42 ± 16.47	<0.001
Categorized by BMI z-scores				
Normal (<85th)	323 (85.0%)	155 (76.7%)	168 (94.4%)	<0.001
Overweight/obesity (≥85th)	57 (15.0%)	47 (23.3%)	10 (5.6%)	
Categorized by WHtR				
Normal (<0.5)	313 (82.4%)	155 (82.4%)	158 (82.4%)	0.002
Abdominal obesity (≥0.5)	67 (17.6%)	47 (17.6%)	20 (17.6%)	

[a] Independent samples *t*-test for continuous variables; Chi-square test for categorical variables.

3.2. Urinary levels of NEOs/metabolites and 8-iso-PGF2α

The urinary levels of NEOs/metabolites and 8-iso-PGF2α are summarized in Table 2. High detection rates were found for six NEOs, including IMI (99.7%), CLO (98.9%), DIN (97.6%), THM (95.5%), ACE (82.9%), and THD (77.6%), with median concentrations of 0.241, 0.258, 0.111, 0.044, 0.003, and 0.006 ng/mL, respectively. The detection rate of NIT was only 36.6%, which was regarded as too low to perform further meaningful analyses. For the two metabolites of NEOs, the median concentrations of N-DMA (detection rate: 100.0%) and 6-CINA (97.9%) were 0.565 and 0.496 ng/mL, respectively. Moreover, the creatinine-unadjusted and creatinine-adjusted median concentrations of 8-iso-PGF2α in our study were 0.725 ng/mL and 1.328 μg/g creatinine (Table 2), which were at a moderate level compared to those of children aged 6–10 years in other countries (Kordas et al., 2018; Selvaraju et al., 2019; Yamano et al., 2015).

3.3. Associations of NEOs/metabolites with adiposity measures and effect modification by sex

The associations of NEOs/metabolites levels with continuous adiposity measures in 7-year-old children are presented in Fig. 1 (Table S2). For NEOs, exposure to IMI was associated with a 0.986-cm increase in waist circumference (95% CI: 0.111, 1.861) and an increase of 0.007 in WHtR (95% CI: 0.001, 0.013). Similarly, exposure to THM was associated with a 0.927-cm increase in waist circumference (95% CI: 0.186, 1.669) and an increase of 0.006 in WHtR (95% CI: 0.001, 0.011). For the two metabolites of NEOs, exposure to 6-CINA was positively associated with body fat percentage (β = 0.565, 95% CI: 0.047, 1.083), while significant negative associations were observed between N-DMA exposure and body fat percentage (β = −1.189, 95% CI: -1.965, −0.414) and fat mass index (β = −0.231, 95% CI: -0.440, −0.022). The results showed that there were sex differences in the associations between NEOs/metabolites and continuous adiposity measures (Table S2). Specifically, sex modified the association between CLO and height z-score ($p_{int} = 0.043$) with a positive association in girls (β = 0.161, 95% CI: 0.032, 0.289), and an insignificant inverse association in boys. Sex differences were also observed in the associations of DIN exposure with body fat mass ($p_{int} = 0.032$), body fat percentage ($p_{int} = 0.009$) and fat mass index ($p_{int} = 0.037$), with negative associations in girls (body fat mass: β = −0.658, 95% CI: -1.143, −0.173; body fat percentage: β = −1.468, 95% CI: -2.512, −0.424; fat mass index: β = −0.352, 95% CI: -0.622, −0.082) and nonsignificant positive associations in boys.

The risk of NEOs/metabolites levels on childhood obesity was further explored, and the ORs and 95% CIs are shown in Table 3. Eeposure to IMI was found to be associated with a higher risk of childhood overweight/obesity (OR = 1.556, 95% CI: 1.100, 2.201) and abdominal obesity (OR = 1.478, 95% CI: 1.078, 2.026). After stratification by sex, similar significant associations were only pronounced among boys, showing that IMI exposure was positively associated with overweight/obesity (OR = 1.504, 95% CI: 1.013, 2.232) and abdominal obesity (OR = 1.498, 95% CI: 1.005, 2.231), and sex modified the association between DIN exposure and the rate of overweight/obesity ($p_{int} = 0.046$), with a significant negative association among girls (OR = 0.356, 95% CI: 0.135, 0.939), and a null association among boys.

