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Analysis of local drinking water for fecal contamination in Solu-Khumbu / Mt. Everest region, Nepal

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ABSTRACT

Background: To evaluate the drinking water quality in the popular trekking area of Solu-Khumbu Mt. Everest region as a possible source for the high incidence of diarrhea.
Material and methods: Drinking water samples (n = 80) were collected from whatever primary source the locals/tourists used at altitudes 2,608 to 5,180m; and where possible, also from inside households. Samples were analyzed for fecal contamination using the DelAgua Dual Incubator at 37 °C and 44 °C to detect the total and thermotolerant coliform bacteria. The pH, temperature, turbidity, smell, and taste were also registered.
Results: No thermotolerant bacteria were found but a significant number of specimens contained many colony forming units (CFU) of total coliform bacteria. Household specimens were more often contaminated compared to the water from the primary source.
Conclusion: Data indicate a significant secondary contamination when water was handled and stored in unhygienic containers. Health education programs on water hygiene, sanitation, and the safe handling and storage of water needs improvement. It is strongly recommended that drinking water is disinfected using filter systems, UV-light dispensers or halogens (e.g. chlorine), or a combination of two methods. Although cooking is a common disinfection method here, fuel is scarce. Water was generally safer when collected directly from the primary source in a clean container than from a lodge.

1. Introduction

Nepalese doctors raised ongoing concerns regarding the high incidence of diarrhea experienced by the tourists and Nepali in the popular Solu-Khumbu region of the Nepal Himalayas (Dr. Kami Sherpa, Khunde/Solu-Khumbu, Nepal; personal communication). This observation of local physicians is in accordance with studies about traveller's diarrhea in remote regions (survey in (Adler et al., 2022)). The area gives access to some of the world's highest mountains including Mount Everest, Nuptse, Lhotse and others, and since 1979 is a World Natural Heritage Site. However, Nepal's urban and rural areas lack basic water treatment facilities and there is poor sanitation in spite of some improvements (Nicholson et al., 2016), (Maharjan et al., 2018), (Murphy and Pandey 2012). Open defecation and sewage waste are often discharged directly into streams or rivers (Nicholson et al., 2016). Most waterborne bacterial pathogens that are potentially introduced into drinking water supplies come via animal or human feces (WHO 2011). These bacteria do not grow in the water, but initiate infection once consumed and in the gastrointestinal tract, and then the feces of these infected humans or animals can then potentially repeat this disease cycle (WHO 2011). Increased tourism to the popular trekking trails in Solu-Khumbu contributes to this cycle, and this may additionally pollute water supplies

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(ie discarded non-biodegradable bottles or batteries (Nicholson et al., 2016), (Murphy and Pandey 2012)). All drinking water sources should be carefully and regularly monitored and managed to protect public health and to secure future sustainability (Nicholson et al., 2016), (Maharjan et al., 2018), (Murphy and Pandey 2012), (WHO 2011).

Epidemiological data from Nepal strongly suggests that fecal contamination of water sources remains a key problem causing diarrheal infections (Nicholson et al., 2016), (Maharjan et al., 2018), (Ansari et al., 2012), (Murphy and Pandey 2012), (WHO 2011). Although diarrhea is often reported as traveller's diarrhea and affects about two thirds of visitors, it is even more a problem for the locals, especially children (Ansari et al., 2012). In Nepal, 30,000 children die every year due to diarrhea ((anonymous) 2019). The under 5-mortality rate in 2012 in Nepal was 42 per 1000 live births (Lamichhane et al., 2017), (Budhathoki et al., 2016) and about 25% of all deaths before the age of 5 are caused by diarrhea (Ansari et al., 2012).

Diarrheal diseases were the fifth leading cause of all Nepali deaths from 1990 to 2017 at 36.12% ((anonymous) 2019). Improvements in sanitation, personal hygiene and water quality are the most important factors to save life (Acharya et al., 2013), to control outbreaks (Kilian 1983), (Lawin and Scherer 1981) and may even avoid epidemic outbreaks as it has happened with typhoid fever in Kathmandu (Karkey et al., 2008), (Basnyat 2015).

Waterborne transmission of human diseases varies in severity from mild gastroenteritis to severe, even fatal, diarrhea, dysentery, hepatitis and typhoid fever (WHO 2011). Different pathogens may cause gastroenteritis (DuPont et al., 2009), (Adachi et al., 2007), (Murphy and Pandey 2012). The most common bacterial infections are caused by *Escherichia coli*, *Campylobacter* species, *Salmonella*, *Shigella* and *V. cholerae*. Viral gastroenteritis is often due to infections involving adeno-, rota- or norovirus. Protozoa parasites like *Giardia lamblia*, *Entamoeba histolytica* or *Cryptosporidia* can also cause intestinal problems. In Southeast Asia, especially Nepal, *Campylobacter* sp. is the most common pathogen causing diarrhea (Pandey et al., 2011). However, since a shift of pathogens was observed recently in several countries worldwide such data is preliminary and continuous epidemiological research is necessary (Adler et al., 2022), (Murphy et al., 2019).

Some severe cases will require antibiotics like azithromycin (Steffen et al., 2015) but the most important therapy in most cases is adequate administration of fluid to avoid dehydration. However, this can be challenging, even critical, where there is no safe drinking water. Several germs in South East Asia have recently changed their resistance profile against antimicrobial drugs (Singh and Mustapha 2013). It should be noted that fluorquinolones which are first-choice drug for traveller's diarrhea in many countries should not be used in South-East Asia, especially Nepal and India, because of the high rate of resistance here, e.g. Camphylobacter 97%, Shigella 78%, and Enterotoxic Escherichia coli (ETEC) 73% (Adler et al., 2022), (Riddle et al., 2021), (Guiral et al., 2019), (Tribble 2017), (Riddle et al., 2017), (Singh and Mustapha 2013), (Pandey et al., 2011)

The survival of pathogens in water differs widely from hours (*Shigella* (Schreiber 1981; Singh and McFeters 1990), (Backer 2007)) or weeks (*E. coli, Campylobacter* (Singh and McFeters 1990), (Backer 2007), ((anonymous) 2018) to months (*Giardia lamblia* cysts (Backer 2007) (deRegnier, Cole et al. 1989),) or to even more than a year (Hepatitis A, polio virus (Backer 2007) (Biziagos et al., 1988),) and depends on the physiochemical condition of the water (McFeters and Terzieva 1991), (Wang and Doyle 1998). However, according to WHO recommendations water should be investigated for thermotolerant coliform bacteria as indicator of germs from recent fecal contamination as this presents the possibility of a transmission of waterborne diarrheal diseases ((anonymous) 2011), (Nicholson et al., 2016), (Farnleitner et al., 2010).

The settlements and villages of the Solo Khumbu region obtain their water directly from different sources including open waterholes, creeks coming down the mountains, or at sources located directly in the village that are sometimes also accessible to animals (e.g. yaks) which may defecate in this water supply (Fig. 1). The location of the primary water source may be close by or travel great distances via exposed tubing as at Gorak Shep (approximately 1800m). Exposed tubes present additional problems as the water can often freeze, and no one is controlling the development of biofilm in a tube several kilometers long.

Contamination of water sources has mostly a shortterm impact on tourists and a long-term influence on the local people and economy. Therefore the topic is relevant for both, travelers and local people. According to the Central Bureau of Statistics there was a total of 4980 households with 20,399 persons living in the Solu-Khumbu District in 2011 (https://cbs.gov.np/population-2011; 6.7.2022), of which about 3,500 were living in the Solu-Khumbu Valley (45.6/km^2). With some variations induced by political reasons, corona-related travel restrictions etc. the region takes an additional seasonal "load" of about 60,000 visitors (https://snp.gov.np/tourism; 6.7.2022).

The first aim of this study was to analyze the primary drinking water sources for fecal contamination from as many water sources as possible in the popular trekking area of Solo Khumbu region, Nepal. The second aim emerged during the study in Nepal: bacterial analyses of water ready to use. In Solu-Khumbu this is normally water stored in canisters in houses or lodges.

2. Material and methods

Water specimens (300 ml) were collected at any source (n = 80) where the locals and tourists collected their drinking water. These locations were at altitudes between 2,608 and 5,180m. Whenever possible samples were also collected from the kitchen of locals, or inside lodges from the water storage containers. For sampling, disinfected polyethylene bottles were used. For transport to the mobile laboratory the bottles were covered to prevent UV light exposure. Characteristics of the source, water temperature, and pH were registered, and all the water sources were documented (Suppl. 1). The locations where the samples were taken are illustrated in Fig. 2.

All samples were incubated the same evening that they were collected using the Dual Incubator Water Testing Kit (DelAgua, Malborough, U.K.). This robust and portable lab testing system was developed to monitor drinking water quality in the field and is used by several international NGOs, WHO and the military. It fulfills the WHO recommendations for water testing guidelines (WHO 2011) and can analyze a total of 32 specimens in each incubation cycle. Following incubation at 37 °C and at 44 °C. For all samples where there was colony growth the colonies of total coliform bacteria and those of thermotolerant bacteria were counted. The group of total coliforms includes fecal bacteria as well as bacteria found in the environment, whereas the thermotolerant coliforms reliably indicate fecal contamination (Maharjan et al., 2018)). Thermotolerant coliform bacteria include *E. coli* and some species of *Klebsiella*, *Enterobacter,* and *Citrobacter*.

Each water specimen used 100 ml for incubation at 37 °C, and another 100 ml at 44 °C. The water was filtered using DelAgua's testing kit which was sterilized by burning methanol in hypoxic environment. This produces formaldehyde, a very potent disinfectant. For incubation sterile MLSB (Membrane Lauryl Sulfate Broth, DelAgua, Malborough, U.K.) was used. The incubation time was 18 h. All CFUs were then counted, and the typical characteristic yellow colonies were recorded as coliform, and all the others (white, pink or other colonies except the typical yellow ones) were registered as "not specified". All countings were performed by the same person to exclude any bias from the method as good as possible.

The pH was measured using a colorimetric system (Phenol red, Tintometer Ltd., Lovibond House, Amesbury, U.K.) covering a range from 6.8 to 8.2. Turbidity was estimated using the measuring tube of the Dual Incubator system. This measurement was assessed semi-quantitatively and covered a range between <5 NTU (nephelometric turbidity units) to >2000.

The postulated correlation between a decrease of contamination and altitude (cold temperatures, less people) was tested by linear

Fig. 1. Yak standing and drinking from the primary water source used by a village in Solo Khumbu (Photo: T.Küpper).

correlation, a possible difference between sources from nature ("primary sources") or probes from households or canisters ("secondary sources") was investigated by Chi square test. $P < 0.05$ was defined as significant.

3. Results

The origin for most of the primary water sources sampled could be located. Primary sources were springs or wells where the specimens could be taken directly. Only in some rare cases this was not possible as the primary source was too far away or was located on a terrain that was too difficult, or even too dangerous, to access. Then we took the water for testing as close as possible to the respective primary source. Since there are several possibilities for contamination between the primary source and the place where the samples were taken such specimens were evaluated separately (hereinafter called "secondary sources").

Most of the water sources were located considerably above the respective village or settlement, but occasionally they were a few kilometers away or required several hours of uphill walking. The one exception to when the water source was not located above the houses or settlements was in Lobuche. Here the water was collected in the center of the village (Fig. 3) from a source enclosed by stone walls to prevent animals from directly contacting the drinking water outlet. There were also no latrines located nearby. The analysis of Lobuche's water showed no bacterial colonies, a pH of 6.8 and a temperature of 1 °C, summarized a very good water quality. However, animal droppings were found near this water source at several points, and may present a source of secondary fecal contamination. All the other water sources were not enclosed in to prevent animal access (e.g. using stone wall enclosures as in Lobuche (Fig. 3)), and only in a few instances was the primary water source enclosed by concrete or flat stones (e.g. at Dingboche).

In total there were 36 probes from lodges of which 25 (69.4%) were positive for coliform bacteria at 37 °C. 48 probes came from "primary sources" somewhere in the mountains and only 22 of them (45,8%) were positive ($P < 0.1$, n.s.).

The handling of water canisters to collect water was another possible source of contamination. It was observed in the villages that the end of the water tube was thrown on the ground in the mud after the canisters were filled with water. Therefore the next person filling their canister might contaminate their water with the soiled end of this water hose. These plastic canisters were generally in an unhygienic condition as they were rarely, if ever, cleaned from the inside. Some of these same storage canisters were additionally used to transport toxic substances such as kerosene or hydrogen peroxide (Fig. 4). Another source of contamination came from the water pipes that grew a significant biofilm (Fig. 5). People also routinely neglected to wash their hands before handling water or food.

The altitude of the water sources was between 2,500 and > 5,100m which were then classified into groups differentiated by 500m of altitude. There were 10 water samples obtained at 2,500-3,000m, 12 at 3,000–3,500m, 23 at 3,500-4,000m, 21 at 4,000–4,500m and 14 higher than 4,500m. The highest water point was at 5,480m. This one provided water for Gorak Shep (approximately 5,300m) for a few hours a day when the temperatures were above freezing.

The pH of the water was between <6.8 and 7.2 with a significant peak between 6.8 and 6.9 (38% of all specimens were pH 6.8) (Fig. 6). The water temperature was between 0 °C and 9 °C, with a peak at 2 °C. All turbidity measurements were <5 TU meaning the water was clear. At all locations the taste and the smell of the water was normal except at Gorak Shep. The expedition team members reported that this water was mouldy, and even after disinfection with chlorine (Micropur®) the taste remained distinctly unpalatable.

The results of the incubated specimens at 44 °C indicated no thermotolerant colonies. This was the case for all sampling points and was independent from the altitude or temperature of the source. However, 46 out of 80 samples (58%) tested positive when incubated at 37 °C for total coliform or non-specified bacteria. From these 46 samples, 24 contained total coliform bacteria (52% of the positive tests or 30% of all samples). Details are given in Table 1. Temperature was lower at altitude and fewer people and animals lived near these water sources at higher elevations and there was a decrease of contaminated specimens with higher altitude. There was no clear correlation, however, a comparison of all specimens from 2,500 to 3,500m vs. those above 4,000m the number of positive specimens from high altitude is significantly lower than the former ($P < 0.05$).

No bacterial contamination was found in the water of the local distribution points in the settlements of Tengboche, Phortse, Machherma, Lhabarma, Thore and Thare. In contrast, the samples taken from inside the lodges, where the water was stored in a container or barrel, these

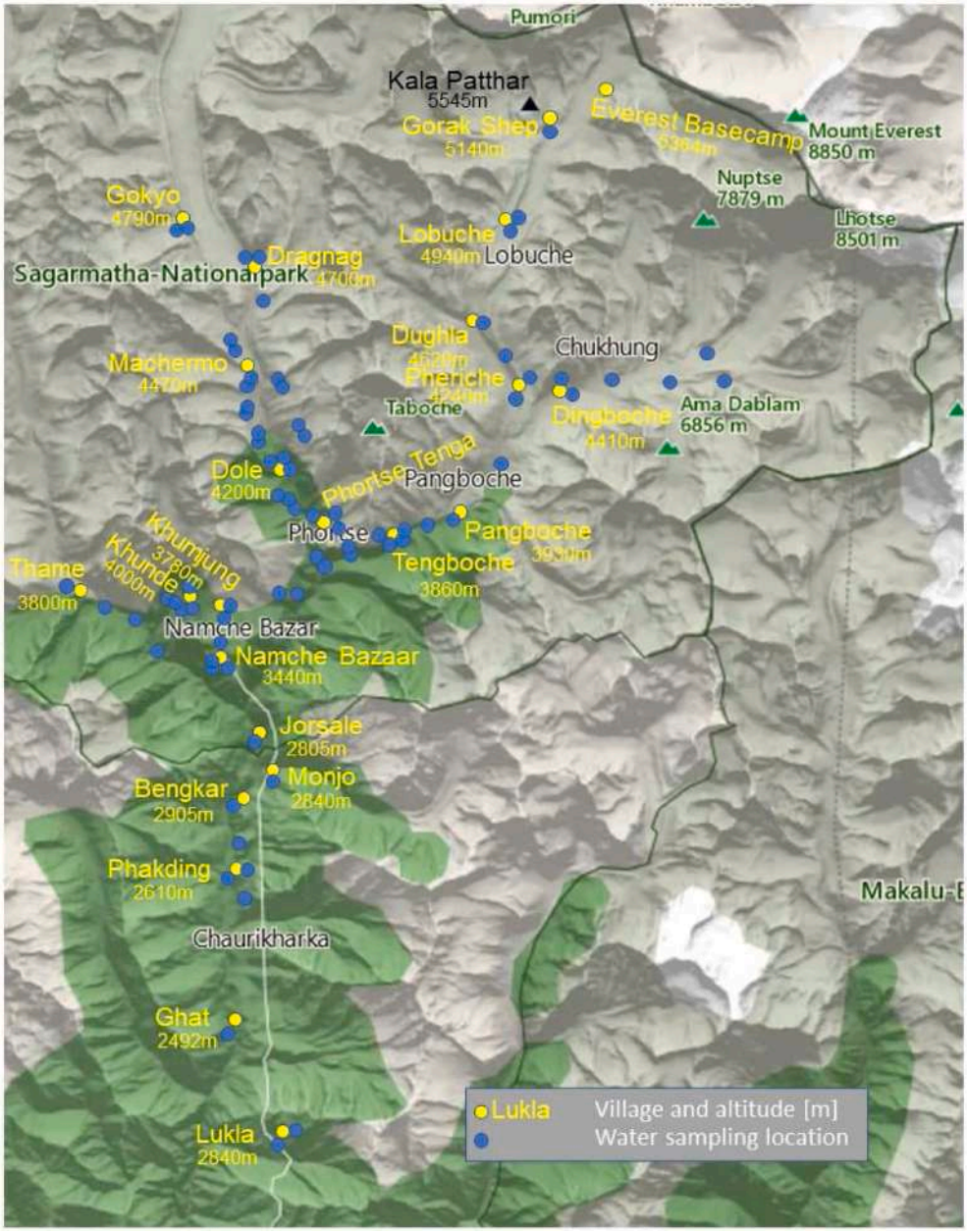

Fig. 2. Locations of where the water specimens were collected from (modified from www.jimmarsh.org).

samples tested positive on total coliforms and had no further differentiated bacteria at 37 °C. Detailed information about all sources is given in Supplement 1.

4. Discussion

We found no thermotolerant bacteria. According to WHO guidelines this indicates that there was no recent fecal contamination in the tested drinking water which could pose a harm to human health. However, it must be stressed that the DelAgua system does not assess every type of bacteria that can result from fecal contamination. It also does not detect viruses or parasites. A detailed differentiation of the coliform bacteria species was not necessary for water testing because all regulations only require that safe drinking water must be free of thermotolerant coliform CFUs (WHO 2011). However, the significant contamination as indicated by total coliform germs and the group "not specified" indicates a potential health risk, although the origin of most of the total coliform germs was from environmental sources.

It must also be mentioned that drinking water quality is not a stable factor and can vary daily dependent on environmental and social reasons and the time between the contamination and the investigation (indicator germs may have died after some time while other bacteria, viruses or parasites may survive longer and even replicate) (McFeters and Terzieva 1991), (Wang and Doyle 1998). When, for example, the clean water from an uphill primary source freezes inside the plastic pipes that serve the village, the locals use alternative sources that are often unsafe. In Phortse's case, they use river water (which we observed the contents of toilets being discharged into), or water from puddles that form on a terrain where pack animals graze. People should be reminded that these sources might represent a risk of obtaining a waterborne infection. On the other hand, a spring with high flow rate and low water temperature will clean itself within short time.

It is possible to heat the water or use chlorine for disinfection to make the drinking water safe, though the latter is rarely practiced in the region. However, after disinfection, care must be taken to not contaminate it again. For example, as lodge owners pass on water to tourists and

Fig. 3. Drinking water source in Lobuche. A stone wall fence prevents lifestock from accessing the water source directly, but they may drink below the outflow. Tubes facilitate the filling of containers. (Photo: T. Küpper).

Fig. 4. Kerosene container actually used for water storage (Photo: T.Küpper).

Fig. 5. Biofilm and mud in a water container (Photo: T. Küpper).

porters, they should have a responsibility to provide safe water, or at least inform the consumers where it comes from. Our results found six locations where the storage of clean water was in unhygienic containers that resulted in contamination with total coliforms that were not further differentiated bacteria. The problem of secondary contamination of water in unhygienic storage containers has been published from other parts of Nepal or other low income countries (Daniel et al., 2020), (Momba and Kaleni 2002), (Lindskog and Lindskog 1988), (Ali et al., 2015). The actual study is limited here. We found a relatively high rate of contaminated containers but because of cultural barriers it was not possible to get valid data. While our female team members sometimes got access to households and the containers this was not possible for the male colleagues and therefore a detailed comparison with primary sources would be significantly biased. However, a frequent and regular cleaning of water containers should prevent the transmission of infections and impede the development of a biofilm.

The point of sampling may explain a difference between our findings and those of some other groups who described a lower contamination in lower or hilly terrain or a general higher contamination by E. coli than in our study (e.g. (Sarkar et al., 2022), (Rai et al., 2012), (Rai et al., 2009)). In contrast to our study the samples were taken at the households while our so-called "primary sources" were located far from any household and high above the villages in cold mountainous terrain. Cold environment and only a few creatures around must be assumed as a good environment for low bacterial occurrence.

Feces as potential source of water contamination is a common occurrence in Solo Khumbu. Fuel for cooking and heating is scant. Petrol-filled jerricans are carried uphill by porters and are quite expensive. To produce extra fuel, Nepali people collect the feces of the pack animals, dry this excrement on stones, and use it to light their fire. All these procedures are performed manually. A problem then arises if the hands are not washed thoroughly afterwards, and before meal preparation, as diarrheal pathogens can spread via smear infection. Improvements in handwashing could reduce diarrheal diseases by 14–40% in developing countries (Ali et al., 2015), (Hoque 2003). Education on hygienic behavior should be extended (Budhathoki et al., 2016). In Kathmandu, the capital city of Nepal, long brick walls are painted with slogans and drawings to educate people to wash their hands after producing combustible material from feces, after defecation, or before preparing food. At Khunde Hospital in Solu-Khumbu, nonverbal drawings are used to inform people about hygiene (Fig. 7). The use of soap might not be an option for some due to the extra cost. A Nigerian study on fecal contamination by street food vendors found that the extra costs associated with buying soap was one of the reasons these vendors did not use soap (Idowu and Rowland 2006). In addition to these educational aspects, some practical considerations here should be noted. The water is very cold in the Himalayans (a peak of 2 °C in our study), liquid water is not always easily available in the high mountains, and most toilets do not have a tap.

In most of our study's visited sites, it would have been possible to get quite good drinking water when the temperature was above the freezing point. We strongly endorse public health education to improve individual hygiene for safe drinking water, especially hand cleaning. Of special importance we would additionally suggest that wherever possible: i) that the primary water source to be as close as possible to the origin of that water supply; ii) that fencing be installed around the primary source to prevent livestock and other animals from contaminating it and; iii) that the water storage containers are regularly cleaned. Since the disinfection cleaning of these containers does not occur, nearly all of them were contaminated inside with residues of chemicals from former use, dirt, and germs (biofilm). This secondary contamination of primarily good water may cause a significant health hazard. This situation is a common finding in several low-income countries (Alemeshet Asefa, Alemu et al., 2021), (Momba and Kaleni 2002), (Lindskog and Lindskog 1988), A rural African study (Momba and Kaleni 2002) had a similar situation to ours whereby the locals collected their undisinfected water from a primary tap or similar source and 97% of them stored their household water (or 100% in our study), mostly in plastic containers, and the majority of these containers were already contaminated from the inside.