3.4. Relationships of 8-iso-PGF2α with NEOs/metabolites and adiposity measures

The relationships of NEOs/metabolites with 8-iso-PGF2α and the relationships of 8-iso-PGF2α with adiposity measures are presented in

Table 2
Concentrations of urinary NEOs/metabolites and 8-iso-PGF2α among 7-year-old children in northern China.

	LOD (ng/mL)	Detection rate (%)	Unadjusted (ng/mL)				Creatinine adjusted (μg/g creatinine)			
			Range	Percentile			Range	Percentile		
				25th	50th	75th		25th	50th	75th
ACE	0.0017	82.9	< LOD - 0.601	0.002	0.003	0.007	< LOD[a] - 1.125	0.003	0.007	0.014
IMI	0.0133	99.7	< LOD - 9.957	0.137	0.241	0.480	< LOD[a] - 12.707	0.249	0.454	0.822
THD	0.0033	77.6	< LOD - 0.071	0.004	0.006	0.009	< LOD[a] - 0.272	0.007	0.010	0.017
CLO	0.0333	98.9	< LOD - 17.004	0.122	0.258	0.664	< LOD[a] - 28.162	0.206	0.488	1.159
THM	0.0067	95.5	< LOD - 5.312	0.021	0.044	0.091	< LOD[a] - 8.798	0.036	0.074	0.150
DIN	0.0133	97.6	< LOD - 2.027	0.054	0.111	0.239	< LOD[a] - 3.508	0.108	0.193	0.376
NIT	0.0067	36.6	< LOD - 1.010	< LOD	< LOD	0.011	< LOD[a] - 1.325	< LOD[a]	< LOD[a]	0.021
N-DMA	0.0067	100.0	0.031–11.364	0.265	0.565	1.114	0.059–36.031	0.434	0.977	2.129
6-CINA	0.0167	97.9	< LOD - 18.724	0.129	0.496	1.887	< LOD[a] - 29.446	0.238	0.967	3.109
8-iso-PGF2α	–	–	0.008–6.371	0.423	0.725	1.528	0.015–51.916	0.985	1.328	1.866

[a] Below the limits of detection for the urinary concentrations were not corrected for creatinine.

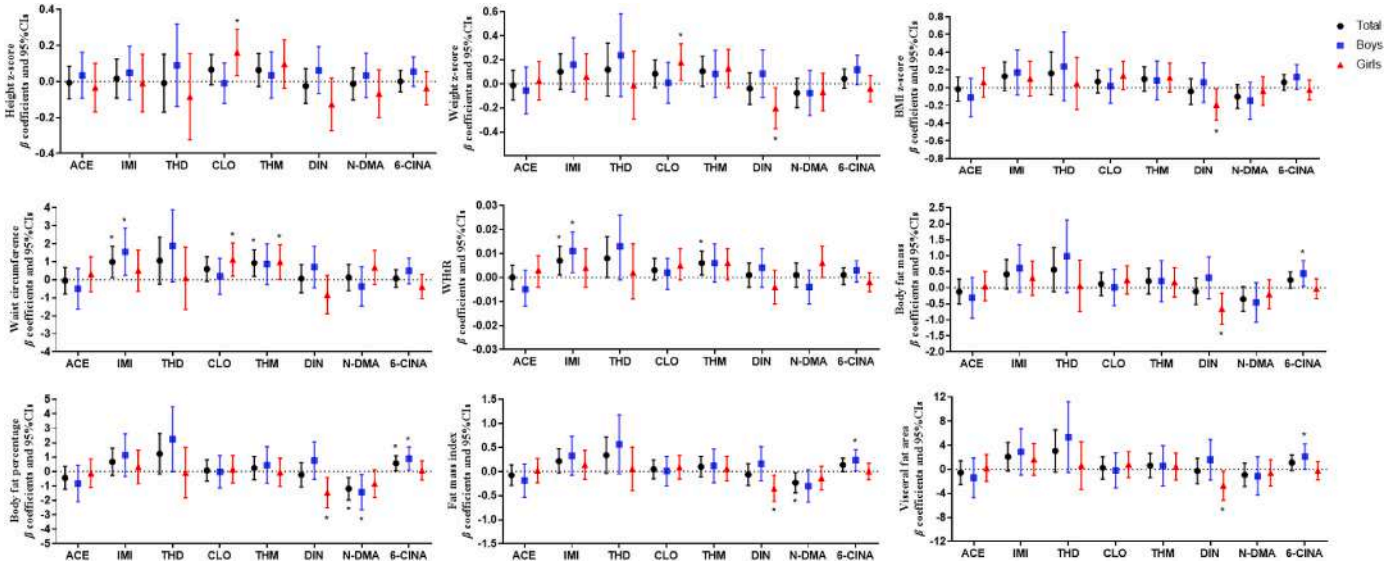

Fig. 1. Changes (95%CI) in adiposity measures associated with NEOs/metabolites based on linear regression models. Concentrations of NEOs/metabolites were ln-transformed. Models in the total population (n = 380) were adjusted for urinary creatinine levels, sex, pediatric age, maternal education, household monthly income, pre-pregnancy BMI, parity and method of delivery. Models in boys (n = 202) or girls (n = 178) were adjusted for urinary creatinine levels, pediatric age, maternal education, household monthly income, pre-pregnancy BMI, parity and method of delivery.
*Significant at $P < 0.05$.

Table S3 and Table S4, respectively. IMI, THD, CLO, THM, DIN and N-DMA were positively associated with 8-iso-PGF2α ($P < 0.001$) (Table S3). Concentrations of 8-iso-PGF2α were associated with increased BMI z-score, waist circumference, WHtR, body fat mass, body fat percentage and fat mass index ($P < 0.05$). A null association of 8-iso-PGF2α with the risk of overweight/obesity and abdominal obesity was observed in the logistic regression model (Table S4).

3.5. Mediation analysis

Mediation analysis was used to estimate the proportions of the effects of NEOs/metabolites (except ACE and 6-CINA) on adiposity measures (BMI z-score, waist circumference, WHtR, body fat mass, body fat percentage and fat mass index) mediated by 8-iso-PGF2α (Table S5). The results showed that a significant mediation effect of 8-isoPGF2α was observed for the associations of IMI (IE: β = 0.002, 95% CI: 0.000, 0.005), THD (IE: β = 0.006, 95% CI: 0.000, 0.012) and THM (IE: β = 0.003, 95% CI: 0.000, 0.006) with WHtR, and the proportions were 27.92%, 69.52% and 35.37%, respectively.

3.6. Sensitivity analyses

Sensitivity analyses demonstrated the robustness of our results, as the overall findings were not affected by adjustments for additional covariates (the frequency of vegetables consumption, the frequency of fruits consumption, and sleep duration), or by removing children with preterm delivery or low birth weight from the total population (Table S6) and the sex-stratified population (Table S7) in all models. However, removing children with preterm delivery or low birth weight resulted in some findings that were no longer significant in the mediation analysis, including the mediation effect of 8-isoPGF2α in the associations of IMI, THD and THM with WHtR (Table S8). Moreover, the RCS model depicted the dose–response relationship between NEOs/metabolites and adiposity measures (Fig. S2). Statistically significant nonlinear associations were found in the relationship of ACE with height z-score and weight z-score, in the relationship of IMI with weight z-score, waist circumference, WHtR, body fat mass, fat mass index and visceral fat area, and in the relationship of CLO with height z-score ($P < 0.05$ for the nonlinear test).