Data is scarce about the point where deterioration of the bacteriological quality of drinking water occurs (source, storage, handling at the point of use). Our observations, although not so systematically assessed

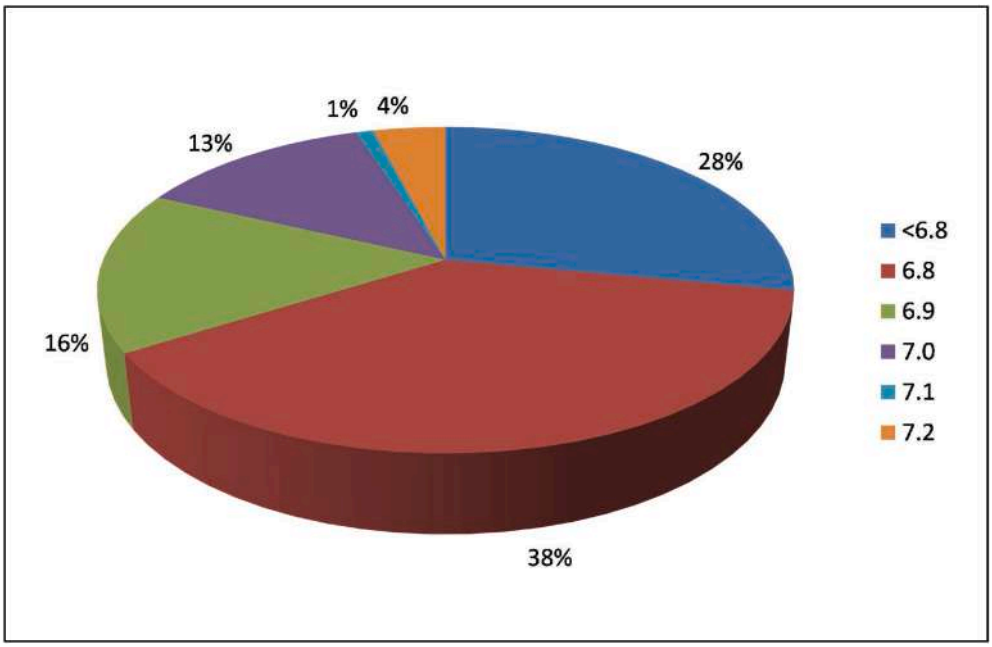

Fig. 6. pH **of the water sources**.

Table 1
Total coliform bacteria and non-specified bacterial samples after incubation at 37 °C.

Altitude [m]	Number of specimens	Number of positive tests for total coliform or non-specified	Total positive tests [%]	Number of positive tests for total coliform bacteria	Total positive tests for total coliform [%]	Number of positive tests for thermotolerant coliform bacteria
2,500–3,000	10	8	80	6	60	0
3,000–3,500	12	8	67	5	42	0
3,500–4,000	23	13	57	3	13	0
4,000–4,500	21	10	48	6	29	0
>4,500	14	7	50	4	29	0
Total	**80**	**46**	**58**	**24**	**30**	**0**

Fig. 7. Water hygiene education at Khunde Hospital.

as those by Alemeshet Asefa et al. (Alemeshet Asefa, Alemu et al., 2021). Our results with 54.5% positive tests from "primary sources" vs. 59.5% from lodges or households differ not significantly, but the tendency indicated by $P < 0.1$ may be interpreted as being in good accordance with their results. They found significantly less thermotolerant coliforms in the primary water sources (21.7%) compared to the household water (83.3%). Water transport, storage and obviously the socioeconomic situation in the households were comparable to Solu-Khumbu. They found the risk of fecal contamination was increased by 9.6-fold for households in the poor tercile compared to rich; 2.8-fold when sanitation facilities were unimproved; 3.7-fold when people and livestock live close together; 3.4-fold when water was not treated and 7-fold when the hands were not washed before water was treated (Alemeshet Asefa, Alemu et al., 2021).

This is easily comparable with Solu-Khumbu. The higher number of CFUs in Alemeshet Asefa's study is simply caused by a higher reproduction rate of bacteria in the much hotter African environment, as opposed to the slower growth rate in Himalayan water just some degrees above the freezing point. Studies from Kote/India and Tamale/Ghana also support these results (Roopavathi and Mamatha 2016), (Boateng et al., 2013).

In summary our data suggest that the main factor for the contaminated drinking water in the Solu-Khumbu region is not the undisinfected water coming from the primary water sources, but rather the handling and storage of the water (Alemeshet Asefa, Alemu et al., 2021), (Mengistie et al., 2013). Health education programs on water hygiene, sanitation and proper storage and handling of water should be improved. Since a significantly higher proportion of water is usually boiled in the Solu-Khumbu region for tea than in India or Africa, the most important topics here are washing the hands before any contact with drinking water, regular cleaning and disinfecting the water containers, avoidance of contact by livestock with the water containers, and improving sanitary facilities.

The use of halogens is an alternative but may be questioned: With actual about 50,000 visitors who stay an average of 11 days (data from the Nepal Ministry of Tourism) there is a need of about 2.4 million litres of clean water per season. If this should be produced by using halogens about 460 kg of substances which have a deteriorating or even toxic

effect on the environment would be set free every season. Therefore we suggest filters or UV light as the ecologically better option.

5. Limitations of the study

The survival of pathogens shows a great variety of time between the different types of organisms (see above). Therefore it is possible that protozoan cysts remain present in cold water over months, while bacteria (e.g. the indicator bacteria *E. coli*) die off after a couple of days. As a result it is only possible to discuss recent fecal pollution using the applied methods. However, since the water was always quite cold, the survival time of the indicator bacteria was increased (Mezrioui et al., 1995) and consequently the possibility of detection covered a longer period. The same holds true for the pH results. As expected the silicates of the region's geology caused a slightly acidic pH at most sources. In such water the survival time of *E. coli* is longer than in alkaline water (Mezrioui et al., 1995). However, in future studies streptococci should be included as they are not pathogenic, but they do indicate old fecal contamination (Mezrioui et al., 1995). Since turbidity was very low in all specimens, this should not have had any influence on the results. Its decrease of efficacy of disinfection procedures was not of interest for our study but turbid water potentially clogs the filter of the DelAgua system or similar incubator techniques. This may cause false-negative results.

The study did not investigate viral contamination. For some "classic" faecal-oral transmitted viral diseases this is neglectable: Polio has been eradicated in Nepal and hepatitis A typically affects young children as a mild disease and then causes life-long immunity. However, there are many others, e.g. enteric viruses with more than 100 species (Ramia 1985) and which cause a public health problem in some regions (Werneck et al., 2017), (Jean et al., 2006), (Tubatsi and Kebaabetswe 2022). Hepatitis E (Vivek et al., 2013), adenoviruses and rotaviruses (Verheyen et al., 2009) or norovirus (Tsang et al., 2018), (Tubatsi and Kebaabetswe 2022). We focused on E. coli as indicator germ according to WHO recommendations to get an idea about possible fecal contamination and because the test for different viruses needs extensive laboratory equipment which was not available in the remote region near Mt. Everest. However, noroviruses are not a typical problem of rural Nepal: It is easy possible to get them in France (Schaeffer et al., 2013) or in any other industrial country but typically they cause outbreaks in enclosed environments like cruise ships (Bert et al., 2014), (Wang et al., 2016), (Qi et al., 2018), (Freeland et al., 2016) or places where many people live very close together (Centers for Disease and Prevention 2009), (Domenech-Sanchez et al., 2011).

However, in future studies it would be interesting to analyze other pathogens, especially *Giardia lamblia*, or to use more precise laboratory, e.g. FISH or PCR methods.

6. Conclusion

No thermotolerant bacteria were detected. This indicated there was no significant recent fecal contamination found in any of the drinking water samples tested. However, water quality is subject to constant change. Our observations still strongly endorse disinfecting all drinking water as best practice. We observed a major problem in the unstable access to safe drinking water (e.g. water frozen in pipes so an alternative water source was used to collect water from puddles located next to pack animals), the poor hygienic conditions (contact with feces and no hand washing with soap), and some toilets are still discharged directly into the rivers providing water to primary drinking water collection points. Another problem we found is the inner contamination of containers used for water transport or storage, a problem reported in many other global studies in developing countries. We also observed biofilm in the water coming from exposed overground pipes.

Therefore, it is highly recommended that visitors of Solu-Khumbu region disinfect their drinking water using filter systems, UV-light dispensers or halogens (e.g. chlorine), or a combination of two methods. However, the total amount of halogens set free at places where many tourists stay should be taken into account with regard to environmental damage. Cooking is another well-applied method, but it is not ecologically worthwhile as fuel is scarce in the mountains. As a rule of thumb water collected directly from the primary source in a clean container is generally safer in the Himalayas than when taken from a lodge.

CRediT authorship contribution statement

Preparation of the research project (Küpper, Apel, Bertsch, M.v.d. Giet)*Assembly of data for the research undertaken (all, except Morrison)*
Conducting of statistical analysis (Risse)
Interpretation of results (Küpper, Risse)
Manuscript preparation (Küpper, Morrison)
Literature review (Küpper, Risse)
Revising the manuscript (Morrison, Küpper)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114043.

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Assessment of multi-chemical exposure using human biomonitoring data from the French Esteban study using exposure load method☆

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ABSTRACT

Exposure to chemical substances is common and comes from several sources (environmental, food, and occupational). It is often studied using a substance-by-substance approach. Although this method helps identify the determinants of exposure to a single chemical, it cannot accurately reflect exposure to multiple chemicals. In this study, we used the concept of exposure load (EL) to evaluate multi-chemical exposure in a representative sample of the general French population. EL corresponds to the number of substances (or metabolites) measured in body fluids above a defined concentration threshold. EL was calculated for adults and children separately for two groups of substances: those currently found in domestic environments (Group A) and pesticides (Group B). Although the EL does not assess the health impact linked to multi-chemical exposure, it does aid in the identification of particularly vulnerable populations. Accordingly, preventive actions specifically aimed at these subgroups could be useful.

In Group A, we found that multi-chemical exposure was generalized since all the adults and children had an EL greater than or equal to 13 (out of 22 substances studied) when the LOQ (limit of quantification) was considered as the discretization threshold. In adults, men, smokers and people of working age (i.e., people under 60 years old) had a higher EL. In Group B, multi-chemical exposure was also generalized, since all the adults (15 substances studied) and children (13 substances studied) had a mean EL almost equal to 6 when the LOQ was considered as the discretization threshold. In adults, persons with occupational exposure to pesticide dust had a higher EL when the P90 was considered as the discretization threshold.

This study highlights widespread multi-chemical exposure in adults and children in France, and the major impact of occupational exposure (Group B) and tobacco smoking (Group A) on EL.

1. Introduction

Exposure to chemical substances is common and comes from several sources (environmental, food, and occupational). Multi-chemical exposure does not necessarily imply simultaneous exposure to several substances. Each substance has a variable half-life in an organism, from a few hours to a lifetime. Sequential exposure, at different times in a person's life, can lead to multi-chemical exposure when some of the substances concerned have long biological half-lives. The magnitude and health effects of multi-chemical exposure are not well-known but they are being increasingly studied, in particular through the concept of the 'exposome' (Julvez et al., 2021; Misra, 2020; Vrijheid et al., 2020), which is a measure of all environmental exposures in a person's life from conception (Wild, 2005).

The European Commission Chemical Strategy for Sustainability indicated that the effect of chemical mixtures needs to be taken into account and integrated more generally into chemical risk assessments (European Commission Chemicals Strategy for Sustainability, 2020). One proposed approach to do this is to introduce a mixture assessment factor (MAF) in single substance risk assessment in order to protect humans from the combined risks of unintentional mixtures (Socianu et al., 2022).

Biomonitoring provides a comprehensive measure of chemical exposure from all sources and routes of uptake (inhalation, ingestion, skin transfer). A French national human biomonitoring (HBM) programme was created in 2009 to estimate the exposure of the French

☆ I confirm that this paper has been read and approved by the authors and by my institution. I also confirm that it has not been published previously, and is not currently being considered by any other peer-reviewed journal.

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population to chemicals, and to better understand the determinants of this exposure. One component of this programme is a cross-sectional survey of the general population in mainland France aged 6–74 years old called Esteban.

Results from Esteban demonstrated that the general population is exposed to everyday pollutants from food, cosmetic products, and personal care sources (Fillol et al., 2021d). Using a substance-by-substance approach, the study found generalized exposure to chemicals. However, although this approach helps identify the determinants of exposure to a single chemical, it cannot accurately reflect exposure to multiple chemicals.

The present study used the concept of exposure load (EL) to assess multi-chemical exposure in the French general population. EL corresponds to the number of substances (or metabolites) measured in body fluids whose concentration is above a defined threshold. The method we employed was based on those used by Health Canada and VITO (Flemish Institute of Technological Research) with minor adaptations (Buekers et al., 2021; Willey et al., 2021) applied to data from the French HBM programme for adults (18–74 years old) and children (6–17 years old) who participated in the Esteban survey. It is important to note that EL does not take into account the toxicity of each substance, so it does not necessarily reflect a health risk.

EL was calculated for adults and children separately for two groups of substances: those currently found in domestic environments (Group A) and in pesticides (Group B).

The main objectives of the present study were therefore to use the concept of EL to describe multi-chemical exposure in the French population for two groups of substances using Esteban data, and to identify subpopulations particularly affected by multi-chemical exposure, with a view to developing improved exposure prevention actions.

2. Material and methods

2.1. Esteban survey

Esteban is a cross-sectional study of the general population living in mainland France aged between 6 and 74 years old. It was conducted between April 2014 and March 2016. In total, 2503 adults (18–74 years old) and 1104 children (6–17 years old) were included during four different periods, in order to take seasonal variation in exposure into account (Balicco et al., 2017).

The study collected data on health, nutrition, exposure to chemicals, and socio-demographic characteristics with the aid of two interview-based questionnaires, four self-administered questionnaires, a 24h dietary recall, and analysis of fasting biological samples (blood, urine, and hair).

Approval for the study was obtained from the French data protection authority and a bioethics committee.

Three-stage cluster sampling was used in Esteban. In the first stage, a stratified sample of primary units (municipality or groups of municipalities) was randomly drawn. In the second stage, households were randomly selected in each primary unit using random generation of landline and cell phone numbers. In the third stage, only one individual (adult or child) was randomly selected to participate in the study among eligible household members, using the Kish method (Kish, 1949). Two samples were drawn separately: one for participating adults and one for participating children. Stratification was performed according to two variables: the region of residence (eight geographical areas) and degree of urbanization (five degrees as follows: rural, <20 000 inhabitants, 20 000–100 000 inhabitants, over 100 000 inhabitants, Paris and its suburbs). The sampling frame of the Esteban study is described in detail elsewhere (Balicco et al., 2017).

2.2. The process of prioritizing biomarkers for Esteban

The process of prioritizing which chemicals to investigate in the French HBM programme, and therefore in Esteban, is described in detail elsewhere (Fillol et al., 2014). Briefly, chemicals were first selected depending on their biomonitoring feasibility, exposure relevance, existing regulations, and priorities in terms of health effects. The Delphi consensus method was then used to prioritize these chemicals and associated biomarkers according to criteria based on i) the scientific contribution these analyses would bring in terms of new knowledge in the French context, ii) the feasibility of preventing exposure, iii) the logistical and analytical feasibility of measuring the biomarkers, iv) the interpretation of results, v) the biomarkers' characteristics (i.e., specificity, intra-individual variability, etc.), vi) the public perception of the substances being measured, vii) exposure characteristics (i.e., origin of the contamination), and viii) hazard identification for each evaluated substance. The positioning of some groups of biomarkers in the prioritized list was debated in discussions during an expert meeting which ended in the production of a consensual, prioritized list of biomarkers to be included in the national HBM programme.

2.3. Biomarker measurements

To ensure relevance of the results, participants were instructed not to consume fish or shellfish in the three days preceding the examination, and not to smoke in the preceding 2 h. Urine samples were collected by participants upon waking (i.e., first morning urine).

Six control samples of ultra-pure water initially packed in a glass ampoule were sent to laboratories to be assayed under the same conditions as the study samples. None of the control samples had a phthalate, bisphenol, metal (cadmium, mercury in urine, chromium, nickel, antimony, cobalt, beryllium and vanadium) or pyrethroid at a quantifiable level, indicating the absence of contamination due to the sample preparation environment or due to the sample collection and cryopreservation equipment.

The laboratories that performed the biomarker measurements were selected by Santé publique France (French Public Health Agency) in a call for tenders based on price, quality, performance, delivery, suitability and experience in HBM studies.

In order to assess the intermediate precision of the analyses, six pairs of replicates were introduced blindly into the analytical series, but not for all biomarkers. In other words, two cryotubes belonging to the same subject with different identifiers were analysed. These six pairs of replicates were analysed, with concordant results for all the substances analysed: metals (arsenic, cadmium, mercury in urine, chromium, nickel, antimony, cobalt, beryllium and vanadium), phthalates, cotinine, and bisphenols. This process was not performed for Group B (urinary pesticides). Details on laboratory procedures and quality control are available elsewhere (Balicco et al., 2019a, b; Chaperon et al., 2021; Fillol et al., 2021a; Fillol et al., 2021b, c; Health Canada; Oleko et al., 2021a; Oleko et al., 2021b, c; Pécheux et al., 2021; Tagne-Fotso et al., 2021).

2.4. Exposure load calculation

The method used to calculate the EL was based on that established by Willey et al. (2021) using data from the Canadian Health Measures Survey (CHMS), and subsequently adapted by Buekers et al. (2021) using data from the 4th Flemish Environment and Health Study (FLEHS-4).We made further adjustments to this adapted method to use it on Esteban data. As adjustment of urinary concentrations by creatinine is no longer the gold standard, individual and threshold concentrations to calculate the EL were expressed in μg/L (Middleton et al., 2016; Slimani et al., 2020; Suwazono et al., 2005).

The Esteban study provided all the descriptive statistics for each biomarker in the study population (Balicco et al., 2019b; Chaperon et al., 2021; Fillol et al., 2021b, c; Oleko et al., 2021a; Oleko et al., 2021b, c; Pécheux et al., 2021; Tagne-Fotso et al., 2021) taking into account all the complex methodological aspects of the study (design

weights, clustering, and stratification). All estimates were made using the survey weights. The weighted analysis was conducted separately in adults (18–74 years old) and children (6–17 years old), for all participants of each biomarker group (i.e., Group A or B). These weights took into account socio-demographic characteristics of the reference population, specifically the general French adult population (18–74 years old) and the French population of children aged between 6 and 17 years old. The statistical analysis was conducted using R software (package 'survey' to take into account the sample design).

To calculate EL, the LOQ, P50 (50th percentile) and P90 in the general population were used as discretization thresholds (threshold values used for all biomarkers for children and adults are available in the Supplement Table S3). The LOQ threshold was used to assess the number of substances quantified per participant. The P50 threshold made it possible to estimate the number of substances for which an individual had concentrations greater than or equal to the median of the general

Table 1
Substances and biomarkers associated, analytical methods, limits of quantification, and quantification frequencies.

Group	Chemical Group	Substance	Urinary Biomarkers	Analytical method	LOQ (μg L^{-1})	% adults>LOQ	% children>LOQ
A	Bisphenols	Bisphenol A (BP A)	BP A total	GC-MS/MS	0.09	100	100
			BP A free	GC-MS/MS	0.09	27.6	16.2
		Bisphenol S (BP S)	BP S total	GC-MS/MS	0.006	100	99.9
			BP S free	GC-MS/MS	0.006	56.2	51.4
		Bisphenol F (BP F)	BP F total	GC-MS/MS	0.02	100	100
			BP F free	GC-MS/MS	0.03	15.6	8.6
A	Phtalates	Di-n-butyl phthalate (DnBP)	Mono-n-butyl phthalate (MnBP)	UPLC-MS/MS	1.30	99.9	100
		Di-iso-butyl phthalate (DiBP)	Mono-isobutyl phthalate (MiBP)	UPLC-MS/MS	0.44	100	100
		Di-methyl phthalate (DMP)	Mono-methyl phthalate (MMP)	UPLC-MS/MS	0.53	94.5	99.6
		Di-ethyl phthalate (DEP)	Mono-ethyl phthalate (MEP)	UPLC-MS/MS	3.30	100	99.8
		Butyl-benzyl phthalate (BBzP)	Mono-benzyl phthalate (MBzP)	UPLC-MS/MS	1.20	93.8	99.2
		Di-cyclohexyl phthalate (DCHP)	Mono-cyclohexyl phthalate (MCHP)	UPLC-MS/MS	0.83	0.2	0.2
		Di-n-octyl phthalate (DnOP)	Mono-n-octyl phthalate (MnOP)	UPLC-MS/MS	0.51	0	0
			Mono-3-carboxypropyl phthalate (MCPP)	UPLC-MS/MS	0.41	80.9	96.8
		Di-isononyl phthalate (DiNP)	Mono-isononyl phthalate (MiNP)	UPLC-MS/MS	1.20	17.2	19.2
		Di (2-ethylhexyl) phthalate (DEHP)	Mono-2-ethylhexyl phthalate (MEHP)	UPLC-MS/MS	0.36	92.1	97.6
			Mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP)	UPLC-MS/MS	0.29	99.9	100
			Mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP)	UPLC-MS/MS	0.63	99.7	100
A	Metals	Arsenic	Total Arsenic	ICP-MS	0.04	100.0	100.0
		Cadmium	Cadmium	ICP-MS	0.005	100.0	100.0
		Mercury	Urinary Mercury	ICP-MS	0.04	95.6	99.4
		Chromium	Chromium	ICP-MS	0.08	97.7	99.9
		Nickel	Nickel	ICP-MS	0.07	97.5	99.1
		Antimony	Antimony	ICP-MS	0.008	99.1	100.0
		Cobalt	Cobalt	ICP-MS	0.004	100.0	100.0
		Beryllium	Beryllium	ICP-MS	0.01	4.2	10.9
		Vanadium	Vanadium	ICP-MS	0.02	99.3	99.9
A	Nicotine	Nicotine	Cotinine	UPLC-MS/MS	0.07	56.6	63.9
B	Pyrethroid	3-PBA	3-PBA	GC-MS	0.015	100	99.6
		F-PBA	F-PBA	GC-MS	0.015	67.3	69.1
		Br2CA	Br2CA	GC-MS	0.015	99.4	99.6
		Cis-Cl2CA	Cis-Cl2CA	GC-MS	0.015	99.8	99.4
		Trans-Cl2CA	Trans-Cl2CA	GC-MS	0.015	98.6	98.6
B	Herbicides	Atrazine	Atrazine	UPLC-MS/MS	0.02	0	0
			Atrazine-desethyl	UPLC-MS/MS	0.005	3.0	0.2
			Atrazine-desethyl-2-hydroxy	UPLC-MS/MS	0.2	0.1	0.2
			Atrazine-desisopropyl	UPLC-MS/MS	0.1	0.2	0.2
			Atrazine-desethyl-desisopropyl	UPLC-MS/MS	0.2	0.3	0
			Atrazine-2-hydroxy	UPLC-MS/MS	0.05	0.7	0
			Atrazine mercapturate	UPLC-MS/MS	0.02	1.5	0.2
		2,4-Dichlorophenoxyacetic acid	2,4-D	UPLC-MS/MS	0.01	55.8	40.4
		Alachlore	Alachlor	UPLC-MS/MS	0.02	0.1	0
			Alachlor mercapturate	UPLC-MS/MS	0.02	0.1	0.4
			2,6-Diethylaniline	UPLC-MS/MS	0.05	0.6	0.4
		Glyphosate	Glyphosate	UPLC-MS/MS	0.05	16.6	14.3
			AMPA	UPLC-MS/MS	0.05	74.0	93.4
		Isoproturon	Isoproturon	UPLC-MS/MS	0.02	0.7	0.0
			IPPMU	UPLC-MS/MS	0.02	0.8	0.2
		Chlortoluron	Chlortoluron	UPLC-MS/MS	0.1	0.1	0.0
		Dimetachlor	Dimetachlor	UPLC-MS/MS	0.05	0.0	0.0
B	Organophosphate pesticides	Chlorpyrifos	Chlorpyrifos	UPLC-MS/MS	0.05	0.66	–
			Chlorpyrifos-oxon	UPLC-MS/MS	0.005	1.72	–
			TCPy	UPLC-MS/MS	0.05	3.1	–
		Chlorpyrifos-methyl	Chlorpyrifos-methyl	UPLC-MS/MS	0.05	1.59	–
			Chlorpyrifos-methyl-oxon	UPLC-MS/MS	0.05	0.0	–
B	Carbamates pesticides	2-Isopropoxyphenol	Propoxur	UPLC-MS/MS	0.05	6.0	6.0
			2-IPP	UPLC-MS/MS	0.05	8.5	11.6

pollution distribution. The P90 threshold indicates the number of substances for which an individual is more exposed than the general population (i.e., a concentration greater than or equal to the 90th percentile of the distribution for at least one of the biomarkers studied for a substance).