Table 3
Odds ratios (OR) and 95% confidence intervals (CIs) for urinary NEOs/metabolites concentrations and childhood obesity.

NEOs/ metabolites	Binary logistic regression OR (95% CI)							
	Overweight/obesity (BMI z-scores ≥85th, n = 57)			P_{int}	Abdominal obesity (WHtR ≥0.5, n = 67)			P_{int}
	Total[a]	Boys[b]	Girls[b]		Total[a]	Boys[b]	Girls[b]	
ACE	0.844 (0.624, 1.141)	0.728 (0.503,1.054)	1.309 (0.752,2.279)	0.112	0.909 (0.697, 1.184)	0.750 (0.522,1.077)	1.216 (0.805,1.837)	0.083
IMI	**1.556 (1.100, 2.201)***	**1.504 (1.013,2.232)***	1.979 (0.865,4.532)	0.800	**1.478 (1.078, 2.026)***	**1.498 (1.005,2.231)***	1.468 (0.871,2.474)	0.983
THD	1.262 (0.758, 2.102)	1.547 (0.851,2.812)	0.836 (0.236,2.960)	0.390	1.335 (0.850, 2.097)	1.670 (0.916,3.045)	1.021 (0.481,2.170)	0.482
CLO	1.074 (0.830, 1.390)	1.100 (0.824,1.469)	1.107 (0.623,1.966)	0.915	1.196 (0.940, 1.520)	1.145 (0.851,1.540)	1.329 (0.876,2.014)	0.486
THM	1.150 (0.864, 1.532)	1.217 (0.878,1.687)	0.994 (0.529,1.869)	0.706	1.284 (0.989, 1.666)	1.316 (0.944,1.835)	1.330 (0.854,2.070)	0.889
DIN	1.016 (0.756, 1.365)	1.236 (0.878,1.739)	**0.356 (0.135,0.939)***	**0.046**	1.119 (0.854, 1.466)	1.394 (0.981,1.981)	0.732 (0.450,1.190)	0.083
N-DMA	0.831 (0.621, 1.113)	0.879 (0.630,1.226)	0.659 (0.341,1.273)	0.488	0.961 (0.738, 1.251)	0.849 (0.605,1.192)	1.183 (0.768,1.824)	0.213
6-CINA	1.067 (0.888, 1.282)	1.156 (0.936,1.427)	0.860 (0.557,1.328)	0.302	1.037 (0.876, 1.228)	1.167 (0.940,1.448)	0.833 (0.616,1.126)	0.141

*Significant at $P < 0.05$.

[a] The models were adjusted for urinary creatinine levels, sex, pediatric age, maternal education, household monthly income, pre-pregnancy BMI, parity and method of delivery.

[b] The models were adjusted for urinary creatinine levels, pediatric age, maternal education, household monthly income, pre-pregnancy BMI, parity and method of delivery.

4. Discussion

In the present study, we detected urinary levels of NEOs/metabolites in 7-year-old children from northern China, estimated their associations with adiposity measures, investigated the role of oxidative stress in these associations, and explored whether sex modifies the effect. We observed relatively high detection rates and concentrations of NEOs/metabolites, positive associations of IMI, THM and 6-CINA with certain adiposity measures, such as waist circumference, WHtR and body fat percentage, and negative associations of N-DMA with body fat percentage and fat mass index. Furthermore, 8-iso-PGF2α may be a potential mediator regulating the relationships of IMI, THD and THM with increased WHtR, and effect modification by sex was observed.