A value was assigned to each substance for each participant: it was equal to 1 if the concentration of at least one of the substance's biomarkers was greater than or equal to the threshold considered, otherwise the value assigned was equal to 0. For example, for glyphosate, if the concentration of AMPA and/or the concentration of glyphosate were greater than or equal to the specified threshold, then the value assigned to the glyphosate substance for this threshold was equal to 1 for this participant.

As the required volume of urine was not available for all Esteban participants, and because of the cost of performing analyses, not all substances were measured for all the participants. Subsamples of participants were randomly drawn in order to analyse a series of biomarkers in their biological matrices. This led to the need to create Group A and Group B for the purposes of exposure load.

The selection of the substances to include in both groups was made by attempting to maximize the number of substances studied (taking into account the series of biomarker used in the draw to assign biological measures to participants), while ensuring a reasonable sample size of adults and children in order to be able to perform data analysis (minimum number of 700 adults and 300 children). This selection led to the exclusion of biomarkers measured in hair and blood, these matrices being less available. Group A comprised 22 substances including metals, cotinine, phthalates and bisphenols. This group was measured for 896 adults and 498 children. Group B comprised 15 substances for adults and 13 substances for children, all of which were urinary pesticides authorized in France at the time of the Esteban study or known to be persistent in the environment. This group was analysed for 742 adults and 497 children.

The list of substances and associated biomarkers studied is available in Table 1. One EL per participant was calculated for Group A and for Group B, corresponding to the sum of the values attributed to each of the substances studied. EL values were calculated separately for adults and for children in both groups.

For each group, all selected biomarkers were taken into account to calculate EL irrespective of the percentage of quantification in Esteban. Therefore, for some biomarkers with low quantification rates, P50 and P90 may have been lower than the LOQ (Table 1). In those cases, participants were only assigned the value 1 if the biomarker was measured strictly above the LOQ.

For Group A, including 22 substances, the following variables were studied: age, sex, educational level, perceived financial situation, and smoking status.

For Group B, including 15 substances for adults and 13 substances for children, the same variables as in Group A were analysed complemented by variables specific to pesticide exposure as follows: season when urine sample was collected, frequency of ventilation of participant's dwelling, proximity to a garden, proximity to a cultivation area, occupational exposure to pesticide dust (adults), consumption of self-produced foods, and consumption of organically-produced foods.

Differences between EL were measured by comparing the confidence intervals around the mean values.

Smoking status was assigned, for adults only, based on responses to questionnaires. In order to assess the impact of cotinine on the identification of multi-chemical exposed population subgroups among adults in Group A, analyses were performed with and without cotinine and its availability in supplements. All graphs presented in the article include cotinine.

2.5. Characterization of EL mixture profile in individuals with the highest multi-chemical exposure

EL allows the number of substances to which individuals are exposed to be quantified. However, it does not provide information on the composition of the mixture. This composition was investigated in detail for the P90 threshold in order to focus on multi-chemical exposure at high concentrations. Individuals with the highest EL were selected to determine whether the observed substance cocktails had similar profiles. Individuals were selected if their EL was greater than the maximum observed EL minus 1 (e.g., if the maximum EL was 10, the selected individuals had an EL of 9 or 10). An exception was made for children in Group A because only one child would have been selected with this rule. They were selected if their El was up to the maximum observed EL minus 3.

3. Results

3.1. Population characteristics

The study population's characteristics are shown in Tables 2 and 3. There were no major differences between the compositions of Groups A and B for adults and children of the Esteban sample as a whole in regard to participant age group, sex, education level and perceived financial situation.

4. Exposure load results

4.1. Group A

4.1.1. Adults

When considering the LOQ as the discretization threshold, the EL distribution was quite narrow. Adults had on average an EL of 18.5 (minimum 13, maximum 20) (Fig. 1A). The 10% of adults most exposed to multiple chemicals had an EL of 20.

Using the 50th percentile exposure as the discretization threshold, a wider EL distribution was observed (Fig. 1B). Specifically, the EL was between 0 and 19, and the mean EL was 9.6. The 10% of adults most exposed to multiple chemicals had an EL of 16.

At the 90th percentile exposure threshold, the EL distribution narrowed again (Fig. 1C). The number of substances to which adults are exposed is low at this threshold with the mean EL was 2 (minimum 0, maximum 10). The 10% of adults most exposed to multiple chemicals had an EL of 5.

Mixtures of substances were assessed in individuals (N = 11) with the highest multi-chemical exposure (i.e., to at least nine substances) at the P90 threshold. Mixtures of substances were very heterogenic. The metals most often found in these individuals were antimony, cobalt, chromium and vanadium. For bisphenols, bisphenol A was the substance most often found. The phthalates most frequently found were the metabolites of DEHP (MEHP, MEOHP, MEHHP), MnBP, MEP and MCPP. Occupational activities of these the 11 individuals were also heterogeneous.

At the LOQ threshold, there was no significant difference in terms of age, sex, smoking status, education level, or perceived financial situation (Supplement Table S1).

Using the 50th percentile exposure as the discretization threshold (Fig. 2), women had an mean EL of 8.9 [8.3; 9.4] versus 10.3 [9.7; 10.9] for men. The EL decreased with increasing age in adults. In addition, smokers had a higher EL than non-smokers and former smokers (Supplement Table S2).

Similarly, when considering the 90th percentile as the threshold (Fig. 3), women, people aged over 60 years, and non-smokers all had significantly lower average EL (Supplement Table S3).

More information on the distribution of EL for each the discretization thresholds used is available in tables in the supplementary material section.

Table 2
Main characteristics of the adult population (18–74 years), CI: confidence interval.

Factor	EL adult population Group A			EL adult population Group B			Esteban study adult population		
	n	%	95%CI	n	%	95%CI	n	%	95% CI
Age (years)									
18–29	55	14.83	[11.08; 18.57]	41	14.49	[10.31; 18.75]	262	16.93	[14.42; 19.63]
30–44	224	29.67	[25.99; 33.23]	183	30.93	[26.72; 35.05]	772	29.00	[27.02; 30.95]
45–59	342	32.32	[28.49; 36.33]	276	32.12	[28.41; 36.03]	1093	31.13	[29.00; 33.37]
60–74	275	23.19	[20.07; 26.53]	242	22.46	[19.11; 26.05]	894	22.94	[21.20; 24.83]
Sex									
Male	406	52.83	[49.10; 56.59]	328	48.64	[43.98; 53.12]	1328	48.37	[46.37; 50.40]
Female	490	47.17	[43.41; 50.90]	414	51.36	[46.88; 56.02]	1693	51.63	[49.60; 53.63]
Education level									
No secondary school certificate or secondary level vocational training certificate; primary school certificate	251	47.81	[42.84; 52.66]	213	48.81	[43.52; 54.02]	863	47.95	[44.46; 51.17]
Upper secondary school certificate	188	20.88	[17.52; 24.30]	150	20.00	[16.51; 23.58]	586	20.60	[18.85; 22.44]
1st cycle university degree	229	14.75	[12.33; 17.31]	182	15.27	[12.05; 18.94]	735	15.15	[13.72; 16.61]
2nd or 3rd cycle university degree	228	16.55	[12.80; 20.64]	197	15.92	[12.61; 19.41]	833	16.3027	[13.25; 20.14]
Perceived financial situation									
I'm comfortable	218	17.61	[14.67; 20.67]	188	19.06	[15.54; 22.81]	698	17.64	[15.76; 19.54]
I get by	344	35.16	[31.62; 38.78]	282	37.03	[32.56; 41.58]	1128	36.02	[34.09; 37.10]
It's tight	97	12.94	[10.01; 16.00]	80	11.49	[8.56; 14.54]	349	13.06	[11.57; 14.52]
I need to be careful/I find it difficult to make ends meet/I am in debt	237	34.29	[30.55; 38.08]	192	32.42	[27.79; 37.29]	824	33.28	[30.90; 35.62]

Table 3
Main characteristics of the child population (6–17 years).

Factors	EL population Group A			EL population Group B			Esteban Study population		
	n	%	95%CI	n	%	95%CI	n	%	95% CI
Age (years)									
6–10	230	41.72	[36.30; 47.18]	204	40.72	[35.48; 46.28]	595	42.14	[38.57; 45.67]
11–14	174	34.84	[30.53; 39.19]	200	35.87	[30.45; 41.08]	502	34.55	[31.33; 37.80]
15–17	94	23.44	[18.30; 28.64]	93	23.41	[18.25; 28.89]	258	23.31	[20.55; 26.07]
Sex									
Boy	242	49.53	[44.33; 54.81]	236	48.31	[43.25; 53.59]	690	49.86	[46.14; 53.55]
Girl	256	50.47	[45.19; 55.67]	261	51.69	[46.41; 56.75]	665	50.14	[46.45; 53.86]
Education level of parent (or legal guardian)									
No secondary school certificate or secondary level vocational training certificate; primary school certificate	145	50.59	[44.38; 56.61]	138	49.11	[43.52; 54.82]	382	49.47	[45.59; 53.32]
Upper secondary school certificate	78	16.98	[13.13; 21.01]	73	17.80	[13.95; 21.68]	217	17.75	[15.44; 20.09]
1st cycle university degree	139	14.90	[11.76; 18.44]	149	15.19	[11.95; 18.60]	366	14.98	[13.05; 16.96]
2nd or 3rd cycle university degree	136	17.53	[13.99; 21.30]	137	17.89	[13.84; 22.47]	387	17.8	[14.91; 20.82]
Parent- perceived financial situation									
I'm comfortable	102	14.27	[10.91; 17.92]	104	15.57	[12.01; 19.13]	272	14.88	[12.60; 17.32]
I get by	187	33.59	[28.53; 38.80]	194	33.17	[28.80; 38.22]	493	32.87	[29.83; 36.04]
It's tight	54	11.17	[7.81; 14.92]	48	10.89	[7.19; 14.97]	140	11.0	[9.03; 13.04]
I need to be careful/I find it difficult to make ends meet/am in debt	155	40.97	[35.47; 46.49]	151	40.37	[34.06; 46.50]	443	41.25	[37.56; 44.84]

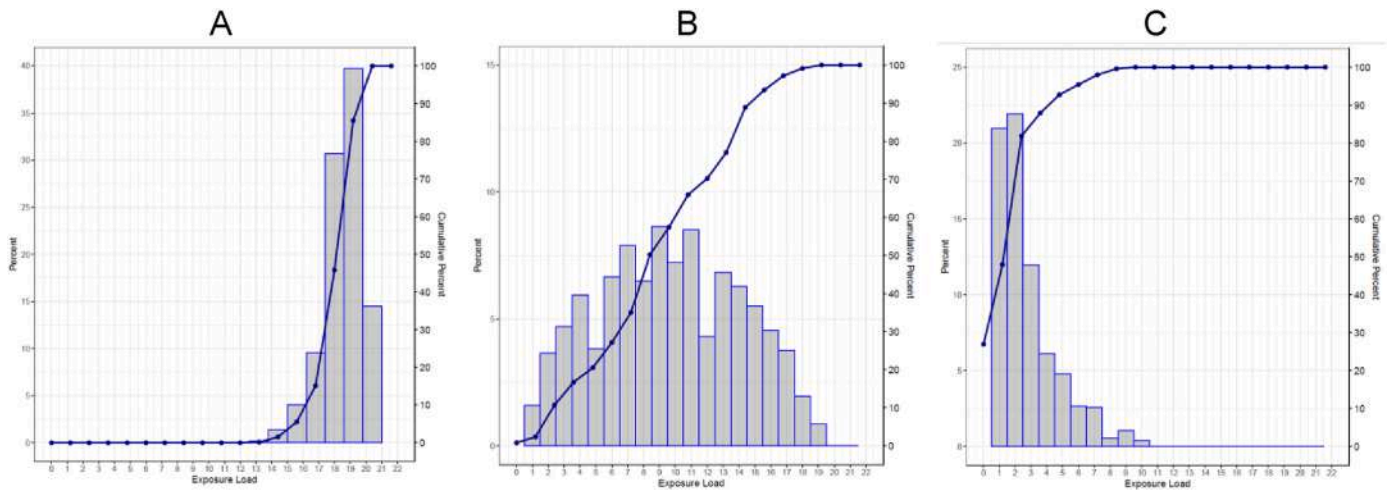

Fig. 1. Distribution of exposure load (EL) in adults for LOQ (A), P50 (B) and P90 (C) as discretization thresholds. The line represents the % of participants (cumulative).

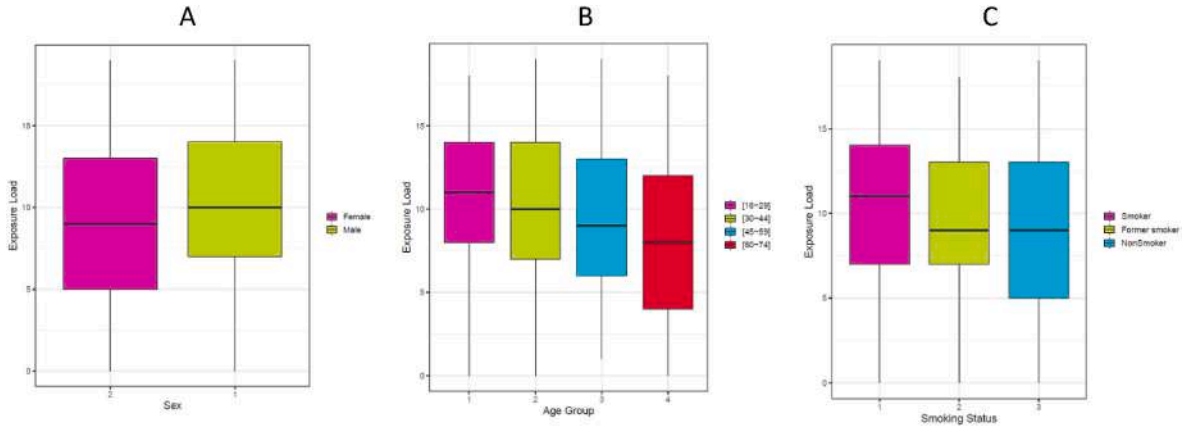

Fig. 2. Boxplot of exposure load in adults using the 50th percentile exposure as the discretization threshold, by sex (A), age (B) and smoking status (C).

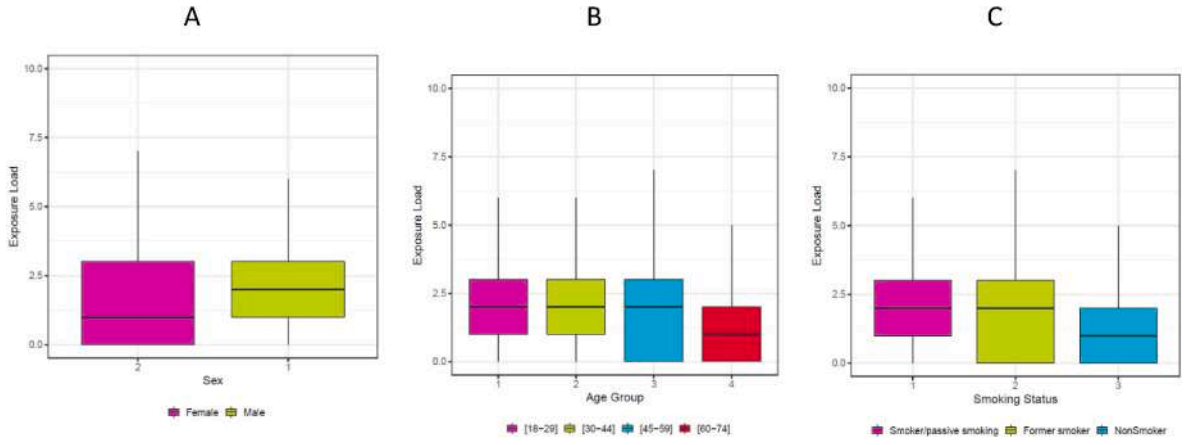

Fig. 3. Boxplot of exposure load in adults using the 90th percentile exposure as the discretization threshold, by sex (A), by age (B) and smoking status (C).

4.1.2. Children

When considering the LOQ as the discretization threshold, the EL distribution was quite narrow. Children had a mean EL of 19.0 (minimum 16, maximum 21) (Fig. 4A). The 10% of children most exposed to multiple chemicals had an EL of 20 (Supplement Table S7).

Just as was observed for adults, the EL distribution was wider when using the 50th percentile exposure as the discretization threshold (Fig. 4B). Mean EL was 9.6 (minimum 0, maximum 19). The 10% of children most exposed to multiple chemicals had an EL of 15 (Supplement Table S8).

At the 90th percentile exposure threshold (Fig. 4C), the EL distribution narrowed again, reflecting what we observed in adults. The

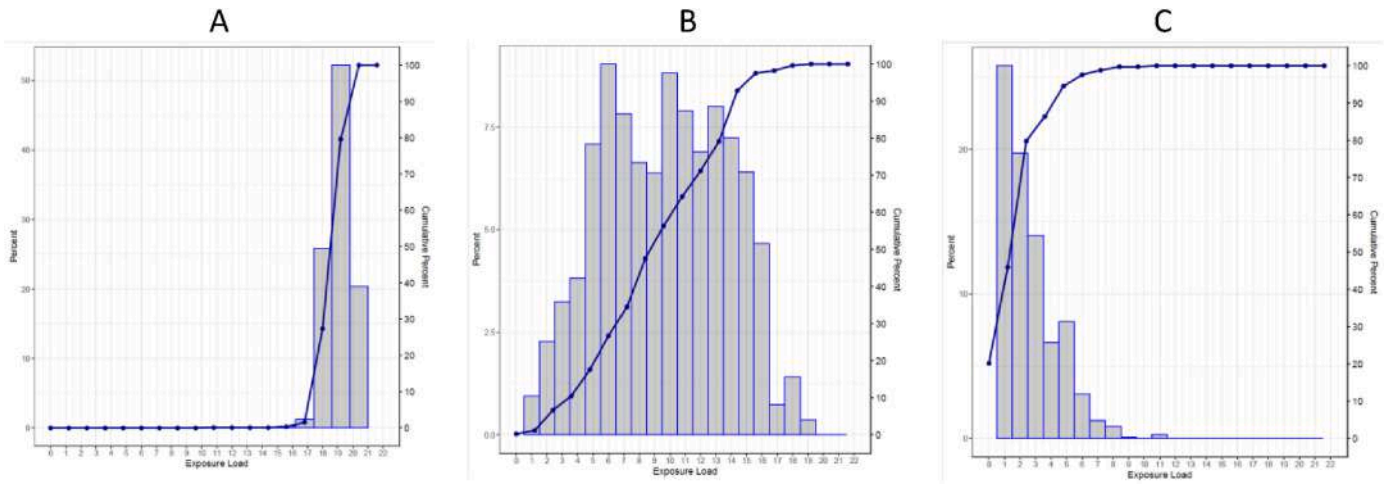

Fig. 4. Distribution of exposure load in children with LOQ (A), P50 (B) and P90 (C) as discretization thresholds. The line represents the % of participants (cumulative).

number of substances to which children are exposed is low at this threshold with a mean EL of 2.1 (minimum 0, maximum 11) (Supplement Table S9). As a comparison, the 10% of adults most exposed to multiple chemicals had an EL of 5. Mixtures of substances were assessed in individuals (N = 9) with the highest multi-chemical exposure (i.e., to at least eight substances) at the P90 threshold. Mixtures of substances were quite homogenous. Phthalates accounted for at least half of the EL, the most frequently found being metabolites of DEHP, MnBP and MiBP. For bisphenols, BPA and BPS contributed to the EL for half of the children. Metals contributed less often to the EL, the most frequent being mercury, nickel, antimony and cobalt.

For children, irrespective of the discretization threshold considered, there was no significant difference in EL according to sex, age, educational level or parents' perception of their financial situation.

4.2. Group B

4.2.1. Adults

When considering the LOQ as the discretization threshold, the EL distribution was quite narrow. Adults had a mean EL of 5.7 (minimum 2, maximum 9) (Fig. 5A). The 10% of adults most exposed to multiple chemicals had an EL of 7 (Supplement Table S4).

Using the 50th percentile exposure as the discretization threshold, the EL distribution was wider (Fig. 5B). The average EL was 3.0 (minimum 0, maximum 6). The 10% of adults most exposed to multiple chemicals had an EL of 6 (Supplement Table S5).

At the 90th percentile exposure threshold, the EL distribution narrowed again (Fig. 5C). The number of substances to which adults are exposed is low at this threshold with a mean EL of 0.79 (minimum 0, maximum 6) (Supplement Table S6). The 10% of adults most exposed to multiple chemicals had an EL of 2. Among all the adults, three had higher EL (5 or 6). They were a legal professional, a salesperson in a sector with no exposure to pesticides, and a professional in the medico-social sector. The pyrethroid metabolites $cisCl_2CA$, $transCl_2CA$ and FPBA were systematically found in all three. Glyphosate, but not its metabolite AMPA, was found in two of them.

At the LOQ, 50th percentile, and 90th percentile thresholds, there was no significant difference according to age, sex, smoking status, consumption of self-produced foodstuffs, consumption of organic food, living near a cultivation area, owning a garden, frequency of housing ventilation, or season when the urine sample was collected.

Using the 90th percentile as the discretization threshold (Fig. 6), participants exposed to pesticides dust in the workplace had an average EL of 1.4 [1.0; 1.9] versus 0.7 [0.6; 0.9] for those unexposed. According to the CI95%, the difference was significant for the mean value.

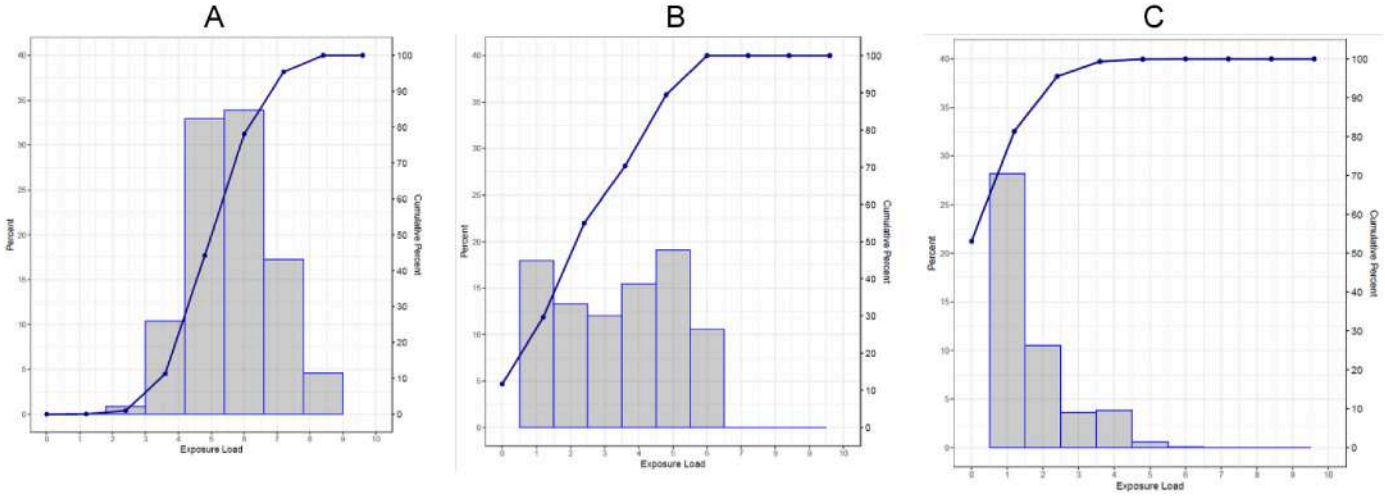

Fig. 5. Distribution of exposure load (EL) in adults with LOQ (A), P50 (B) and P90 (C) as discretization thresholds. The line represents the % of participants (cumulative).

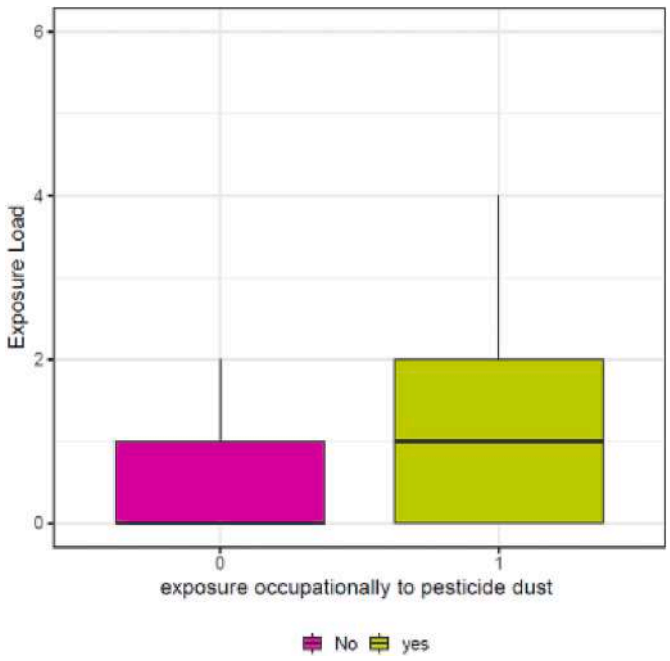

Fig. 6. Boxplot of exposure load using 90th percentile exposure as discretization threshold, according to occupational exposure to pesticide dust.

4.2.2. Children

When considering the LOQ as the discretization threshold, the EL distribution was quite narrow. Children had on average EL of 5.9 (minimum 1, maximum 8) (Fig. 7A). The 10% of children most exposed to multiple chemicals had an EL of 7 (Supplement Table S10).

Just as was observed in adults, the EL distribution was wider when using the 50th percentile exposure as the discretization threshold (Fig. 7B). Specifically, the average was 3.5 (minimum 0, maximum 7). The 10% of children most exposed to multiple chemicals had an EL of 6 (Supplement Table S11).

At the 90th percentile exposure threshold, the EL distribution narrowed again (Fig. 7C). The number of substances to which children are exposed was low at this threshold with a mean EL of 0.9 (minimum 0, maximum 6) (Supplement Table S12). The 10% of children most exposed to multiple chemicals had an EL of 2. Among all the children, four had high EL (i.e., 5 or 6). For these participants, the pyrethroid metabolites cisCl_2CA, transCl_2CA and 3-PBA were systematically found above the P90 threshold; FPBA was identified in three of them. Herbicides (2,4-D and glyphosate or AMPA) were also partly responsible for the high EL in three of these children.

Considering the LOQ as the discretization threshold (Fig. 8), teenagers aged between 15 and 17 years old had a mean EL of 5.6 [5.4; 5.9] versus 5.9 [5.6; 6.1] for children aged between 11 and 14, and 6.2 [6.0; 6.4] for those aged 6–10. The number of adolescents in the 15-17 year-old group was lower (N = 93) than in the other two age groups.

No other significant difference in EL was observed, irrespective of the discretization threshold considered, between children when considering sex, age, consumption of self-produced foodstuffs, consumption of organic food, living near a cultivation area, parents owning a garden, frequency of housing ventilation, and season when the urine sample was collected.

5. Discussion

5.1. Group A

Most individuals were exposed to multiple chemicals at low concentrations. Multi-chemical exposure was generalized since all the adults and children included had an EL greater than or equal to 13 (out of 22 substances studied) when the LOQ was considered as the discretization threshold. As expected, the EL decreased when the discretization threshold increased.

In adults, being male, a smoker and being of working age were associated with a higher EL for the P50 and P90 discretization

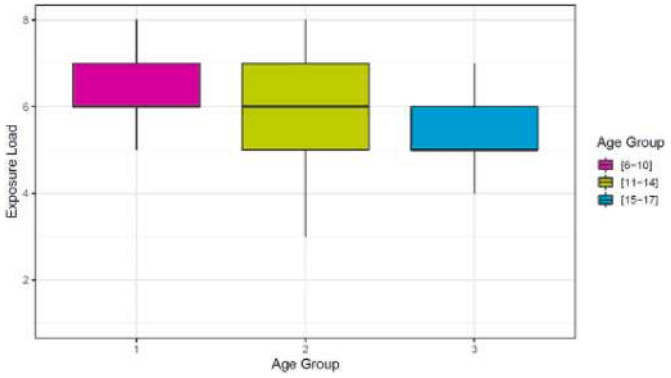

Fig. 8. Boxplot of exposure load in children using LOQ as discretization threshold according to age group (age group 1 = 6–10 years old, age group 2 = 11–14 years old, age group 3 = 15–17 years old).

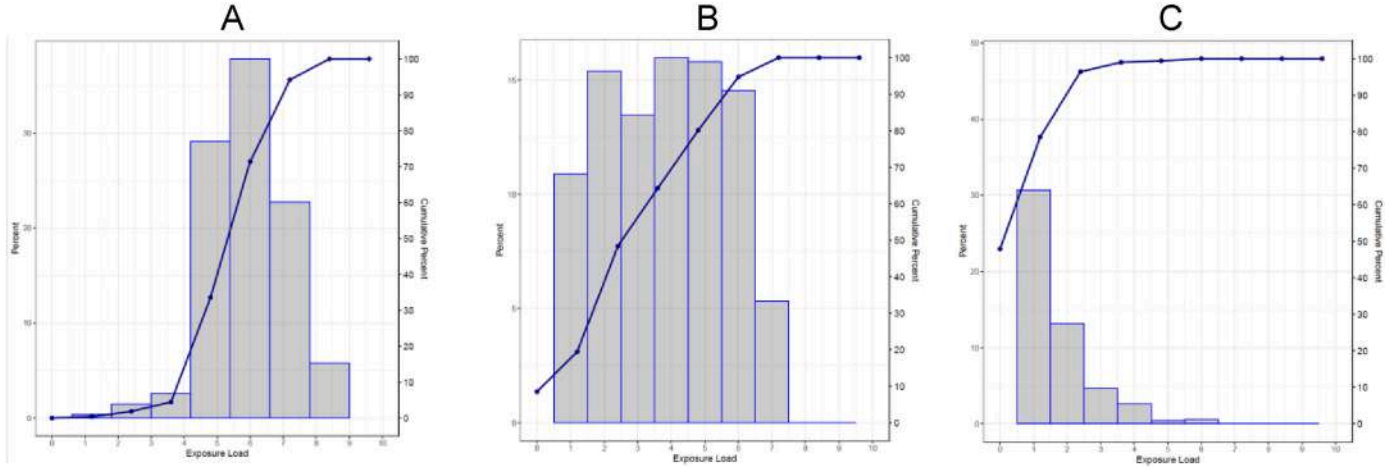

Fig. 7. Distribution of exposure load in children with LOQ (A), P50 (B) and P90 (C) as the discretization thresholds. The line represents the % of participants (cumulative).

thresholds. There is no obvious explanation for the influence of age on EL, because although some substances in Group A were cumulative (lead, arsenic, bisphenols, etc.) others were not (phthalates, etc.). Smokers' exposure to chemicals occurs directly through inhalation of a wide variety of substances contained in cigarettes, but also by frequently bringing one's hands to one's mouth (Kegel et al., 2014). Sex can differentially influence multi-chemical exposure through lifestyle, occupational exposure and physiological parameters (Kennedy and Koehoorn, 2003).

The eleven adults with a high EL value (i.e., 5 or 6) at the P90 threshold were exposed to a very heterogeneous mixture of substances. Their occupations were very different from one another.

Children had EL values of the same order of magnitude as adults. No child subpopulation (i.e., age group, sex, etc.) was identified as particularly at risk of multi-chemical exposure. The nine children with a high EL at the P90 discretization threshold were exposed to a homogeneous mixture of substances (metabolites of DEHP, MnBP, MiBP, BPA and BPS). Particular attention should therefore be given to these substances to improve prevention of exposure.

5.2. Group B

Multi-exposure to pesticides was also generalized since all the adults (for 15 substances tested) and children (for 13 substances tested) included had a mean EL value close to 6 (for 15 substances tested) when the LOQ was considered the discretization threshold. As expected, the EL value decreased as the exposure threshold increased.

Among adults, persons with occupational exposure to pesticide dust had a higher EL for the P90 threshold. This is to be expected since professionals are often in direct contact with pesticides when applying them to plants). In contrast, proximity of one's dwelling to croplands was not associated with higher EL.

For children, the numbers of participants for each modality were often very low for the specific variables for pesticide exposure, which clearly limits interpretation of EL values in terms of these variables.

The EL value did not vary significantly with organic food consumption in adults or children. This may be due to the way the Esteban study information was collected. The modalities for the question regarding consumption of organic food proposed were as follows: 4 to 7 times/week, 1 to 3 times/week, 1 to 3 times/month, and never or less than 1 time/month. It was therefore difficult to distinguish participants with a predominantly organic diet from those who consumed little organic food on a daily basis.

The three adults with a high EL (5 or 6) at the P90 threshold were exposed to a homogeneous mixture of substances (pyrethroid and glyphosate). Particular attention should therefore be given to reducing exposure to these substances.

Children had EL values of the same order of magnitude as adults. No child subpopulation was identified as being at high risk of multi-chemical exposure. The four children with high EL (5 or 6) at the P90 threshold were exposed to a very homogeneous mixture of substances (pyrethroid, glyphosate, AMPA and 2,4-D). Particular attention should therefore be given to these substances to improve prevention of exposure.

5.3. Global

Our calculation of EL did not take into account the toxicity of each of the substances investigated. It is therefore important to consider the number of substances to which people are exposed, but also the chemical composition within multi-chemical exposure. Furthermore, having equal EL values does not necessarily mean that the same composition of chemicals is present or that the concentrations of the different chemicals are the same.

The EL approach is limited by the number of available substances measured in the study. Multi-chemical exposure of children and adults was highlighted for the two groups of substances considered.

The method we used to calculate the EL was based on that established by Willey et al. (2021) using data from the Canadian Health Measures Survey (CHMS), and subsequently adapted by Buekers et al. (2021) using data from the 4th Flemish Environment and Health Study (FLEHS-4). One of the main differences between our study and theirs was that we assessed multi-chemical exposure in subsamples of the general population and in a limited set of chemical groups. This limits our evaluation of multi-chemical exposure in the general French population. Similarly, any comparison of our EL with those determined by other HBM programmes is difficult. However, it should be noted that Willey and al. also found a higher EL in smokers. Similarly, Buekers et al. also highlighted an increase in EL with smoking but only for the P90 threshold (total smokers in young teenagers was limited) (Buekers et al., 2021). With regard to sex, EL levels in Canadians males and females highlighted similar exposure burdens while Flemish boys had higher EL than girls. Similar EL patterns were observed, with Canadians and Flemish studies, between the discretization thresholds: EL distributions at P50 are wider than at P90 and the majority of people had very low EL considering P90 as the discretization threshold. The substances considered had little overlap with those selected by Health Canada and VITO for the calculation of EL.

Performing an assay of all biomarkers in all participants in future French studies would ensure greater accuracy when measuring both chemical exposure burden and multi-chemical exposure. A complete evaluation of multi-chemical exposure is however unlikely, given the very large number of substances which persons are exposed to and the fact that we do not have validated biomarkers for all of them. One possibility would be to create groups of individuals with chemical analyses of substances that may induce the same health effect and to establish EL related to this effect.

Future improvements in biological analyses thanks to new biomarkers could lead to better characterization of the EL. Such developments could also have an impact on the LOQs of substances currently analysed. Finally, lower quantification limits would impact the calculation of the EL at the LOQ threshold and could lead to a higher EL for this discretization threshold. Another possibility would be to define a threshold for each of the substances that would correspond to health-based guidance values in order to better take into account toxicity through EL. This would however be limited to the small number of substances for which we have these health-based thresholds.

6. Conclusion

This study quantified multi-chemical exposure burden of French population using data from the ESTEBAN study. Although EL does not assess the health impacts linked to multi-chemical exposure, it does allow the identification of particularly vulnerable populations. Accordingly, preventive actions specifically aimed at these subgroups could be useful. We highlighted widespread multi-chemical exposure in adults and children in the general population of France, and the major impact of occupational exposure (Group B) and tobacco smoking (Group A) on EL. This result may help guide public health policy when designing interventions to prevent multi-chemical exposure and smoking.

For the children most exposed to the highest concentrations (P90) in group A, multi-chemical exposure to phthalates and bisphenols has been demonstrated. These substances have endocrine disrupting effects that significantly impact children's health. The prevention of exposure to these substances is therefore crucial.

Our study also highlighted that the adults with the highest multi-chemical exposure at the highest concentrations (P90) of pesticides were primarily exposed to pyrethroids and glyphosate. The same was true for children, who were additionally exposed to AMPA and 2,4-D. These substances may therefore require special attention.

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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.2022.114054.

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Cadmium exposure in adults across Europe: Results from the HBM4EU Aligned Studies survey 2014–2020

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ABSTRACT

The objectives of the study were to estimate the current exposure to cadmium (Cd) in Europe, potential differences between the countries and geographic regions, determinants of exposure and to derive European exposure levels. The basis for this work was provided by the European Human Biomonitoring Initiative (HBM4EU) which established a framework for alignment of national or regional HBM studies. For the purpose of Cd exposure assessment, studies from 9 European countries (Iceland, Denmark, Poland, Czech Republic, Croatia, Portugal, Germany, France, Luxembourg) were included and urine of 20–39 years old adults sampled in the years 2014–2021 (n = 2510). The measurements in urine were quality assured by the HBM4EU quality assurance/quality control scheme, study participants' questionnaire data were post-harmonized. Spatially resolved external data, namely Cd concentrations in soil, agricultural areas, phosphate fertilizer application, traffic density and point source Cd release were collected for the respective statistical territorial unit (NUTS). There were no distinct geographic patterns observed in Cd levels in urine, although the data revealed some differences between the specific study sites. The levels of exposure were otherwise similar between two time periods within the last decade (DEMOCOPHES - 2011–2012 vs. HBM4EU Aligned Studies, 2014–2020). The age-dependent alert values for Cd in urine were exceeded by 16% of the study participants. Exceedances in the different studies and locations ranged from 1.4% up to 42%. The studies with largest extent of exceedance were from France and Poland. Association analysis with individual food consumption data available from participants' questionnaires showed an important contribution of vegetarian diet to the overall exposure, with 35% higher levels in vegetarians as

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opposed to non-vegetarians. For comparison, increase in Cd levels due to smoking was 25%. Using NUTS2-level external data, positive associations between HBM data and percentage of cropland and consumption of Cd-containing mineral phosphate fertilizer were revealed, which indicates a significant contribution of mineral phosphate fertilizers to human Cd exposure through diet. In addition to diet, traffic and point source release were identified as significant sources of exposure in the study population. The findings of the study support the recommendation by EFSA to reduce Cd exposure as also the estimated mean dietary exposure of adults in the EU is close or slightly exceeding the tolerable weekly intake. It also indicates that regulations are not protecting the population sufficiently.

1. Introduction

Cadmium (Cd) is widespread in the environment and its presence is a consequence of both natural and anthropogenic sources. Anthropogenic sources include industrial emissions, urban pollution and pollution by cadmium-containing fertilizers and may result in elevated levels of Cd in soil (Nordberg et al., 2015, 2018). Most of agricultural soil contamination occurs by the use of Cd-containing phosphate fertilizers leading to elevated levels of Cd in crops and for example, in France, mineral phosphate fertilizers have been identified as the main source of Cd in agricultural soil in arable farming regions (Carne et al., 2021). Consistent with the very slow turnover of Cd in soils (Nordberg et al., 2018), several studies indicated gradual increases in Cd content in soil after long-term application of mineral phosphate fertilizers (summarized by Park et al., 2021). However, in addition to phosphate fertilizers, contributors to Cd deposition in agricultural soils are also atmospheric pollution, sewage sludge and compost material (EFSA, 2009; Park et al., 2021).

Depending on the Cd mobility in soils (speciation, pH, organic matter, etc.), Cd is taken up by plants which results in increased levels of Cd in food and feeds (EFSA, 2009), and the use of phosphate fertilizers and sewage sludge facilitates the mobilization of Cd in the environment and its accumulation in crops (Nordberg et al., 2018). The primary source of Cd exposure in humans is therefore through ingestion of food crops grown on soils that are either Cd-contaminated or are naturally rich in this metal (Park et al., 2021). In populations with high consumption of rice, the main source of Cd is rice; while cereal products, grains, and root vegetables are important sources in many populations worldwide (EFSA, 2012; Nordberg et al., 2018). Meat and fish normally contain lower Cd levels. Animal offal such as kidney and liver can exhibit high Cd concentrations, as these are the organs in animals in which Cd concentrates, but these are normally consumed in lower quantities (EFSA, 2009, 2012; Nordberg et al., 2018). In addition to diet, smoking is another major source of Cd exposure in the general population as tobacco plants tend to accumulate high levels of Cd in their leaves (Ganguly et al., 2018).

In humans, Cd is widely distributed in the body, where it accumulates over time, with a biological half-life ranging from 10 to 30 years. As Cd is mainly stored in the liver and kidneys (EFSA, 2009; ATSDR, 2012) it affects the kidneys in particular and can cause renal failure after long-term exposure, even at low exposure levels (Nordberg et al., 2018). In addition, Cd is classified as a human carcinogen, which is mainly based on occupational studies of lung cancer. Epidemiological studies in general populations have also reported significant associations with a number of adverse health effects at low exposures, but more evidence is needed in order to establish causality (Nordberg et al., 2018). A recent study showed that at low levels of exposure, Cd can contribute to the risk of osteoporosis, with 28% of cases of osteoporosis in women over 55 years of age being attributable to Cd exposure (Ougier et al., 2021a).

In order to ensure a high level of protection to all consumers, including exposed and vulnerable subgroups of the population, the Panel on Contaminants in the Food Chain within EFSA (2012) proposed a tolerable weekly intake (TWI) of 2.5 μg/kg body weight. Maximum levels of Cd in foodstuffs are set by the Regulation (EC) No. 1881/2006, and furthermore, the use of Cd is restricted in certain products (Annex XVII of REACH), among them recycled PVC, which is currently under review. There is also an ongoing discussion regarding the allowable maximum levels in mineral phosphate fertilizers with a link to maximum levels in food. In spite of improved regulations and guidelines it is not clear whether Cd exposures in human populations have increased, decreased, or stayed unchanged in the last decades, with varying trends being reported for different regions or countries (summarized by Nordberg et al., 2018).

The European Human Biomonitoring Initiative (HBM4EU) provided a framework for alignment of national and regional studies based on existing HBM capacity and the development of new capacities. Cd was included as one of the priority substances (Gilles et al., 2021, 2022) identified based on the needs of EU institutions, participating countries and stakeholders (Ougier et al., 2021b). In line with the identified knowledge gaps, the objective of the present study was to assess the current exposure to Cd in Europe, potential differences between the countries and/or regions in Europe and to evaluate whether the HBM values reflect environmental levels (Cd in soil) or not, particularly whether Cd variability in soil, and consequentially food, explains Cd variability in HBM data.

2. Methodology

2.1. Study design and sampling frame

The study is part of the European Human Biomonitoring Initiative (HBM4EU) conducted in 21 European countries, the so-called HBM4EU Aligned Studies. It builds on existing HBM capacity in Europe by aligning national or regional HBM studies targeting the general population (Gilles et al., 2021). The studies were aligned with respect to the sampling period (2014–2021), age groups (children, teenagers and young adults), sampling size (240–300 per study) with a 1:1 male to female ratio, biomarkers of interest, and questionnaire data available per age group. Residents in hotspots, patient groups, or specific occupational groups were excluded. Studies of all 4 geographical regions of Europe according to the United Nations geo-scheme (North, West, South, East) were included, with at least two countries per region. Key aspects of recruitment, sampling, questionnaire development and sample transport were quality assured following the standard operating procedures for each study phase, which were developed within the HBM4EU project. Details on the framework of the HBM4EU Aligned Studies and the approach that has been applied to align European HBM initiatives across Europe are provided by Gilles et al. (2021, 2022).

For the purpose of the present paper, data on adults (20–39 years of age) of the following studies were used: CPHMINIPUB (parents)/DYMS (Denmark, n = 292), Diet_HBM (Iceland, n = 203), (C)ELSPAC: YA (Czech Republic, n = 300), POALES (Poland, n = 228), HBM in Croatia (Croatia, n = 300), INSEF-ExpoQuim (Portugal, n = 295), ESTEBAN (France, n = 393), Oriscav-Lux2 (Luxembourg, n = 210), and ESB (Germany, n = 289). Detailed information for each study is provided by Gilles et al. (2022). Cadmium concentrations were available in first morning (n = 1288), random spot (n = 933) and 24 h urine samples (n = 289) (Table 1).

2.2. Chemical analysis

According to the framework of the HBM4EU Aligned Studies, determination of Cd in urine samples was performed in laboratories that successfully passed the HBM4EU quality assurance quality control (QA/QC) program (Esteban López et al., 2021; Nübler et al., 2021), except for the ESTEBAN study (France), for which measurements were performed before the establishment of the HBM4EU QA/QC program and were therefore not quality assured by HBM4EU. Seven laboratories were involved in the determination of Cd concentrations in urine samples for the presented study and all of them used Inductively Coupled Plasma Mass Spectrometry (ICP-MS) for the measurements. More details on the analytical procedures used by different laboratories are provided in Table 2. Limit of detection (LOD) ranged from 0.002 μg/L to 0.01 μg/L (not available for Luxembourg and Germany studies) and limit of quantification (LOQ) between 0.0016 μg/L and 0.1 μg/L.

Creatinine concentrations in urine were available for all data collections (n = 2500), however specific gravity was only available for one data collection (Czech study, n = 300), and the latter was therefore not considered for normalization of urinary Cd concentrations in the present study. All urine samples had creatinine levels above 5 mg/dL limit set by Lauwerys and Hoet (2001) for biomonitoring in the U.S. workplace to exclude too diluted samples for the screening of selected drugs of abuse. According to the WHO criteria for valid urine samples for occupational monitoring (WHO, 1996), the number of samples with creatinine levels below the lower limit of 30 mg/dL were 77 (3.5%), and the number of samples above the higher limit of 300 mg/dL were 92 (4.2%).

2.3. Questionnaire and ancillary data

Accompanying data were available from the questionnaires and spatially resolved data extracted from the available European databases. Since the HBM4EU Aligned Studies aligned both new, ongoing and recently conducted studies, a post-harmonization approach was applied to harmonize the collected questionnaire data.