Past studies of human exposure to NEOs/metabolites mainly focused on adults, and evidence is still limited for children. To date, only nine studies have reported urinary levels of NEOs/metabolites in children worldwide, including four Chinese studies (Wang et al., 2020a; Yue et al., 2022; Zhao et al., 2022; Zhou et al., 2021), three Japanese studies (Ikenaka et al., 2019; Osaka et al., 2016; Oya et al., 2021), one U.S. study (Ospina et al., 2019), and one European study (Laubscher et al., 2022) (Table S9). In general, relatively higher detection rates and concentrations of urinary NEOs/metabolites were observed in Chinese children than in children from other countries. For instance, a Japanese study reported the detection rates of IMI, ACE, THM and CLO as 13%, 9%, 28%, and 41% in 3- to 6-year-old children, respectively (Ikenaka et al., 2019). However, relatively higher detection rates of IMI (95.7%), ACE (95.1%), THM (99.7%) and CLO (93.4%) were reported in 305 Chinese children aged 8–11 years (Zhao et al., 2022), with levels similar to those in our study. Moreover, the median concentrations of CLO (0.258 ng/mL) detected among 7-year-old children in this study were slightly higher than those reported in American children aged 6–11 years (n = 416, <0.20 ng/mL) (Ospina et al., 2019) and in Japanese children (n = 1036, <0.13 ng/mL) (Oya et al., 2021). Similarly, compared with the Japanese study (Oya et al., 2021), our study had a relatively higher median concentration of IMI (China vs. Japan = 0.241 vs. < 0.07 ng/mL). Additionally, the median concentration of N-DMA (0.565 ng/mL) was highest in our study, which was higher than that in Japanese children (0.39 ng/mL) (Ikenaka et al., 2019) and that of Swedish children (0.324 ng/mL) (Laubscher et al., 2022). Compared with developed countries (i.e., the USA and Japan), China has higher NEOs usages for agricultural production (Li et al., 2019; Tao et al., 2019). Thus, Chinese children might have more chances for exposure to NEOs via the route of vegetables and fruits intake (Zhang and Lu, 2022; Zhang et al., 2019a), which could partly explain the higher urinary concentrations of NEOs/metabolites in the current study compared with studies in other areas.

The associations between NEOs/metabolites and children's adiposity measures were mixed in our study. At present, the results of associations between NEOs levels and obesity are inconsistent in mammalian experiments. Some animal studies have indicated positive relationships between NEOs and obesity (Tanaka, 2012; Yan et al., 2020). For example, after 30 days of exposure to NEOs (4 mg/kg bw/day), significantly increased body weight was observed in ICR mice compared with the control groups (Yan et al., 2020). However, some animal studies have reported inverse associations (Mosbah et al., 2018; Sheets et al., 2016). For instance, wistar rats aged 8–12 weeks old with ACE (27 mg/kg/day) in their diet for over 45 days exhibited a greater decrease in weight (Mosbah et al., 2018). Unlike existing animal studies, only four epidemiological surveys investigated the relationships between NEOs/metabolites and adiposity measures, with two focusing on children. A biomonitoring-based study reported that N-DMA concentrations were associated with higher odds of obesity in school-age children from Shanghai, China, though the association was only of marginal significance (β = 2.03, 95% CI: 0.99, 4.17) (Wang et al., 2020a). A null association between 6-CINA and BMI z-score was reported among 11- to 12-year-old children in Cyprus (Makris et al., 2019). Among US adults, detectable levels of ACE were associated with decreased BMI, waist circumference, body fat percentage and fat mass index, but exposure to the metabolite of IMI was associated with greater rates of overweight/obesity (Godbole et al., 2022). In addition, Peng et al. observed that concentrations of IMI in hair samples were positively associated with BMI (β = 0.03, 95% CI: 0.02, 0.04) among Chinese women (Peng et al., 2020). In short, exposure to NEOs may be closely correlated with adiposity measures, but the results of existing studies are mixed. Considering that the obesogenic effect of NEOs/metabolites remains uncertain, more studies should be conducted in the future.