Questionnaire data included personal information (sex, age, educational level, country of birth), socio-demographic information on the participant (socio-economic status - income, current occupation status, occupational sector and occupational activities), information on residential environment (country of residence, NUTS level 1, 2, and 3, type of residence/degree of urbanization, residential history, density of traffic in the residential area, farmlands, orchards or vineyards in vicinity, frequency of dusting and vacuuming), information on dietary habits (vegetarian diet, consumption of local food, seafood, fish, meat, poultry, vegetable and fruit, offal and cereals, type and source of drinking water), lifestyle information (smoking status, number of cigarettes smoked per day, passive smoking exposure), personal care and health (height, weight, body mass index, chronical illness, physical activity, and pregnancy status and parity if the subject was female). Educational level was used as a surrogate for socio-economic status. The classification was based on the International Standard Classification of Education (ISCED) (Gilles et al., 2021).

Additional supporting data for the statistical analyses were obtained from various data sources available at European level. Two sources with different spatial resolution were used for Cd distribution in soil. Distribution of Cd in topsoil mapped based on the FOREGS (Forum of European Geological Surveys) geochemical database with 5 km resolution (Lado et al., 2008), and a map of Cd concentration in the topsoil of the European Union produced based on the LUCAS (Land Use/Land Cover Area Frame Survey) data and available in 1 km resolution (Tóth et al., 2013, 2016). Moreover, the following ancillary data were extracted from the statistical office of the European Union (Eurostat): percentage of agricultural areas and cropland, respectively; annual application of phosphate fertiliser reported as consumption by countries or estimated in tonnes; and the density of motorways and other road network in km per km^2. The data were extracted for the year(s) most closely corresponding to the year of conduct of the respective study. Annual releases of Cd to air and water from industrial facilities located within the individual NUTS regions as reported in the European Pollutant Release and Transfer Register (E-PRTR) (https://prtr.ec.europa.eu/) in the period 2008–2015 were also considered. All data were extracted and assigned to the HBM data at NUTS unit level at the finest resolution possible, depending on the spatial resolution of the source data (EUROSTAT, 2018). Visualization of the acquired external data is provided in the Supplemental material (Fig. S1).

2.4. Statistical analysis

In case of Cd levels below LOD/LOQ, random values were imputed between 0 and LOD, between 0 and LOQ, or between LOD and LOQ (depending on the values reported by the data provider) using a truncated lognormal distribution. Urinary Cd concentrations were standardized by creatinine as estimator of urine density, as it was available for all studies. The descriptive statistics (N, geometric mean (GM) and 95% confidence intervals (CIs) and percentiles P05, P10, P25, P50, P75, P90, P95) were calculated using unadjusted data for each country. The levels are reported per volume (μg/L) and standardized for creatinine (μg/g creatinine). As the studies differed in the type of urine sample (Table 1), creatinine-standardized Cd concentrations were used in data interpretation. European exposure values were calculated using survey procedures to take into account the complex survey design when calculating variance estimates (Park and Lee, 2004), for the pooled population, and stratified by sex, educational level, smoking, degree of urbanization and geographic region (North, South, East, West). We calculated the GM and 95th percentile (P95), and their 95% confidence interval. The geometric mean and its confidence limits were obtained by

Table 1
Basic information for the participating studies.

Country	Study	N	Representativeness	Sample type	Reference
Denmark	CPHMINIPUB (parents)/DYMS	292	Regional	Spot random	Busch et al. (2021)
Iceland	Diet_HBM	203	National	Spot random	–
Czech Republic	(C)ELSPAC: YA	300	Regional	First morning	Piler et al. (2017)
Poland	POALES	228	Regional	Spot random	–
Croatia	HBM in Croatia	300	National	First morning	–
Portugal	INSEF-ExpoQuim	295	National	First morning	–
France	ESTEBAN	393	National	First morning	Balicco et al. (2017); Fillol et al. (2021)
Luxembourg	Oriscav-Lux2	210	National	Spot random	Alkerwi et al. (2019)
Germany	ESB	289	Regional	24 h	Kolossa-Gehring et al. (2012); Lemke et al. (2021); Lermen et al. (2014)

CPHMINIPUB(parents)/DYMS = Copenhagen Minipuberty study (parents)/Danish Young Men Study; Diet_HBM = Icelandic National Dietary Survey; (C)ELSPAC:YA = Central European Longitudinal Studies of Parents and Children: Young Adults; POALES = Polish Aligned Environmental Study; HBM in Croatia = Human biomonitoring survey in adults in Croatia; INSEF-ExpoQuim = Exposure of the Portuguese Population to Environmental Chemicals: a study nested in the 1st Portuguese National Health Examination Survey (INSEF) conducted in 2015; ESTEBAN = Etude de santé sur l'environnement, la biosurveillance, l'activité physique et la nutrition; Oriscav-Lux2 = Observation des Risques et de la Santé Cardiovasculaire au Luxembourg; ESB = Environmental Specimen Bank.

Table 2
Basic information on the chemical analytical procedures used in different laboratories.

Country of the study	Urine dilution ratio	Isotopes used for quantification	Analysis mode	LOD (μg/L)	LOQ (μg/L)
Denmark	1:9	^{111}Cd, ^{113}Cd, ^{114}Cd	Helium-mode	0.015	0.05
Iceland	1:9	^{111}Cd	Collision cell, Helium	0.01	0.03
Czech Republic	1:9	^{111}Cd	Collision cell, Helium	0.004	0.013
Poland	1:9	^{114}Cd	DRC, methane	0.004	0.008
Croatia	1:9	^{111}Cd	Collision cell, Helium	0.004	0.013
Portugal	1:4	^{111}Cd	Standard mode	0.0075	0.025
France	1:9	^{103}Rh, ^{202}Hg	Standard mode	0.002	0.005
Luxembourg	1:9	^{111}Cd	Collision cell, no gas and Helium	–	0.1
Germany	4:15	^{111}Cd, ^{113}Cd, ^{114}Cd	Collision cell, no gas and Helium	–	0.0016–0.0184

LOD = Limit of detection for cadmium in urine; LOQ = Limit of quantification for cadmium in urine; DRC = Dynamic Reaction Cell.

taking the antilog of the estimated mean and its upper and lower confidence limit of the log-transformed biomarker values. European exposure values for cadmium were calculated in μg/L and in μg/g creatinine.

With relevance to health risk, we calculated proportion of study population which exceeded the available health-based guidance levels (HBM GVs) for Cd in urine. We used HBM-I value (1 μg/L), which was derived by the German HBM Commission and presents the concentration below which there is no risk for adverse health effects, and consequently, no need for action (Apel et al., 2017). Because HBM-I is based on the kidney effects in women above 50 years of age, the HBM4EU produced age-dependent values which considered accumulation of Cd in the human body: 0.2 μg/g crt (11–20 years of age), 0.3 μg/g crt (21–30 years of age), 0.5 μg/g crt (31–40 years of age) and 0.8 μg/g crt (41–50 years of age) (Lamkarkach et al., 2021).

For geographic comparison survey procedures were used as well, with linear regression adjusting for main characteristics of the study population that could be of influence on the observed exposure values and differ between the countries. Namely these are creatinine, age, sex, smoking, educational level, sampling year and urine sample type (first morning, random spot or 24 h urine).

In order to identify determinants of cadmium exposure, Cd concentrations in urine were studied in relation with possible determining factors using analysis of variance (ANOVA), bivariate linear regression and multiple mixed linear regression models, with country as a random factor. For this purpose, ln-transformed imputed Cd data standardized for creatinine were used. Creatinine was additionally forced into the regression model. Sensitivity analysis was done for variables in a limited set of studies (e.g. applying the WHO creatinine exclusion criteria; exclusion of smokers; subsets of data with availability of specific dietary variables). The obtained linear regression coefficients were reverse log transformed and were therefore expressed as the fold change in Cd concentration for unit increase of the covariate. Regression diagnostics of the final models included linearity, normality, multicollinearity, and independence.

Biomarker data that did not successfully pass the HBM4EU QA/QC program (ESTEBAN, France), was excluded from the calculation of European exposure values and geographical comparison. However, it was included in the calculation of HBMGVs exceedance proportion and analysis of exposure determinants.

Statistical analysis was performed using STATA SE 12.0 and R software 4.1.2. Spatial analyses and visualizations were performed using the QGIS geographic information system application version 3.22.7.

3. Results

3.1. General characteristics of the study population

Studies selected for the present work were conducted between 2014 and 2021 (Table 3), with only four participants (from Iceland) sampled in 2021. The majority of the participants were sampled in 2017, 2018 and 2019 (n = 413, 449 and 787, respectively), followed by year 2020 (n = 309), 2015 (n = 259), 2014 (n = 145) and 2016 (n = 144). There were some differences according to the four geographic regions - the studies from the North covered the period 2017–2021, studies from the East years 2017 and 2019, South 2019–2020, and West 2014–2018. Overall, the sampling campaigns were more or less evenly distributed across different seasons, however, there were certain differences between the countries and regions (Table 3). Males and females were also more or less equally represented, with exception of the Polish study, where almost 70% of participants were women. The age of the participants ranged between 20 and 39 years and it was more or less evenly distributed between countries and regions. The lowest mean age was in the German study (24 years), and the highest in the Portuguese (almost 35 years) (Table 3).

Most of the participants lived in cities (overall 65%), which was the case in seven out of nine studies and in all four geographic regions. Among all study participants, 18% reported to be smokers. The percentage was lowest in Iceland (7%) and highest in Croatia (30%), and according to the regions lowest in the North and highest in the South. As usually experienced in HBM studies, the education of the study participants was skewed towards higher levels (level 5 or higher, according to the International Standard Classification of Education, ISCED). The skewness was less obvious in the Portuguese study (Table 3).

3.2. Cadmium levels in urine

The concentrations of Cd in urine samples in the pooled European population and in each participating country are presented in Table 4. Overall, there were 16 samples (0.6%) with Cd levels below the given limits of detection, and 85 (3.4%) below the given limits of quantification (both LOD and LOQ for the respective study are provided in Table 2). Among the latter, 54 values were reported to be between the LOD and LOQ. Although the measurements for the Luxembourg study showed markedly higher LOQ than other studies, they were not excluded from the data analysis as the number of participants below the LOQ was sufficiently low (7%) and lower than P50 of all participating studies.

Comparing unadjusted Cd levels in urine among the participating countries (Table 4), the lowest mean urinary Cd level was observed in the Portuguese study (GM 0.09 μg/g crt, 95% CI 0.09–0.10 μg/g crt), and did not differ statistically from the levels in the Danish (0.10 μg/g crt, 0.09–0.11 μg/g crt) and Czech studies (0.10 μg/g crt, 0.10–0.11 μg/g crt) ($p = 1.000$ for all comparisons, according to the ANOVA Bonferroni adjustment). The highest mean levels were observed in the Polish (0.36 μg/g crt, 0.34–0.39 μg/g crt) and French studies (0.39 μg/g crt, 0.36–0.42 μg/g crt), and the two did not differ statistically ($p = 1.000$, according to the ANOVA Bonferroni adjustment). The levels did not differ statistically also between Denmark, Czech and Iceland, and between Iceland and Croatia (all cross-comparisons $p = 1.000$, except Denmark vs. Iceland $p = 0.464$). All other differences were statistically significant ($p < 0.05$).

Mean concentrations of Cd in urine on the level of NUTS2 geographic units are presented in Fig. 1. The tendency toward higher urinary Cd levels in the studies from East and West can be seen from the raw

Table 3
General characteristics of the study population.

Country of the study	Sampling year	Sampling season (%)				Sex (%)		Age (years)		Degree of urbanization (%)			Smoking (%)		ISCED (%)		
		Spring	Summer	Autumn	Winter	Female	Male	Mean	Min-max	Cities	Towns/ suburbs	Rural	No	Yes	Low (ISCED 0–2)	Medium (ISCED 3–4)	High (ISCED ≥5)
ALL	2014–2021	16.3	22.8	38.3	22.6	53.8	46.2	30.7	20–39	64.9	17.45	17.6	82.0	18.0	5.0	27.7	67.3
Denmark	2017–2019	23.3	42.8	32.2	1.4	41.8	58.2	30.3	20–39	90.4	8.6	1.0	90.6	9.4	11.6	23.5	64.9
Iceland	2019–2021	0.0	35.0	52.7	12.3	56.2	43.8	30.8	20–39	76.4	11.6	12.1	93.0	7.0	6.0	29.5	64.5
Czech Republic	2019	39.7	29.7	26.7	4.0	51.7	48.3	27.3	20–37	74.7	10.4	14.9	87.9	12.1	0.7	23.4	75.9
Poland	2017	0.0	0.0	100.0	0.0	69.3	30.7	33.5	20–39	100.0	0.0	0.0	86.8	13.2	0.0	41.7	58.3
Croatia	2019–2020	0.0	0.0	59.7	40.3	53.0	47.0	30.6	20–39	58.0	13.7	28.3	69.7	30.3	0.3	36.0	63.7
Portugal	2019–2020	5.4	51.5	25.4	17.6	58.0	42.0	34.6	28–39	27.1	37.0	35.9	74.9	25.1	20.0	36.3	43.7
France	2014–2016	21.1	19.9	34.4	24.7	55.0	45.0	32.3	20–39	45.0	28.5	26.5	70.9	29.1	2.3	29.5	68.2
Luxembourg	2016–2018	20.5	27.1	30.0	22.4	52.9	47.1	33.6	25–39	19.1	45.2	35.7	82.9	17.1	4.8	32.9	62.4
Germany	2014–2018	27.7	0.0	0.0	72.3	49.8	50.2	24.1	20–29	100.0	0.0	0.0	90.0	10.0	0.0	0.0	100.0
Region																	
NORTH	2017–2021	13.9	39.6	40.6	5.9	47.7	52.3	30.5	20–39	84.7	9.8	5.5	91.6	8.4	9.3	26.0	64.7
EAST	2017–2019	22.5	16.9	58.3	2.3	59.3	40.7	30.0	20–39	85.9	5.8	8.3	87.4	12.6	0.4	31.3	68.3
SOUTH	2019–2020	2.7	25.5	42.7	29.1	55.5	44.5	32.6	20–39	42.7	25.2	32.1	72.2	27.8	10.1	36.1	53.8
WEST*	2014–2018	24.7	11.4	12.6	51.3	51.1	48.9	28.1	20–39	65.9	19.0	15.0	87.0	13.0	2.0	13.8	84.2

Remarks: NORTH: Denmark, Iceland; EAST: Czech Republic, Poland; SOUTH: Croatia, Portugal; WEST: Luxembourg, Germany; *ESTEBAN study (France) excluded for calculation European exposure values and geographical comparison.

(unadjusted) data, with some distinct regional differences within France (Fig. 1a). However, the concentrations adjusted for basic influencing factors (sample type, sex, age, smoking, education and sampling year) showed more unified concentrations across Europe, with smaller differences in GMs between the respective study areas (Fig. 1b). The adjusted GMs were 0.14 μg/g crt and 0.13 μg/g crt in Denmark and Iceland, respectively; 0.15 μg/g crt in Luxembourg; 0.22–0.24 μg/g crt in France; 0.19–0.22 μg/g crt in Germany; 0.149 μg/g crt in Poland and 0.168 μg/g crt in Czech; 0.16–0.17 μg/g crt in Croatia; and 0.20–0.21 μg/g crt for Portugal.

In accordance with the unadjusted mean Cd levels, the highest proportion of study participants exceeding the HBM-I value of 1 μg/L (Apel et al., 2017) was observed in the Polish and French studies (10.1% and 8.7%, respectively). In other countries, the percentage spanned between 0% and 2.4%, and the overall exceedance in the pooled population was 2.9% (Table 4). The age-dependent values (Lamkarkach et al., 2021) for relevant age groups of our study participants were exceeded by 16.4% participants of the total (pooled) population. The highest proportion of exceedances was again observed in Poland (33%) and France (43%), but also in Germany (36%). In one area in France, the percentage exceeded 50%. In other countries, the exceedance spanned between 1.4% (Denmark) and 8.7% (Czech Republic) (Fig. 2, Table 4).

If smokers were excluded from the study population (data not shown), the exceedance percentage was only slightly lower (15.3% in the pooled database), the highest was in France (39%), followed by Germany (35%) and Poland (33%). In the rest of the studies, the percentage of exceedances in non-smokers ranged from 0.4% in the Danish to 9.6% in the Czech study.

3.3. European exposure values

For derivation of European exposure values, we used survey design data analysis with country as a primary sampling unit (PSU). Because of the fact that the biomarker data in the French study was not quality assured by HBM4EU, this study was excluded from the calculation of European exposure values. The calculated levels are expressed per volume (μg/L) as well as per creatinine (μg/g creatinine) in order to make comparison with exposure values from other studies worldwide possible.

3.4. Geographic comparison

In order to compare the four geographic regions (North, South, West, East), survey data analysis was used as well (country = PSU), and additionally the models were adjusted for sample type, creatinine, sex, age, smoking, education and sampling year in order to take into account basic factors that could influence the difference in exposure levels between the countries/regions. According to linear regression, the difference between Western and Northern studies in adjusted estimated Cd levels in urine (μg/g crt) was 1.53-fold, and between Western and Southern studies 1.65-fold, however, the adjusted levels did not differ significantly between the regions ($p = 0.157$ and $p = 0.168$, respectively). Similarly, the estimated levels for the studies in Eastern Europe did not differ significantly from the Northern (1.42-fold difference, $p = 0.296$) and Southern studies (1.53-fold difference, $p = 0.265$). The difference between the West and East was 1.08-fold ($p = 0.832$), and the same between the North and South (1.08-fold, $p = 0.804$). The adjusted GMs with 95% CI are shown in Fig. 3.

3.5. Determinants of exposure

3.5.1. Basic determinants of exposure

The main influencing factors relevant for Cd exposure that were identified prior to the study were firstly checked for statistical significance and trends in the bivariate analysis (data not shown). In the second step, the influencing factors were included in the study-specific

Table 4
Cadmium concentration in urine in μg/L and μg/g creatinine in the pooled European database (All) and in each of the participating studies, indicated by the country names.

	N	N < LOD	N < LOQ	GM	95% CI	Min-Max	P5	P10	P25	P50	P75	P90	P95	N > HBMGV
Cd in urine (μg/L)														[a]**HBM-I**
Denmark	292	4	45	**0.123**	0.112–0.350	<LOD-1.39	0.033	0.041	0.075	0.127	0.217	0.325	0.433	3
Iceland	203	0	11	**0.135**	0.119–0.153	0.010–1.38	0.029	0.040	0.080	0.150	0.250	0.410	0.530	2
Czech R	300	0	0	**0.132**	0.122–0.142	0.019–0.801	0.046	0.053	0.087	0.138	0.205	0.303	0.336	0
Poland	228	0	0	**0.408**	0.369–0.450	0.021–2.67	0.115	0.158	0.261	0.435	0.705	1.01	1.16	23
Croatia	300	0	0	**0.175**	0.160–0.192	0.013–2.10	0.048	0.066	0.106	0.177	0.298	0.467	0.722	5
Portugal	295	12	14	**0.109**	0.098–0.120	<LOD-0.760	0.028	0.039	0.076	0.120	0.170	0.290	0.400	0
France	393	0	0	**0.365**	0.340–0.391	0.053–2.98	0.115	0.153	0.229	0.352	0.591	0.890	1.20	34
Luxembourg	210	–	15	**0.316**	0.288–0.347	<LOQ-1.30	<LOQ	0.120	0.210	0.350	0.520	0.675	0.890	5
Germany	289	–	0	**0.199**	0.186–0.213	0.053–1.11	0.077	0.096	0.131	0.200	0.285	0.451	0.593	2
Cd in urine (μg/g creatinine)														[b]**Age-dependent HBMGVs**
Denmark	282	4	45	**0.101**	0.094–0.109	<LOD-0.700	0.042	0.051	0.066	0.102	0.141	0.209	0.266	4
Iceland	203	0	11	**0.118**	0.109–0.128	0.026–0.758	0.051	0.059	0.075	0.116	0.172	0.231	0.316	7
Czech R	300	0	0	**0.104**	0.095–0.114	0.010–1.28	0.025	0.034	0.061	0.106	0.180	0.280	0.353	26
Poland	228	0	0	**0.364**	0.340–0.390	0.066–1.48	0.164	0.187	0.254	0.362	0.520	0.684	0.856	76
Croatia	300	0	0	**0.123**	0.114–0.133	0.017–0.785	0.042	0.050	0.080	0.125	0.189	0.277	0.361	9
Portugal	295	12	14	**0.094**	0.086–0.103	<LOD-0.771	0.023	0.041	0.064	0.104	0.147	0.218	0.280	4
France	393	0	0	**0.389**	0.360–0.420	0.055–8.86	0.123	0.155	0.241	0.369	0.598	1.08	1.71	168
Luxembourg	210	–	15	**0.183**	0.170–0.197	<LOQ-0.788	0.066	0.091	0.134	0.187	0.258	0.349	0.395	12
Germany	289	–	0	**0.267**	0.253–0.282	0.097–2.32	0.134	0.150	0.194	0.258	0.347	0.526	0.643	105

Remark: GM – geometric mean; CI – confidence interval; P5 – 5th percentile, P10 – 10th percentile, etc.; HBMGV – health-based guidance value.
[a] HBM-I value: concentration below which there is no risk for adverse health effects, and consequently, no need for action (Apel et al., 2017).
[b] Age-dependent alert values were derived by HBM4EU (Lamkarkach et al., 2021) to prevent exceeding the guidance value of 1 μg/g creatinine (crt) at later age (>50 years) and are based on kidney toxicity as critical target. Namely these are 0.2 μg/g crt for 11–20 years, 0.3 μg/g crt for 21–30 years and 0.5 μg/g crt for 31–40 years old adults.

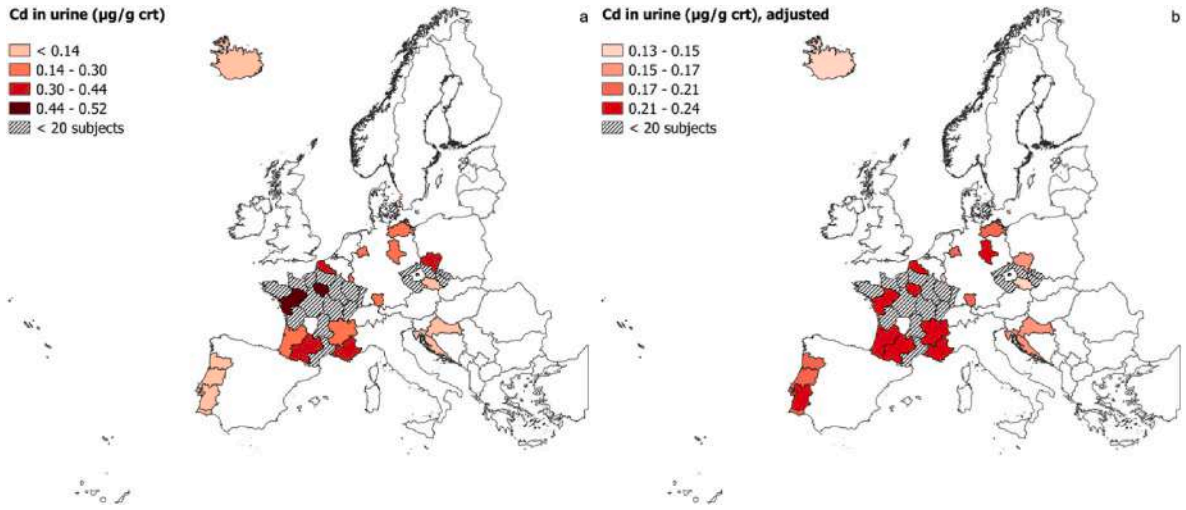

Fig. 1. Geometric means (GM) for Cd in urine (μg/g creatinine) per NUT2 area: (a) non-adjusted (n = 2500) and (b) adjusted for the main influencing factors (sample type, sex, age, smoking, education and sampling year) (n = 2088). GMs are given only for those NUTS2 with 20 or more participants.

multiple regression models (Table S2). The models revealed higher levels in women than in men and increasing Cd urinary levels with age in the majority of the studies. However, in the Polish study, association with sex and age was not significant (p = 0.119 and 0.178, respectively). Higher levels of urinary Cd in smokers than in non-smokers were observed only in three studies (Portugal, p < 0.001, France, p < 0.001, and Luxembourg, p = 0.022). Decrease in Cd levels with higher educational level was observed only in the Portuguese study, where the most obvious difference was between the low level of education and the other two levels (p = 0.009 and 0.059, respectively). With regard to the sampling year, the increasing trend in Luxembourg (p = 0.001) and decreasing trend in Germany (p = 0.002) were confirmed. In the other studies, no significant differences or trends were revealed with regard to the educational level or year of sampling, which might be due to unevenly distributed educational level and narrow sampling time range (Table 2). All study-specific models, except Polish, were highly statistically significant (p < 0.001) with 19%–34% of variability explained by the variables in the model. The model for the Polish study was insignificant (p = 0.103, R^2 = 0.04) (Table S2).

3.5.2. *Food-related determinants of exposure*

In addition to the basic influencing factors, some potential explanatory variables were checked for association with Cd levels in urine in a study-specific manner. They are summarized in Table S1. These variables were added to the basic models, presented in Table S2. Among the available variables, vegetarian food was observed as positively and at least marginally significantly associated with Cd levels in urine in the Czech and German studies (p = 0.071 and < 0.001, respectively).

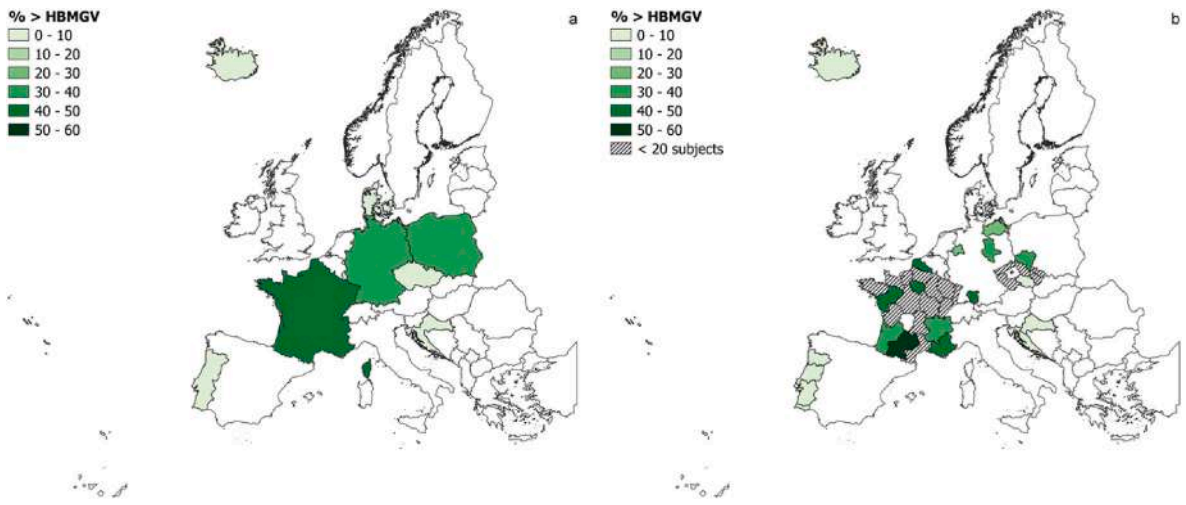

Fig. 2. Exceedance of age-dependent HBM-GVs in percentage of study population (a) per country and (b) per NUTS2. Percentages are given only for those NUTS2 with 20 or more participants.

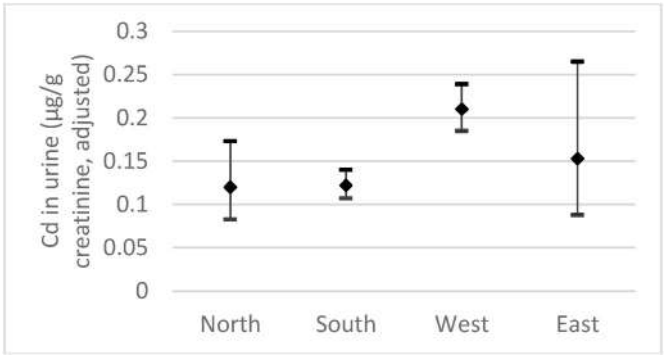

Fig. 3. Geographical comparison of adjusted Cd levels in urine (µg/g creatinine): GMs and 95% CI. The levels are adjusted for creatinine, sample type, sex, age, smoking, education and sampling year. Survey design was used (PSU = country). The French study (ESTEBAN) was excluded from the geographic comparison, because biomarker data was not assured by HBM4EU QA/QC.

Consumption of cereals was significantly associated with urine levels in the Polish study ($p = 0.028$). A significant trend was revealed also for organ meat consumption in the studies from Croatia and Luxembourg, but the trend was negative. However, it has to be noted, that there was limited availability of food-related data across different studies (Table S1). Frequency of local food consumption was available only for the Croatian study, and frequency of cereals consumption for the Icelandic, Czech, Polish, Croatian, and Luxembourg studies. Most covered was data for vegetarian diet, available in studies from Iceland, Czech Republic, Croatia, Portugal, France and Germany. Among these, very few participants reported to be vegetarians (3%), with the highest percentage in the German (7%), and the lowest in the Portuguese (0.7%) and French (1%). Organ meat consumption was available in studies from Czech Republic, Poland, Croatia, France, Luxembourg and Germany. Along with the dietary data, drinking water data was checked against Cd levels in urine, namely source (public, private well) and type of drinking water consumed by the subject (bottled, tap, ground or other type of drinking water). Marginal significance was found only in the French data, where the participants drinking water from 'other' sources had higher urinary Cd levels than those drinking bottled or tap water (Table S2).

3.5.3. Traffic-related determinants of exposure

As a potential traffic-related source of exposure, density of traffic in the residential area (no traffic, light traffic, intense traffic) was available from the questionnaire data for the Croatian and French studies. However, it was not found to be significantly associated with Cd levels in urine in none of the two studies (data not shown). Degree of urbanization was further checked in the models as a proxy for traffic density, and although available for all studies, it did not show any significance in relation to Cd levels in urine.

3.5.4. Pooled multilevel mixed model

In the last step, a multiple regression model was built for the pooled European population, which included all nine studies (Model 1, Table 6). Sex, age and smoking were confirmed to be highly statistically significantly associated with Cd levels in urine. The levels in women were 33% higher than in men, and they increased by 3% each year of life within the age range of the study participants. Smokers had 25% higher levels than non-smokers. Additionally, the levels were 14% lower in the participants with medium or high educational level in comparison to the ones with low level of education. With regard to the sampling year, a slight decreasing trend, 4% each year, was observed (Model 1, Table 6). As the participating studies differed in type of the urine sample collected, this factor was also included in the pooled model to control for potential influence of the sample type. Cadmium concentrations in daily and morning urine samples were somewhat higher than in spot urine samples, and concentrations in daily urine higher than the ones in morning urine, but none of the differences was statistically significant ($p = 0.214$, 0.126 and 0.260, respectively) (not shown).

Despite the fact that only 3% of the subjects declared themselves as vegetarians (among those that provided data on vegetarian food), vegetarians had 35% higher levels of Cd in urine than non-vegetarians with strong statistical significance (Model 2, Table 6). Other dietary variables were not found to be significant in the pooled model, which is partially also due to the above-mentioned fact that the data had very limited availability. However, organ meat consumption which had similar availability as vegetarian food (variable available in six studies, $n = 1976$) did not show any association with Cd levels in urine ($p = 0.906$). Traffic density was also not found to be significantly associated with Cd in urine in the pooled model ($p = 0.896$), and neither was the degree of urbanization ($p = 0.869$) (data not shown).

As part of sensitivity analysis, the model was applied on the subset of database with complete data on vegetarian diet ($n = 1631$) (not shown). The coefficients stayed exactly the same as shown in the Model 2 (Table 6). An additional model was checked applying the WHO creatinine criteria for urine levels exclusion based on creatinine levels (not shown). This model showed similar coefficients as shown in Model 2,

unless for vegetarian diet where the coefficient was even slightly higher (1.40, n = 1545). We also stratified the model based on the type of the urine sample - spot random sample and spot morning sample, while the model with daily urine sample type was not considered as it was only available for one country. The model with Cd concentrations measured in morning urine samples (n = 1264) showed the same trends and significance and also similar estimates of change (coefficients) as Model 1 (Table 6). The model with Cd concentrations measured in spot random samples (n = 922) showed somewhat different estimates of change, particularly for education and sampling year, where the trend was reversed in comparison with the Model 1 (Table 6) (not shown).

3.6. NUTS2 level-based variables

The variables collected at the NUTS2 level, namely Cd concentration in topsoil, percent cropland, application of phosphate fertilizer, Cd release to air and water, road density, and Cd point source release were added to the basic model. Step-wise inclusion of the listed variables was not possible, as they were not available for all studies and all NUTS2 areas. Therefore, the external (NUTS2) variables were checked for statistical significance by adding each one of them independently into the Model 1 shown in Table 6. As a result, five additional models were generated (Models 4–8, Table S3). The regression coefficients for associations between these variables and urinary Cd concentrations are presented in Fig. 4. Vegetarian diet is added to the figure for the sake of comparison of all potential sources of Cd exposure. All, except Cd concentrations in soil, were associated positively and significantly with the urinary Cd levels. Each 10 percent of cropland were associated with 3% increase in urinary Cd, but the association was marginally significant ($p = 0.060$), and 100 kg of phosphate fertilizer application per km^2 with 5.5% increase ($p < 0.001$). Road density and Cd release from point sources were also revealed significant for Cd exposure, with 27% increase for each km of roads per km^2 ($p < 0.001$), and 10% increase for each 1 kg Cd released per 100 km^2 ($p = 0.032$). Despite the substantial differences in sample size between the models, the models were robust with the main influencing factors having stable coefficients across the models (Table S3).

Finally, the external (NUTS2-based) variables were added to the Model 3 (Table 6). Unfortunately, among the statistically significant variables identified in Table S3, phosphate fertilizer data could not be used, as the sample size would be reduced to n = 215, with only two countries included. The estimated change due to the vegetarian diet was

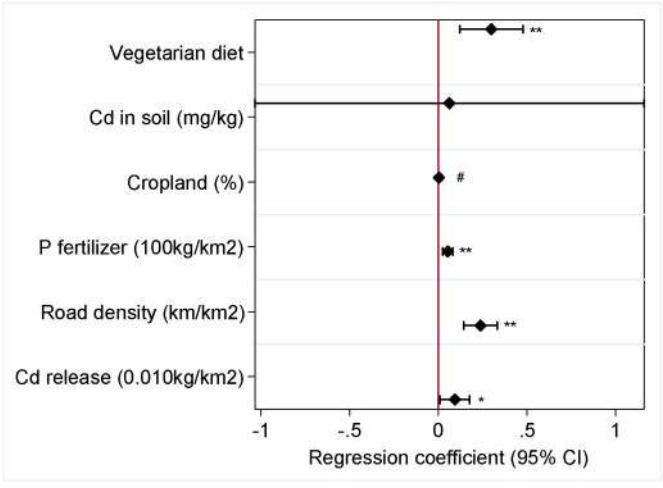

Fig. 4. Regression coefficients (95% CI) for association between potential explanatory variables and Cd concentrations in urine (ln μg/g crt) from the independent statistical models, each adjusted for creatinine, sample type, sex, age, smoking, education and sampling year. Level of significance marked as $^{\#}p<0.1$, $^{*}p < 0.05$, $^{**}p \leq 0.001$.

52%, and the coefficients for road density and Cd releases were 27% and 10%, respectively, the latter being marginally significant in the model. Coefficients for sex, age, smoking and sampling year were similar to the Models 1 and 2, while the educational level no longer showed significance with the urinary Cd levels in the Model 3. Without accounting for external information (Models 1 and 2), around 40% of variability explained by the variables in the model was between the countries, while in the Model 3, which included also external information, almost all variability was within the countries (Table 6).

4. Discussion

The paper deals with the assessment of current Cd exposure in the European population of adults and it is based on a study population which has been harmonized in terms of basic population characteristics: age (20–39 years), sex (female to male ratio 1:1), sample size (240–300), sampling period (2014–2021), exclusion of hotspots and patients, biomarkers of exposure (Cd concentration in urine), and partially also questionnaire data (socio-demographic characteristics, life style, health status, residential environment and diet). The harmonization process has been established within the HBM4EU project, which also coordinated the quality control procedure for measurement of Cd in urine to ensure comparability of analytical (biomarker) data. To ensure geographical comparability, two studies per region (North, East, South, West) were included. For identification of main sources and determinants of exposure, questionnaire data was used along with external data sources available at European level on the basis of the NUTS level (cropland percentage, phosphate fertilizer application, concentrations of Cd in soil, traffic density and point source release of Cd).

4.1. General characteristics of the study population

The study population of adults for assessment of Cd exposure (n = 2510) included 53.8% women and 46.2% men 20–39 years of age. According to the residential area 64.9% live in cities, 17.45% in towns/suburbs and only 17.65% in rural areas. The percentages are only slightly different from the percentages for the total HBM4EU Aligned Studies adult population (n = 3522) for which the characteristics were concluded to approach the characteristics of the general European population based on age matched EUROSTAT EU-28, 2017 data (Gilles et al., 2022). However, participants with no or low level of education (ISCED 0–2) were largely underrepresented (5%). The percentage of smokers in the study population was 18%, which is very similar to the percentage of every day smokers in the general European population in 2014 (19.9%) or 2019 (19.3%) (EUROSTAT EU-27). On a study level, some characteristics deviated from the country-level EUROSTAT statistics, namely level of urbanization, ISCED and particularly smoking. However, when comparing the characteristics on the regional level, the percentages better reflected the European situation (lower percentage of smokers in the northern countries and higher in some southern countries).

4.2. Exposure levels

The HBM4EU Aligned Studies is the second HBM survey that was harmonized on a European level in terms of performing comparable measurements of Cd in urine. The first pan-European HBM project - DEMOCOPHES produced harmonized measurements of Cd in urine in mother–child pairs from 16 European countries sampled in 2011–2012 (Berglund et al., 2015; den Hond et al., 2015). Results of the population group of women from the DEMOCOPHES study (n = 1632, 24–52 years) (den Hond et al., 2015) were compared to the results of women from the present study (n = 1350, 20–39 years) for those countries that participated in both studies (Table S4). The GMs for the overall group of women were the same in both surveys: being 0.20 μg/g crt. Comparing only non-smoking mothers, GM for the HBM4EU Aligned Studies was

0.20 μg/g crt and for DEMOCOPHES 0.18 μg/g crt (Berglund et al., 2015). In both surveys, the highest levels were observed for Poland and the lowest for Denmark.

The DEMOCOPHES Polish study reported a GM of 0.38 μg/g crt in all mothers and 0.36 μg/g crt in non-smoking mothers (Berglund et al., 2015; den Hond et al., 2015). Practically the same GM levels were observed in the HBM4EU Polish dataset (0.38 μg/g crt, both all and non-smoking women) (Table S4). On the lower exposure end, Denmark reported a GM of 0.12 μg/g crt for all mothers in DEMOCOPHES and 0.11 for non-smoking ones, while in HBM4EU the GM for all female participants was 0.14 μg/g crt and 0.13 μg/g crt for non-smoking ones (Berglund et al., 2015; den Hond et al., 2015) (Table S4). Similar to the Polish and Danish studies, also Luxembourg shows stagnating levels in comparison to the DEMOCOPHES assessment. In the latter, all participating mothers had exactly the same GM for Cd in urine as HBM4EU female participants from Luxembourg (0.22 μg/g crt), and the situation repeated for non-smoking women (both 0.21 μg/g crt) (Berglund et al., 2015; den Hond et al., 2015) (Table S4).

Another country that participated in both harmonized surveys was Czech Republic. Results for this country are consistent with the decreasing time trend reported from the observations of blood Cd concentrations in the period from 1996 to 2009 (Černá et al., 2012). The levels of 0.4 μg/g crt in women and 0.3 μg/g crt in men were reported for the period 2005–2009 (n = 1227), 0.21 μg/g crt in women in the DEMOCOPHES study (2011–12) (Berglund et al., 2015; den Hond et al., 2015) and in the present study 0.11 μg/g crt (in both studies GMs were equal in all and in the non-smoking women) (Table S4). A slight decreasing trend can be observed also for Portugal, where the GM for women was 0.16 μg/g crt in DEMOCOPHES and 0.11 μg/g crt in HBM4EU (Table S4).

The German DEMOCOPHES Cd data was re-analyzed later to achieve comparability with the DEMOCOPHES European study population, and reported lower levels for mothers (0.18 μg/g crt) (Schwedler et al., 2017) as were found in the HBM4EU Aligned Studies female participants of the German study (Table S4). The aligned studies population is part of the Environmental Specimen Bank (ESB) program active from 1980 and publicly available data of the ESB indicate that Cd in 24-h urine has generally decreased between 1990 and 2019 (UPB, 2022), but unchanged concentrations (expressed per volume) were observed for the last two decades, with slight increase in 2020 and 2021 (Becker et al., 2013; UPB, 2022). A similar situation was observed in a recent comparison of urinary Cd data for children in the German Environmental Survey (GerES), where overall, participants in GerES V (2014–2017) had about 15% lower Cd concentrations than GerES II (1990–92) children and adolescents, but not lower then GerES IV participants (2003–2006) (Vogel et al., 2021).

France was not part of the harmonized DEMOCOPHES survey, but as apparent from the literature data, the levels in this country seem to stagnate as well. Nisse et al. reported a GM of 0.39 μg/L Cd in urine of participants from Northern France for the period 2008–2010 (n = 1992), which is similar to the French study included in the aligned survey (0.37 μg/L, Table 3) (Nisse et al., 2017).

The above listed comparisons show somehow varying trends between the countries, but there is firm evidence indicating that overall Cd exposure levels in the general population are currently not decreasing markedly. The latter can be further observed from another HBM program, the Flemish Environment and Health study (FLEHS) representative for Flanders, the northern part of Belgium. Although in adults, urinary Cd levels decreased from the first (FLEHS I, 2002–06) to the third cycle (FLEHS III, 2012–15) (Schoeters et al., 2017), teenagers showed a decrease in blood Cd concentrations from FLEHS I to FLEHS II (2007–11) (Vrijens et al., 2014), but further comparison with succeeding cycles conducted up to year 2020 (FLEHS III and FLEHS IV) showed stable levels in blood with 40% teenagers exceeding the established health based HBM guidance values (HBM-GVs) for urinary concentrations and the corresponding age in the last sampling period, 2016–2020 (Schoeters et al., 2022). The authors refer to continuous efforts to reduce the sources and limit the exposures in humans (regulations) that were initially reflected in decreasing internal Cd levels (Schoeters et al., 2017, 2022), while stagnation or even increase in recent decade may be ascribed to unchanged nutritional habits and persisting Cd levels in soil, particularly in historically contaminated or industrial sites (Becker et al., 2013; Schoeters et al., 2022). Moreover, the data on phosphate fertilizer consumption per countries available for years from 2000 to 2018 (Eurostat) showed initial marked decrease in consumption from 2000 to 2009 for all countries except Poland, followed by somewhat varying trends from 2009 onwards (Fig. S2). Although the reason(s) for the observed trends could not be definitely explained, the observations provide firm evidence that the regulations are still required to protect humans from elevated exposure to Cd.

Moreover, the exceedance of HBM-GVs was evident in all studies of the HBM4EU Aligned Studies, in the total study population 16.4% of participants presented levels above the age-dependent HBM-GVs, recently derived by Lamkarkach et al. (2021) to protect from kidney dysfunction at later ages. The exceedance was particularly high in the Polish, French and German studies, with over one third of participants exceeding the value that is protective for risk of adverse kidney effects (Fig. 2a). According to the NUTS2 level information (Fig. 2b) we can see that in all areas (with n > 20) of these three countries, there is one third of the population or more at hypothetical risk for kidney effects: in the French study the exceedances ranged from 31% to 56%, in the German study from 28% to 43%, while the Polish study was represented only by one NUTS2 area (33%). According to the exceedances observed in the DEMOCOPHES (den Hond et al., 2015) and HBM4EU Aligned Studies (subgroup of women) based on the HBM-I values (1 μg Cd/L urine) the percentage has stayed at the same level in the last decade (2.9% and 3.0%, respectively).

These results are in line with the latest EFSA report, where a medium estimate for weekly intake for adults was 1.77 μg/kg b.w. (range 1.50–2.23 μg/kg b.w.) and P95 3.13 μg/kg b.w. (range 2.47–4.81 μg/kg b.w.). The latter is above the current tolerable weekly intake (TWI) of 2.5 μg/kg b.w. and confirms that adults at the 95th percentile exposure could exceed health-based guidance values on account of dietary intake of Cd (EFSA, 2012).

When the levels were adjusted for the main influencing factors (sample type, age, sex, smoking, education and sampling year) and compared geographically, smaller differences in mean levels were revealed between countries and/or regions (Fig. 1). Given that the 95% confidence intervals were sufficiently narrow, the results provide evidence of more or less uniform exposure across Europe, with somewhat lower levels in the North.

On a global scale, the HBM4EU population mean level for Cd in urine (0.15 μg/g crt, Table 5) was equal to the mean for the Canadian population group of 20–39 years old adults sampled in 2018–2019 (GM 0.15 μg/g crt, 95% CI 0.12–0.18 μg/g crt, n = 329), and somewhat higher than the mean for the adults of the same age sampled in 2016–17 (0.12 μg/g crt, 95% CI 0.10–0.14, n = 372) (Health Canada, 2021). Here, a similar situation can be observed as for Europe – initial decrease of concentrations from the beginning of the HBM program, but stagnation or even increase in the most recent campaigns as evidenced from the 6th survey cycle report (Health Canada, 2021). In the USA, the stagnating trend is evident for the last decade, with mean level in adults of 20 years or more being higher than in the HBM4EU population in the two latest sampling periods: 2015–2016 (0.190 μg/g crt, 95% CI 0.175–0.205, n = 1792) and 2017–2018 (0.189 μg/g crt, 95% CI 0.175–0.205, n = 1707) (CDC, n.d.). In the Korean National Environmental Health Survey, a downward trend since 2008 has been observed based on Cd concentrations in blood (Seo et al., 2015), however in the last cycle (2015–2017) the GM for spot urine was 0.36 μg/L (≥19 years of age) (Jung et al., 2022), which is considerably higher than in Europe or North America. The authors explain the levels with very frequent consumption of rice, which is a notable staple of the East Asian diet, and the Korean

Table 5
Exposure levels for the European population calculated using survey data analysis with country as primary sampling unit (PSU). The levels were **not** adjusted for basic influencing factors.

		n	Cd in urine (μg/L)		n	Cd in urine (μg/g creatinine)	
			GM (95% CI)	**P95 (95% CI)**		**GM (95% CI)**	**P95 (95% CI)**
Unstratified	All	2117	0.17 (0.12–0.25)	0.75 (0.69–0.81)	2107	0.15 (0.10–0.22)	0.53 (0.50–0.58)
Region	North	495	0.13 (0.12–0.14)	0.49 (0.43–0.57)	485	0.11 (0.09–0.12)	0.29 (0.26–0.33)
	South	595	0.14 (0.09–0.21)	0.52 (0.47–0.59)	595	0.11 (0.08–0.14)	0.33 (0.29–0.36)
	West	499	0.24 (0.16–0.36)	0.70 (0.62–0.78)	499	0.23 (0.16–0.32)	0.57 (0.51–0.64)
	East	528	0.21 (0.08–0.58)	0.94 (0.81–1.13)	528	0.18 (0.06–0.54)	0.69 (0.62–0.80)
Degree of urbanization	Cities	1441	0.18 (0.12–0.27)	0.77 (0.70–0.85)	1437	0.16 (0.10–0.27)	0.59 (0.54–0.65)
	Towns/suburbs	323	0.17 (0.10–0.30)	0.67 (0.56–0.80)	318	0.12 (0.09–0.17)	0.35 (0.31–0.40)
	Rural areas	336	0.16 (0.09–0.27)	0.60 (0.51–0.70)	336	0.12 (0.09–0.16)	0.36 (0.32–0.41)
ISCED	Low	117	0.15 (0.12–0.20)	0.74 (0.44–1.26)	113	0.11 (0.08–0.15)	0.39 (0.27–0.60)
	Medium	575	0.17 (0.10–0.29)	0.77 (0.67–0.89)	575	0.13 (0.08–0.22)	0.56 (0.47–0.67)
	High	1414	0.17 (0.13–0.24)	0.71 (0.65–0.78)	1408	0.16 (0.10–0.24)	0.54 (0.50–0.59)
Sex	Female	1134	0.18 (0.12–0.27)	0.79 (0.71–0.88)	1134	0.17 (0.11–0.27)	0.61 (0.56–0.67)
	Male	983	0.16 (0.12–0.22)	0.67 (0.60–0.76)	973	0.12 (0.08–0.18)	0.40 (0.37–0.44)
Smoking	No	1763	0.17 (0.11–0.24)	0.71 (0.65–0.78)	1756	0.14 (0.09–0.22)	0.53 (0.49–0.57)
	Yes	335	0.21 (0.15–0.30)	0.87 (0.72–1.06)	332	0.16 (0.11–0.22)	0.62 (0.50–0.77)

Remark: ESTEBAN data collection (France) excluded from calculations of European exposure values.