Sex differences were observed in the influence of certain NEOs on adiposity measures in this study. Specifically, significant negative associations were observed between DIN exposure and body fat mass, body fat percentage, fat mass index and the risk of overweight/obesity among girls, while nonsignificantly positive associations were observed

among boys. Differences in these associations between girls and boys could be explained by some potential biological mechanisms. For example, a protective effect generated by estrogen (Sun et al., 2017) and the activation of PPARα (a major receptor involved in fatty acid metabolism) may be responsible for the decrease in the weight of female mice (Anderson et al., 2004). The adipogenic effect of NEOs in male mice might be related to the increased oxidative stress and higher levels of triglycerides in the liver (Lukowicz et al., 2018). Given the limited evidence and relatively small sample size of the present study, more research is warranted to verify the potential sexually dimorphic traits of NEOs.

We also observed that positive associations of NEOs/metabolites with adiposity measures were mediated by 8-iso-PGF2α. Several epidemiological studies have documented that EDCs (He et al., 2020; Steffensen et al., 2020; Tran et al., 2017) including NEOs could induce peroxidation of lipid and subsequent excessive generation of 8-iso--PGF2α (Li et al., 2020; Makris et al., 2019), which are consistent with our results. Moreover, previous studies reported that 8-iso-PGF2α levels were associated with increased adiposity measures, e.g., weight and BMI (Arogbokun et al., 2021; Jia et al., 2019). It is noteworthy that 8-iso--PGF2α has been documented to be associated with adipose tissue dysfunction, consequentially playing a critical role in the development of obesity and metabolic syndrome (Soldo et al., 2022). Therefore, it is biologically plausible that 8-iso-PGF2α may be involved in associations between NEOs/metabolites exposures and adiposity measures. However, due to the cross-sectional study design, our findings regarding the potential role of 8-iso-PGF2α in the aforementioned associations should be interpreted with caution and it should be confirmed by future prospective studies and experimental studies.

Our study examined the levels of a relatively large number of NEOs/metabolites and provides a possible clue for future research on the mechanism of their effect on adiposity measures. However, the limitations in this study should also be acknowledged. First, the half-lives of NEOs/metabolites (e.g., IMI, CLO, DIN and N-DMA) are short (0.17–1.45 days) in the human body (Harada et al., 2016); therefore, our collection of single-spot urine samples may cause exposure misclassification. Repeated or 24-h urine samples should be collected to better characterize average/integrated exposure of NEOs/metabolites over time in future studies (Xu et al., 2021). Second, we only used creatinine for urine dilution correction, and information about specific gravity was not available, which may be more preferable in children (Wang et al., 2015). Third, the cross-sectional study design in the current study restricted the ability to determine causal relationships between NEOs/metabolites and adiposity measures and the potential mediation of oxidative stress. Our results need to be further verified by longitudinal studies. Fourth, the influence of some confounding factors that are correlated with childhood obesity, such as other environmental chemicals (e.g., heavy metals, organochlorines, pyrethroids) (Nasab et al., 2022; Pinos et al., 2021) and nutritional status (e.g., breast feeding, calorie intake) (Ma et al., 2020) could not be excluded, but the consistent results in sensitivity analyses suggested the robustness of our findings. Finally, selection bias may be a concern due to the relatively high rate of loss to follow-up. However, no significant difference was observed in demographic characteristics between the study population and the excluded population, indicating that our study population could generally represent the whole cohort.

5. Conclusions

As shown in this study, 7-year-old children in northern China are widely exposed to NEOs/metabolites. Our results suggested mixed associations between NEOs/metabolites and adiposity measures in children, with a promoting effect of IMI, THM and 6-CINA and a reverse effect of N-DMA. Oxidative stress may be one of the underlying mechanisms of the effect of NEOs on childhood obesity. Moreover, there were sex differences in the influence of specific NEO exposure (such as DIN) on adiposity measures. Cautious interpretations are recommended until our findings can be replicated in other larger longitudinal studies.

Declaration of competing interest

The authors declare that they have no actual competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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