Table 6
Multilevel mixed regression model for the pooled European population with study as a random factor. The estimates are expressed as the fold change in Cd concentration for unit increase of the covariate.

Cd in urine (μg/g crt)	Estimate (95% CI), Model 1	Estimate (95% CI), Model 2	Estimate (95% CI), Model 3
N	*2475 (9 studies)*	*1631 (6 studies)*	*424 (3 studies)*
overall p-value	*<0.001*	*<0.001*	*<0.001*
var $_{BETWEEN}$	*43%*	*39%*	*<0.01%*
Age (years)	1.03 (1.02–1.04) **	1.04 (1.03–1.04) **	1.02 (1.00–1.03) **
Sex (F vs. M)	1.33 (1.26–1.40) **	1.32 (1.24–1.41) **	1.35 (1.21–1.50) **
Smoking (yes vs. no)	1.25 (1.17–1.33) **	1.26 (1.16–1.36) **	1.24 (1.09–1.41) **
ISCED - low	1.00	1.00	1.00
ISCED - medium	0.86 (0.76–0.97)*	0.75 (0.64–0.88) **	0.67 (0.30–1.45)
ISCED - high	0.86 (0.76–0.96)*	0.75 (0.64–0.87) **	0.55 (0.25–1.20)
Sampling year	0.96 (0.93–1.00)*	0.94 (0.91–0.98) **	0.93 (0.89–0.97) **
Vegetarian (yes vs. no)	–	1.35 (1.13–1.61) **	1.52 (1.19–1.93) **
Road density (km/km^2)	–	–	1.37 (1.22–1.54) **
Cd release (kg/100 km^2)	–	–	1.10 (1.00–1.20) #

#p < 0.10, *p < 0.05, **p < 0.01.
Model 1 = adjusted for creatinine, sample type, age, sex, smoking, education, and sampling year (all studies).
Model 2 = Model 1 + additionally adjusted for vegetarian diet (CZ, DE, FR, CRO, IS, PT).
Model 3 = Model 2 + additionally adjusted for road density and Cd release (CZ, DE, FR).

general population consuming even more frequently than populations from other Asian countries (Jung et al., 2022).

4.3. Determinants of exposure

The basic demographic and life-style factors of Cd exposure, namely sex, age, smoking and education are well recognized from previous HBM studies and (inter)national programs and were selected as the main potentially influencing factors of exposure. As such, they were included in the simple bivariate statistical analysis to check significance and trend for each study separately and further on in the basic regression models, both study-specific and pooled. They were also applied in the geographical comparison of exposure levels to better reflect dietary and environmental determinants of exposure, which might differ between the countries/regions in Europe. The type of urine sample (spot random urine, morning urine, daily urine) was also included in the statistical models and geographical comparison in order to control for potential influence of the sample type on Cd levels, but it was not our aim to explore the influence itself.

As expected, higher levels were observed in women than in men (33%), difference that is typically observed in adult healthy populations, and reflected by the urine as well as blood concentrations of Cd (CDC, n.d.; Černá et al., 2012; Health Canada, 2021; López-Herranz et al., 2016; Snoj Tratnik et al., 2019). The main reason are the lower iron stores (expressed as lower ferritin concentrations) in women in comparison to men, which enhances uptake of Cd as the two metals are known to have similar absorption mechanism (Lee and Kim, 2014). The sex-related difference was well established for all participating studies, with borderline significance in the Polish study, where women represented only one third of the study population.

Increasing levels of Cd in urine or blood with age are expected due to the accumulation throughout the lifetime (ATSDR, 2012) and have been observed in various national or regional HBM studies (Castaño et al., 2012; CDC, n.d.; Health Canada, 2021; Nisse et al., 2017; Snoj Tratnik et al., 2019), including the pan-European DEMOCOPHES survey (den Hond et al., 2015), as well as in the HBM4EU Aligned Studies (overall 3% increase per life year) where the age range was smaller (20–39 years) than in the DEMOCOPHES (20–49 years). Within the HBM4EU, only the Polish study did not show significant association between urinary Cd levels and age of participants.

Among the basic influencing factors, smoking was confirmed to be the major determinant with 25% higher urinary levels in smokers than in non-smokers (Table 6). This is consistent with the fact that tobacco plants hyperaccumulate Cd, resulting in high concentrations in their leaves independent of the soil Cd content and reports of many other

regional, national and international studies, including DEMOCOPHES where smoking mothers had 31% higher levels in urine than non-smoking mothers (den Hond et al., 2015). However, the significance on the study level was only evident in the studies with one of the highest proportion of smokers - Portugal, France and Luxembourg (Table 3), which were also the only studies with observed association between urinary Cd concentrations and number of cigarettes smoked per day (data not presented). The lack of significant associations in other participating studies could be explained by insufficient statistical power due to low percentage of smokers and/or the fact that urinary Cd concentrations reflect long-term accumulation of Cd in kidney cortex, while blood Cd concentrations better reflect current exposure (Nordberg et al., 2015) and as it was demonstrated on the basis of USA NHANES data (Adams and Newcomb, 2014), there is a noticeable overlap between the two. In fact, some previous studies reported the absence of difference in urinary Cd levels between smokers and non-smokers, while for blood levels the difference was significant (Baeyens et al., 2014; Snoj Tratnik et al., 2019). Moreover, while in the DEMOCOPHES study group of mothers, passive smoking at home was found to be associated with higher Cd levels in urine of non-smokers (Berglund et al., 2015), this was not reproduced in the present study, neither in the total population of non-smokers ($p = 0.608$), the group of non-smoking women ($p = 0.802$), nor in the study specific models (data not presented). This might be explained partially by the above-mentioned discrepancy between the two biomarkers of exposure, but also by the fact that data on passive smoking was available only for six studies and data on frequency of exposure to passive smoking only for three, and among them very few were exposed to passive smoking daily (2%). For example, Vogel et al. (2021) reported absence of association in case urinary Cd was used, but association was found with blood Cd levels in those who stayed daily in rooms at home where other people smoked. Clearly, blood Cd concentrations appear to be more reliable in relation to smoking and/or passive smoking, particularly in cases of limited statistical power or lack of fully harmonized questionnaire data.

Smoking has been discussed as a factor that might result in higher Cd exposure in people with lower educational level due to a higher proportion of smokers (Baeyens et al., 2014) and indeed significance of educational level did not persist after adjusting for smoking in the recent Flemish study including adolescents (Schoeters et al., 2022). However, at the same time increased Cd levels can be associated with high education due to increased consumption of vegetables (Vogel et al., 2021). In the present study, independently of smoking, lower levels of Cd in urine were associated with higher levels of education (Table 6). The effect of education was not evident on a study level, except in Portugal, which makes sense as in the latter the education was more equally distributed among the three levels than in the other participating studies (Table S2, Table 3). Similar results were reported in other studies (e.g. den Hond et al., 2015; Berglund et al., 2015) and could be that the variability in urinary levels is shared by both factors, but also some other factors related to diet might be part of the cause.

Among other potential explanatory variables relevant for Cd exposure that were checked for significance in the multiple regression models, vegetarian diet was the only dietary variable available from questionnaires that was revealed as strongly significantly associated with Cd in urine in the pooled population – vegetarians had 35% higher levels than non-vegetarians (Table 6). Vegetables are one of the main sources of Cd exposure in non-smokers (EFSA, 2009; Nordberg et al., 2018), which, together with presumption that vegetarians consume higher quantities of plant-based food than non-vegetarians, well explains our observation. However, there are only a few HBM studies reporting vegetarian-relevant results. Just recently, a similar observation was reported for children participating in the German GerES V program, with vegetarians having 35–41% higher levels of Cd in urine (Vogel et al., 2021). The authors stated that the participating children with a vegetarian diet had a significantly lower ferritin, which increases Cd absorption (EFSA, 2009). The most recent EFSA report on the cadmium dietary exposure in the European population (EFSA, 2012) provides estimates based on the Cd levels in food items and detailed frequencies of consumption and shows that food consumed in larger quantities had the greatest impact on dietary exposure to cadmium. The highest contribution to exposure across different age groups was on account of grains and grain products (27%), followed by vegetables and vegetable products (16%) and starchy roots and tubers (13%). For adults the respective percentages were 27%, 17% and 12%, whereas meat and edible offal accounted for 8.7% contribution to the overall exposure (EFSA, 2012). From these estimates it is clear that plant-based dietary items are by far the major exposure source, particularly if the diet is exclusively vegetarian and includes higher quantities of plant-based food as opposed to meat-including diet.

Among specific dietary sources available from the questionnaire data, only cereals in the Polish study were identified as significantly associated with the urinary Cd levels (Table S2). Cereals belong to the EFSA food category with largest contribution to exposure with bread and rolls identified as the major source (EFSA, 2012). Unfortunately, the reasons behind study-specific findings cannot be discussed further and with reference to country specifics/characteristics as the data were not available for all participating studies and also EFSA estimates are not provided for all countries.

In line with the hypothesis that application of mineral fertilizer based on phosphate may contribute to Cd exposure in the general population via diet, the association between urinary Cd concentrations and data on phosphate fertilizer application in kg/km^2 was confirmed, and with marginal significance also percentage of cropland in the area (Fig. 4). Direct association between soil and urine concentrations was not revealed, but it has to be noted that the range of mean Cd concentration in soil in the areas that were included in the present analysis was narrow, i.e. 0.05–0.20 mg/kg, and also that the levels are not given exclusively for agricultural land. Cropland and fertilizer data appear to be more relevant as they are directly linked with the exposure of consumers.

Fertilizers have been recognized as one of the main sources of Cd contamination through the food chain, and as demonstrated by some recent studies, plant-available Cd concentrations in soils amended with P fertilizer (and compost) gradually increase over decades of application (Park et al., 2021) and that the control of Cd input into the food chain is needed to stop the increase over time due to Cd accumulation in the part of the population likely to be overexposed to Cd through food (Carne et al., 2021). By the use of probabilistic mass-balance model, the authors simulated the transfer of Cd from agricultural soil to food consumed by the French population and account for variability in French soils, local specificities and agricultural practices and estimated that content of Cd in mineral phosphate fertilizers should not exceed 20 mg/kg P_2O_5 in order to achieve reduction of Cd content in agricultural soils and ensure the exposure of consumers does not exceed the health threshold values (Carne et al., 2021). The level is 3-times lower than the current concentration for phosphate fertilizers of 60 mg Cd/kg P_2O_5, adopted by the EC in view of a potential application of this regulation in 2022 (Regulation (EU) No 2019/1009).

Although the data analysis in the present paper does not exclusively deal with population groups consuming locally-grown crops and intake of crops imported from other regions cannot be excluded, the observed combined associations based on HBM and external data clearly indicate the importance of phosphate fertilizers contribution within the dietary exposure pathway in the general population. Together with the considerable share of population above the HBMGVs, these results support the findings from the probabilistic modelling (Carne et al., 2021) and confirm the need for control/reduction of Cd input into the food chain.

In addition to the diet-related intake, our results demonstrated traffic and industrial releases as significant contributors to Cd exposure in the general population. Traffic intensity close to home has been shown as a statistically significant determinant for blood Cd in children from six European countries, whose Cd measurements had also undergone strict

QA/QC procedures and were performed centrally and varied little between the countries (Hrubá et al., 2012). The authors explanation follows the fact that, although in small amounts, vehicle tires contain zinc contaminated by Cd. In the present study, traffic density in the participant's residential area was available through the post-harmonized questionnaire data with three categories (no traffic, light traffic, intense traffic) and through the Eurostat database (km roads/km^2). Statistically significant association with Cd concentrations in urine was observed for the latter, which demonstrates that NUTS2 level external information is superior to the data from questionnaires, reported by participants. Industrial point-source release of Cd to air and water is an additional potential source of Cd exposure, which is available from the E-PRTR database, and has been used as an external dataset for interpretation of HBM data before – in the DEMOCOPHES study (Smolders et al., 2015). There, the association between annual releases on a NUTS2 levels and mother's or child's urine Cd concentrations has not been observed. The association has been re-analyzed in the present study together with other potential sources of exposure, and it was revealed as significant (Fig. 4, Table 6). Furthermore, it showed that differences in exposure were mostly on account of variability within the countries/studies.

It is important to note that for a more realistic exposure characterization at environmentally-relevant levels of exposure as observed for Cd in Europe and also globally, it is crucial that all potential sources of exposure from different pathways are included in the assessment, as HBM data provides cumulative exposure (Knudsen et al., 2012; Smolders et al., 2009). Moreover, use of external data that was based on a NUTS2 spatial resolution rather than national (Fig. S1), provided data with higher precision on potential sources of exposure, and therefore enabled a refined association study also from a spatial perspective.

4.4. Strengths and limitations

Clearly, the most important strengths of the present study are comparable measurements of Cd in urine, which have undergone strict QA/QC procedures, harmonized study population characteristics across the participating studies, sample size and the fact that all four geographical regions (North, South, West, East) of Europe were covered. Another advantage is that we were able to perform refined analysis of exposure, by considering multiple sources of exposure (socio-demographic determinants, dietary and external environment-related determinants) simultaneously obtaining more realistic assessment of exposure.

Despite the achieved high degree of harmonization on a European level, there are still some important limitations which remain to be further addressed. The first is partial (post)harmonization of questionnaire data, resulting in limited availability of questionnaire data across studies, and the second is a lack of external data, which is not available at the same level of resolution and not for all NUTS areas. As a consequence, the statistical models were not entirely comparable between each other (each was built on a different dataset) and it was not possible to include all available potential sources of exposure into a single model. Another issue is related to urinary Cd concentrations and standardization of the levels for urine dilution and the risk of underestimating the exposure due to creatinine over-compensation (Hoet et al., 2016). We could overcome this issue by using standardization based on specific gravity, which appears to be a more reliable alternative in the context of environmental exposures, without the risk of over-adjustment and with fewer uncertainties associated with its use (Hoet et al., 2016; Stajnko et al., 2017). Unfortunately, measurements of specific gravity could not be used in the present study, as they were only available for one study. Availability of Cd concentrations in blood would allow further refinement of exposure assessment, with emphasis on recent exposures.

5. Conclusions

The HBM data of nine countries harmonized at the European level showed a small degree of variability in mean urinary Cd concentrations in adults across the four geographic regions of Europe, particularly when adjusted for the main influencing factors (sex, age, smoking, education and year of sampling). There was no clear time trend in exposure observed and the HBM-GVs for Cd in urine were exceeded by 16% of the study participants. Exceedances differed across the study areas, with the largest percentage of exceedance observed in the French and Polish studies, where approximately one third of the study population was above the age-dependent values.

To our knowledge, this study was the first to confirm, based on the HBM data, that the mineral phosphate fertilizers are a significant source of Cd exposure through the diet in Europe. Furthermore, vegetarian diet might increase exposure to Cd even more than smoking, which calls for attention in case of promoting vegetarian diet. In addition to EFSA's estimates on the food categories contributing the most to the exposure (EFSA, 2012), higher degree of traceability of plant-based dietary items is needed on a sub-national (regional) level. Besides the smoking and diet-related sources, traffic and Cd releases from industrial facilities remain a significant source of exposure to Cd in the general population.

The findings of the study support the recommendation by EFSA to reduce Cd exposure as also the estimated mean dietary exposure of adults in the EU is close or slightly exceeding the tolerable weekly intake. Results also indicate that current regulations are not protecting the general population sufficiently.

Author contributions

Conceptualization, J.S.T, D.K., M.H., L.G., E.G., O.S. and G.S.; data curation, L.R.M., L.G.; data analysis and visualization, J.S.T., D.K.; supervision, E.G., G.S., M.H.; writing—original draft, J.S.T, D.K.; writing—review and editing, S.N., M.R., A.V.N, M.K.-G., T.W., M.E.-L, L.G., G.S., E.G.; study PIs, A.-M.A., A.J., E.J., K. Ó., J.K., L.A., B.J., W.W., N.J. H., S.N., I.C., L.R., M.R., A.V.N, B.A., M.K-G., T.W. All authors have read and agreed to the published version of the manuscript.

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Informed consent statement

Informed consent was obtained from all subjects involved in the study. The detailed information on the ethics committees is provided by Gilles et al. (2022).

Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114050.

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Cumulative risk assessment of five phthalates in European children and adolescents

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ABSTRACT

The European Human Biomonitoring Initiative (HBM4EU) assessed human biomonitoring data on phthalates in children and adolescents, that were sampled between 2014 and 2021, in a harmonised way. These so-called "HBM4EU Aligned Studies" revealed that almost all children and adolescents were exposed to multiple phthalates concurrently. Some phthalates have been shown to act in a dose-additive manner, thus, a mixture risk assessment is warranted. In our study, we determine the risk from combined exposure to five anti-androgenic phthalates, namely DEHP, DiBP, DnBP, BBzP and DiNP by making use of the hazard index (HI) approach. Toxicologically-based human biomonitoring guidance values (HBM-GVs) derived within the framework of HBM4EU served as basis. Our results show that exposures of 17% of children and adolescents from twelve European countries resulted in hazard indices (HI) > 1 with an HI of 1.77 at the 95th percentile (geometric mean,

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GM = 0.44). Main drivers for the mixture risk are DnBP and DiBP. Generalized Linear Model (GLM) analysis including four major exposure determinants (age, sex, European region, sampling year) simultaneously reveal differences for the European regions and between sampling years. Children and adolescents living in the Eastern region of Europe have on average, higher HIs (GM=0.58) than in the Southern region (GM = 0.36) and Western region (GM = 0.42). Moreover, participants from which urine samples were taken in the earlier years (2014–2016) seem to have higher average HI levels than participants from studies with later sampling periods. Strikingly, the majority (63%) of participants with HIs > 1 would have gone unnoticed in single substance risk assessments as individual phthalates levels were below corresponding HBM-GVs. Thus, our results underline the importance of mixture risk assessment approaches to adequately address risks from concurrent chemical exposure.

Abbreviations

3xG	Health - Municipalities - Birth Study (Belgium, BE)
BEA	Biomonitoring in Adolescents Study (Spain, ES)
CELSPAC:TE	Central European Longitudinal Study of Parents and Children: Teenagers (Czech Republic, CZ)
CROME	Cross-Mediterranean Environment and Health Network Study (Greece, EL)
ESTEBAN	Health study on environment, biomonitoring, physical activity and nutrition (France, FR)
FLEHS IV	4th cycle of the Flemish Environment and Health Survey (Belgium, BE)
GerES V-sub (unweighted)	5th cycle of the German Environmental Survey (subsample, unweighted data, Germany, DE)
GM	Geometric mean
HBM	Human Biomonitoring
HBM4EU	The European Human Biomonitoring Initiative
HBM-GV	Human Biomonitoring Guidance Value
HI	Hazard index
ICI/EQUAS	European interlaboratory comparison investigations (ICI) and external quality assurance schemes (EQUAS)
InAirQ	Transnational Adaption Actions for Integrated Indoor Air Quality Management Study (Hungary, HU)
MCR	Maximum Cumulative Ratio
MRA	Mixture Risk Assessment
NEB II	Norwegian Environmental Biobank II (Norway, NO)
OCC	Odense Child Cohort (Denmark, DK)
PCB cohort follow-up	Endocrine disruptors and health in children and teenagers in Slovakia study (follow-up study, Slovakia, SK)
RQ	Risk Quotient
SLO CRP	Exposure of children and adolescents to selected chemicals through their habitat environment study (Slovenia, SI)
SPECIMEn-NL	Survey on Pesticide Mixtures in Europe (The Netherlands, NL)
SVHC	Substances of Very High Concern

1. Introduction

HBM4EU is a joint project funded under the Horizon 2020 programme designed to advance and harmonise Human Biomonitoring (HBM) in Europe (HBM4EU, 2017–2022). A main achievement is the so-called "HBM4EU Aligned Studies". A sampling frame has been developed and later been implemented by aligning existing national and regional HBM studies to meet a common goal, that is the assessment of human biomonitoring data on environmental chemicals in a harmonised way (Gilles et al., 2021, 2022).

One of the first prioritised chemical substance group under HBM4EU were phthalates (Ougier et al., 2021). Phthalates are used as plasticisers to soften poly vinyl chloride (PVC) and are or used to be applied in a variety of consumer products, such as cosmetics, food packages, medicinal products, textiles, toys, and footwear (European Chemicals Agency (ECHA) and Danish Environmental Protection Agency, 2016; EFSA, 2019). Several phthalates have endocrine disrupting properties and are classified in the European Union as reproductive toxicants, category 1B ("May damage fertility and/or the unborn child") under regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures. As a result, they are identified as substances of very high concern (SVHC) (ECHA, 2022) and are subject to various regulations in the European Union. Animal studies have revealed that exposure to certain phthalates affects fertility and reproduction of both sexes (US CPSC, 2014; European Chemicals Agency (ECHA) and Danish Environmental Protection Agency, 2016; NRC, 2008; Yost et al., 2019). Most susceptible for phthalate toxicity is the male offspring if exposed prenatally: *in utero* exposure to e.g. diethylhexyl phthalate (DEHP), diisobutyl phthalate (DiBP), di-n-butyl phthalate (DnBP) and butyl benzylphthalate (BBzP) during the critical window of sexual development induces various irreversible malformations and alterations of the reproductive tract of the male rat offspring, which are summarized under the term "phthalate syndrome" (Conley et al., 2021; European Chemicals Agency (ECHA) and Danish Environmental Protection Agency, 2016; ECHA, 2017a; EFSA, 2019; German HBM Commission, 2011; NRC, 2008; US CPSC, 2014). Observed effects include hypospadias, cryptorchidism, testicular and epididymal malformations, but also reduced sperm count and reduced anogenital distance (Gray et al., 2000; Gray and Butterworth, 1980; Hannas et al., 2011; Howdeshell et al., 2008a, 2015). The occurrence of the same effects after exposure to different phthalates observed in animal experiments led to the assumption that this group of substances may act via the same mechanism and thus might have cumulative effects. Phthalates suppress testosterone and insulin-like 3 hormone production, androgens crucial for male sexual development (Gray et al., 2000; Howdeshell et al., 2008a). More than 10 years ago, the National Academies of Science National Research Council Committee (NRC) recommended to assess the risk from exposure to reproductive phthalates together by using a dose-addition approach (NRC, 2008). Phthalate mixture studies in rodents confirmed the cumulative effects of phthalates already at low doses for the individual chemical (Conley et al., 2021; Furr et al., 2014; Hannas et al., 2011; Howdeshell et al., 2007, 2008b, 2015, 2017). It can be assumed that the assessment of cumulative mixture effects is relevant for humans exposed to several phthalates at the same time. Besides the concurrent exposure to reprotoxic phthalates, scientist have expressed their concerns about the vast number of endocrine disrupting chemicals the general population is exposed to (Howdeshell et al., 2017; Kortenkamp, 2007, 2008; Orton et al., 2014). It has been shown in animal studies that beyond the group of phthalates also other anti-androgenic substances that disrupt male reproductive tract development act in a dose-additive manner and thus contribute to the cumulative risk. This is even true if the individual substances act via different mechanisms of action to disrupt the androgen-mediated pathway or even via

completely different pathways (Conley et al., 2018, 2021; Howdeshell et al., 2017; Rider et al., 2010). To evaluate the risk of possible health effects from exposure to reprotoxic phthalates in European children and adolescents, within HBM4EU, health-related human biomonitoring guidance values (HBM-GVs) for the general population (HBM-GV_{GenPop}) were derived for five phthalates, namely DEHP, DnBP, DiBP, BBzP and di-(2-propylheptyl)phthalate (DPHP) (Lange et al., 2021). These values refer to the urinary concentration of the specific biomarker(s) of a phthalate at and below which, according to current knowledge, no risk of health impairment is anticipated. HBM-GVs can directly be compared with the urinary biomarker concentrations gathered in HBM studies (Apel et al., 2020b). Since the developing organism is most sensitive to the toxicological effects from phthalates, it is necessary to prevent the foetus from phthalate exposure, but also to protect children and adolescents as these are among the most vulnerable populations. Therefore, in the present study, a mixture risk assessment (MRA) was conducted by using the hazard index (HI) approach as straightforward approach making use of harmonised European human biomonitoring data and the HBM-GVs already derived in HBM4EU. Generically, the HI is the sum of risk quotients of the individual substances (RQ_i), which represent the exposure level divided by a toxic potency measure. For HI < 1, it is assumed that there is no concern for cumulative mixture effects (EFSA Scientific Committee et al., 2019; NRC, 2008; Teuschler and Hertzberg, 1995). The five phthalates, included in the MRA (DEHP, DnBP, DiBP, BBzP and DiNP), were selected based on their common reprotoxic properties and on their co-occurrence in the European subpopulations (Cullen et al., 2017; Hond et al., 2015; Husøy et al., 2019; Santé Publique France, 2019; Schoeters et al., 2017; Schwedler et al., 2020). HBM-GV_{GenPop} were utilised to assess the cumulative risk posed by the five reprotoxic phthalates in HBM data of European children and adolescents from the HBM4EU Aligned Studies. We also apply and discuss the use of additional "precautionary factors" on the HI to account for concurrent exposures to other anti-androgenic phthalates and other substances not included in the phthalate MRA as previously suggested by Apel et al. (2020a) and Kortenkamp and Koch (2020).

2. Methods and materials

2.1. Study designs and fieldwork

A sampling strategy for a Europe-wide human biomonitoring survey was established to collect harmonised and quality-controlled HBM data. As few countries already run HBM programs on a regular basis, this strategy intended to build on existing national and regional HBM capacities and infrastructures as much as possible. Therefore, the strategy set up inclusion and exclusion criteria for HBM studies to be harmonised (Gilles et al., 2021). Studies were eligible that i) were already completed, but would provide biobanked samples; ii) that were already initiated before HBM4EU; and iii) studies that hadn't yet started. In addition, the eligible studies were aligned with respect to age group, biomarkers of interest, sampling period (between 2014 and 2021), and were provided with guidelines on post-harmonisation of questionnaires data, matrix, sample population, sample size (maximum n = 300), and sampling process (Gilles et al., 2021). The HBM4EU Aligned Studies include recent phthalate exposure data from two age groups: (i) children aged 6–11 years, and (ii) adolescents aged 12–18 years. Three studies, included children at the age of 12 years in the sample, i.e. GerES V-sub (unweighted), ESTEBAN and PCB cohort (children) as some participants turned 12 at the time of urine collection and the interview in which also age was obtained was conducted prior to sample collection (Gilles et al., 2022). In total, 12 and 11 studies delivered exposure data on up to 15 exposure biomarkers (metabolites) for 10 phthalate diesters in children and adolescents, respectively (Gilles et al., 2021, 2022). Per design recommendation, each study should have sampled as many girls as boys and individuals with different socio-economic status living in cities, towns/suburbs and rural areas should be represented. Different urine sampling types were collected in the participating studies, i.e. random spot urine samples (SU) as well as first morning urine (MU) samples. In the harmonisation processes within the HBM4EU Aligned Studies, MU samples, were defined as samples collected between 6 and 12 o'clock am. Urine samples collected outside this window, were considered as SU. Therefore, for some studies (SLO CRP, GerES V-sub, 3xG, BEA, CELSPAC:TE), both sampling types are applicable as few participants fall outside the time window set for MU. For more details on the study characteristics, see Gilles et al., (2022). To ensure a wide European coverage the sampling strategy stratified Europe into four geographical regions (North, South, West, East) and proposed that the number of studies assigned to a geographical region is proportional to the number of inhabitants for the respective region. It was suggested setting the minimum of studies per European region to be included as follows: at least 2 studies for the Northern region; at least 3 studies for the Southern region; 3–4 studies for the Western region; and at least 1 study for the Eastern region (Gilles et al., 2021). Informed consent was given by all study participants and all studies were approved by ethical committees. For more details, please see Gilles et al. (2022) and Supplementary Material, Table S15.

2.2. Chemical analysis

Prior to chemical analyses, best suitable biomarkers, analytical methods and human matrices for the phthalate substance group was determined by an experts group from the HBM4EU consortium (Vorkamp et al., 2021). Exposure to phthalates were determined by measuring their metabolites (specific exposure biomarker(s)) excreted in urine (Table 1). Within HBM4EU a European network of HBM laboratories was built and a quality assurance/quality control (QA/QC) program was set up, in which HBM laboratories from partner countries were qualified, among others, for phthalate measurements (Esteban López et al., 2021). To ensure the comparability and reliability of the measurements data, chemical analysis should be performed by laboratories that successfully participated in the ICI/EQUAS (European interlaboratory comparison investigations/external quality assurance schemes) of HBM4EU. As the analytical laboratory could choose for which metabolite it participated in the ICI/EQUAS, each metabolite measurement is given a data quality label (A-D). Data that were analysed by an analytical laboratory that passed the ICI/EQUAS were labelled "Biomarker data quality assured by HBM4EU QA/QC" (data quality label A). If participation was not successful, the data quality label "Biomarker data not quality assured by HBM4EU QA/QC" (data quality label C) was given. For some studies, sample analyses were performed prior to the HBM4EU project. To use as much data as possible, the quality assurance unit (QAU) within HBM4EU were asked how to evaluate the analytical results gathered outside HBM4EU. If the analytical laboratory that analysed the samples outside HBM4EU did later successfully participated in the ICI/EQUAS within HBM4EU, the data quality label "Biomarker data generated before HBM4EU QA/QC program but deemed comparable by HBM4EU QAU" (data quality label B) was assigned, otherwise it was labelled "Biomarker data generated before HBM4EU QA/QC program but comparability not guaranteed by HBM4EU QAU" (data quality label D) (Gilles et al., 2021). All laboratories determined phthalate metabolites in urine by liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Vorkamp et al., 2021).

2.3. Study and biomarker selection for the MRA

Only exposure data on metabolites for the five selected phthalates (DEHP, DiBP, DnBP, BBzP and DiNP) included in the MRA were considered (Table 1). As the exposure (i.e. metabolite concentration) is directly compared to HBM-GV_{GenPop} derived for the respective metabolite or metabolite combination (Table 1), only studies that delivered data on a minimum set of metabolites were included: mono-benzyl

Table 1
Phthalate compounds and respective exposure biomarkers included in the mixture risk assessment measured in the HBM4EU Aligned Studies and their corresponding HBM-GV_{GenPop}.

Parent compound	Abbreviation	CAS-Number	Specific exposure biomarker (s)	Abbreviation	HBM-GV_{GenPop} children in mg/L	HBM-GV_{GenPop} adults incl. adolescents in mg/L
Di(2-ethylhexyl) phthalate	DEHP	117-81-7	Mono(2-ethyl-5-hydroxyhexyl) phthalate	5-OH-MEHP	For the sum of 5-oxo- & 5-OH-MEHP:	For the sum of 5-oxo- & 5-OH-MEHP:
			Mono(2-ethyl-5-oxohexyl) phthalate	5-oxo-MEHP	0.34	0.5
			Mono(2-ethyl-5-carboxypentyl) phthalate	5-cx-MEPP	For the sum of 5-cx-MEPP & 5-OH-MEHP: 0.38	For the sum of 5-cx-MEPP & 5-OH-MEHP: 0.57
Butylbenzyl phthalate	BBzP	85-68-7	Mono-benzyl phthalate	MBzP	2.0	3.0
Di-n-butyl phthalate	DnBP	84-74-2	Mono-n-butyl phthalate	MnBP	0.12	0.19
Diisobutyl phthalate	DiBP	84-69-5	Mono-iso-butyl phthalate	MiBP	0.16	0.23
Diisononyl phthalate	DiNP	28553-12-0, 68515-48-0	Mono(4-methyl-7-hydroxyoctyl) phthalate	OH-MiNP	pHBM-$GV_{GenPop\text{-}MRA}$* for the sum of cx- & OH-MiNP:	pHBM-$GV_{GenPop\text{-}MRA}$* for the sum of cx- & OH-MiNP:
			Mono(2,7-methyl-7carboxy-heptyl) phthalate	cx-MiNP	0.34	0.51

CAS = Chemical Abstract Service, HBM-GV_{GenPop} = Human biomonitoring guidance value for the general population.
*These are not HBM-GVs derived within HBM4EU and therefore did not undergo a consolidation process with experts from the member countries. For this reason, there are labelled provisional (p) and with MRA (mixture risk assessment). These values were only derived for the purpose of a mixture risk assessment and cannot be used in single substance risk assessment.

phthalate (MBzP), mono-iso-butyl phthalate (MiBP), mono-n-butyl phthalate (MnBP), mono(2,7-methyl-7carboxy-heptyl) phthalate (cx-MiNP), mono(4-methyl-7-hydroxyoctyl) phthalate (OH-MiNP), mono(2-ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP), and either mono(2-ethyl-5-carboxypentyl) phthalate (5-cx-MEPP) or mono(2-ethyl-5-oxohexyl) phthalate (5-oxo-MEHP). For DiNP and DEHP, the HBM-GV_{GenPop} is given for a sum of two oxidised metabolites (in μg/L), i.e. ∑OH-MiNP, cx-MiNP and either ∑5-OH-MEHP, 5-oxo-MEHP or ∑5-OH-MEHP, 5-cx-MEPP with a preference given to the former metabolite concentration. Therefore, the exposure to DiNP and DEHP expressed as the sum (in μg/L) of the two oxidised metabolite concentrations and calculated as such. For three participants, information on 5-oxo-MEHP

Table 2a
Children data sets and main study characteristics included in the phthalate cumulative risk assessment.

European Region	Country	Study Name	N	Sampling Year	Urinary sample type
North	Norway	NEB II	300	2016–2017	First morning urine
	Denmark	OCC	300	2018–2019	Random spot urine
South	Slovenia	SLO CRP	149	2018	First morning urine, Random spot urine
	Greece	CROME	161	2020–2021	First morning urine
East	Hungary	InAirQ	262	2017–2018	Random spot urine
West	France	ESTEBAN	286	2014–2016	First morning urine
	Germany	GerES V-sub (unweighted)	300	2015–2017	First morning urine, Random spot urine
	Belgium	3xG	133	2019–2020	First morning urine, Random spot urine
	The Netherlands	SPECIMEn-NL	89	2020	Random spot urine

N = number of study participants. 3xG = Health – Municipalities – Births study (BE), CROME = Cross-Mediterranean Environment and Health Network study (EL), ESTEBAN = Health study on environment, biomonitoring, physical activity and nutrition study (FR), GerES V-sub (unweighted) = German Environmental Survey 2014–2017 subsample (DE), InAirQ = Transnational Adaption Actions for Integrated Indoor Air Quality Management study (HU), NEB II = Norwegian Environmental Biobank II (NO), OCC = Odense Child Cohort (DK), SLO CRP = Exposure of children and adolescents to selected chemicals through their habitat environment study (SI), SPECIMEn-NL = Survey on PEstiCide Mixtures in Europe (NL).

Table 2b
Adolescents data sets main study characteristics included in the phthalate cumulative risk assessment.

European Region	Country	Study Name	N	Sampling year	Urinary sample type
North	Norway	NEB II	181	2016–2017	Random spot urine
South	Greece	CROME	150	2020–2021	First morning urine
	Slovenia	SLO CRP	96	2018	First morning urine, Random spot urine
	Spain	BEA	300	2017–2018	First morning urine, Random spot urine
East	Czech Republic	CELSPAC: TE	300	2019–2020	First morning urine, Random spot urine
	Slovakia	PCB cohort follow-up	287	2019–2020	Random spot urine
West	Belgium	FLEHS IV	300	2017–2018	Random spot urine
	France	ESTEBAN	304	2014–2016	First morning urine
	Germany	GerES V -sub (unweighted)	300	2015–2017	First morning urine, Random spot urine

N = number of study participants. BEA = Biomonitoring in Adolescents study (ES), CELSPAC:TE = Central European Longitudinal Studies of Parents and Children: Teenagers (CZ), CROME = Cross-Mediterranean Environment and Health Network study (EL), ESTEBAN = Health study on environment, biomonitoring, physical activity and nutrition study (FR), FLEHS IV = Flemish Environment and Health Study IV (BE), GerES V-sub (unweighted) = German Environmental Survey 2014–2017 subsample (DE), NEB II = Norwegian Environmental Biobank II (NO), PCB cohort follow-up = Endocrine disruptors and health in children and teenagers in Slovakia (SK), SLO CRP = Exposure of children and adolescents to selected chemicals through their habitat environment study (SI).

was not available, thus, the sum of 5-OH-MEHP and 5-cx-MEPP was calculated instead.

A prerequisite for the HI approach is the availability of concurrent exposure data for all substances included in the MRA. Therefore, three datasets for children (Poland, Slovakia, Italy) and two for adolescents (Sweden, Poland) had to be excluded as they did not measure all required metabolites. As a result, the European coverage as suggested by the HBM4EU sampling strategy is not fully met neither for children, nor for adolescents. For the children subsample, only two studies from the Southern region (instead of three) and for the adolescent subsample, only one study from the Northern region (instead of two) met the requirements. An overview of studies in children and adolescents included in the current MRA and their main study characteristics can be found in Table 2a,b, respectively. For our study to ensure a European coverage, it was therefore decided that at least one study per European region must be included. In very few exceptional cases single metabolite measurements which did not meet HBM4EU QA/QC criteria and/or data were obtained before organising the HBM4EU QA/QC program, and methods were not included (data quality label C&D) were used. Otherwise, the complete data set of that study would have to be excluded although all other metabolite measurement data were quality assured by HBM4EU's QAU. It was decided to include these measurements for an exemplary assessment of the phthalate mixture risk in European children and adolescents to make use of as much quality-assured data as possible. The majority of biomarker data was quality assured by HBM4EU QA/QC (data quality label A&B) with 97.2% of the metabolite measurements in children and 94.1% in adolescents. Analytical results from GerES V-sub (unweighted) were obtained prior to HBM4EU and were deemed comparable by the HBM4EU QAU. Data included in the current analyses with data quality labels C and D were i) for children: MBzP measurements from the OCC cohort (DK) and cx-MiNP measurements from the NEB-II cohort (NO); and for ii) adolescents: OH-MiNP measurements in the PCB cohort follow-up (SK) and CELSPAC:TE cohort (CZ), cx-MiNP measurements in NEB-II (NO) and MiBP measurements from CELSPAC:TE (CZ) and PCB cohort follow-up (SK).

2.4. Human biomonitoring guidance values (HBM-GVs)

Consolidated HBM-GVs for the general population (HBM-GV_{GenPop}) are available for four of the five substances addressed in the current MRA, namely DEHP, DnBP, DiBP and BBzP. Detailed information regarding their derivation can be found in Lange et al. (2021). Briefly, HBM-GV_{GenPop} were based on the most sensitive endpoint of each substance. Effects were within the anti-androgenic effect spectrum seen in rat offspring after prenatal exposure. HBM-GV_{GenPop} were derived for two different age groups: children (age 6–13 years) and adults including adolescents (≥14 years). In this study, HBM-GV_{GenPop} for children were applied to individuals of the age 13 and younger, and the HBM-GV_{GenPop} for adults were used for participants aged 14 years and older. For four adolescents (12-18-year old) the exact age in years was not available, thus the HBM-GV_{GenPop} for adults was applied.

To include DiNP in the current MRA, provisional HBM-GV_{GenPop} (each for children and adolescents), solely for the purpose of the present MRA were derived and termed pHBM-$GV_{GenPop\text{-}MRA}$ to make the distinction clear. DiNP has been shown to affect male sexual development in the rat, i.e. suppression of foetal testis testosterone after prenatal exposure (Clewell et al., 2013; Furr et al., 2014; Hannas et al., 2011). The potency of DiNP to decrease foetal testis testosterone, however, was lower compared to DEHP (Hannas et al., 2011). DiNP has been suggested to be included in a MRA for male reproductive health as it is regarded to add to the cumulative risk by decreasing foetal testosterone (Apel et al., 2020a; EFSA, 2019; Kortenkamp, 2020; Kortenkamp and Koch, 2020; US CPSC, 2014). EFSA (2019) and Kortenkamp and Koch (2020) identified the study by Clewell et al. (2013) as critical for reproductive effects on DiNP (EFSA, 2019; Kortenkamp and Koch, 2020). Clewell et al. (2013) exposed pregnant rats by gavage to 0, 50, 250 or 500 mg DiNP/kg bw/d from gestational day 12–19. At a dose of 250 mg/kg bw/d decreased foetal testis testosterone production and multinucleated gonocytes were observed and the dose was identified by the authors as lowest observed effect level (LOEL) and 50 mg/kg bw/day as no observed effect level (NOEL). Kortenkamp and Koch (2020) proposed a reference dose for male reproductive toxicity suitable for a phthalate MRA (RfD_{AA}) of 59 μg/kg bw/d based on a benchmark dose lower bound (5% benchmark response: testosterone suppression; $BMDL_{05}$) of 5.9 mg/kg bw/d for foetal testis testosterone synthesis suppression observed in the study by Clewell et al. (2013) (Kortenkamp and Koch, 2020). According to the derivation strategy of HBM-GV (Apel et al., 2020b), pHBM-$GV_{GenPop\text{-}MRA}$ for DiNP were derived for children and adults including adolescents using i) the $BMDL_{05}$ of 5.9 mg/kg bw/d from Kortenkamp and Koch as TRV-like value; ii) toxicokinetic data (i.e. fractional urinary excretion factors for OH-MiNP and cx-MiNP) from Anderson et al., 2011); and iii) a factor of 10 each for inter-and intra-individual variability. It is important to note, that for the derivation of consolidated HBM-GV_{GenPop}, the most sensitive endpoints for the respective compounds were chosen, while for the pHBM-$GV_{GenPop\text{-}MRA}$ derived for DiNP this is not the case. Instead, a common anti-androgenic reprotoxic endpoint was chosen (i.e. suppression of foetal testicular testosterone synthesis).

2.5. Mixture risk assessment and the use of precautionary factors

For the risk assessment, the concentration of a metabolite or of the sum of metabolites (in μg/L) of each individual phthalate (i.e. ∑DEHP metabolites, MiBP, MnBP, MBzP, ∑DiNP metabolites) was divided by their respective HBM-GV_{GenPop}/pHBM-$GV_{GenPop\text{-}MRA}$ to obtain a risk quotient (RQ) for each substance. RQs for each phthalate included in the MRA were then summed to gain the hazard index (HI) per study participant i according to the following formula:

$$HI_i = RQ_{DnBP,i} + RQ_{BBzP,i} + RQ_{DiBP,i} + RQ_{DEHP,i} + RQ_{DiNP,i}$$

Several different classes of chemicals that act by dose addition in mixture models have recently been compiled and reviewed by Howdeshell et al. (2017). Orton et al. (2014) have compiled 24 current use and environmentally relevant pesticides and 17 non-pesticidal pollutants (e.g. parabens, benzophenones, perfluorooctane sulfonate (PFOS), galaxolide, tonalide, BDE100, 4-MBC, and PCB13) that have anti-androgenic properties and produce combination effects. Kortenkamp (2020) concluded, that a minimum set of chemicals to be assessed together with phthalates includes certain pesticides (vinclozolin, prochloraz, procymidone, linuron), pain killers (paracetamol, aspirin and ibuprofen), some pharmaceuticals (finasteride, ketoconazole, and the lipid-lowering drug simvastin), poly-chlorinated dibenzo-dioxins and other dioxin-like pollutants and phenolics (bisphenol A, butylparaben). Thus, "precautionary factors" of 5 and 10 to account for co-occurring anti-androgenic substances that contribute to the risk of adverse effects on reproduction have previously been applied to the HI (Apel et al., 2020a; Kortenkamp and Koch, 2020), yielding adapted HIs of 0.2 and 0.1. As the evaluation of a mixture risk from all these substances goes beyond the scope of our study, we not only evaluated our data towards the HI of 1 but also to adapted HIs of 0.2 and 0.1. The percentage of participants exceeding these adapted HIs were calculated.

The maximum cumulative ratio (MCR) for each participant was calculated by dividing each participant's HI by their individual maximum RQ (RQ_{max}) as introduced by Price et al. (2012) and applied for phthalate mixture risk assessments by Apel et al. (2020a). Scatterplots depicting MCR vs. HI were created. MCRs are calculated to understand whether the risk from combined exposure is driven by a single chemical or multiple chemicals. For participants with MCR < 2, one phthalate contributes to the majority of the combined risk, whereas the opposite is true for participants with MCR > 2.

Finally, to estimate the effect of major exposure determinants (age, sex, European region, sampling year) on individual HI levels a

multivariate linear regression analysis was done using the Generalized Linear Model (GLM) including all four predictors simultaneously. The model was specified with a log link function to account for the non-normal distribution of HIs (not shown) and survey procedures to account for higher similarity of individuals within each study.

Statistical analyses were performed on individual data in RStudio (RStudio Team, 2021). Values below limits of detection (LOD) and limits of quantification (LOQ) and values between LOQ and LOD were imputed per biomarker and study when there were at least 30% of detected values (applicable to all biomarkers reported here) (Lubin et al., 2004). Details will be found in Govarts et al., (*submitted*).

3. Results and discussion

In our study, exposure data from eight phthalate metabolites analysed in urine samples of 4,198 children and adolescents aged 6–18 years from 12 countries is used (see Table 2a,b). Male and female participants are equally represented, but there are slightly more adolescents (n = 2,218) than children (n = 1,980) (see Table 3). In children, quantification frequencies (QFs) are very high (87% or higher ≥ LOQ) for all, except for DiNP metabolites in Belgian (Flemish) children (see supplementary material, Table S1). In European adolescents QFs are even higher, with each phthalate metabolite quantified in at least 91% of the study subsamples (Table S1). The high QFs confirm the concurrent exposure and thereby verify the selection of DEHP, DnBP, DiBP, DiNP and BBzP for our MRA.

3.1. Mixture risk of phthalates in European children and adolescents

A summary of descriptive statistics on the hazard indices per subsample, including GM, 95th percentiles, corresponding 95th confidence intervals and range can be found in Table 3. On average, exposures of European children and adolescents result in HI at GM of 0.44, suggesting no risks from combined exposure to the five phthalates. At the 95th percentile, however, HI are above the limit of acceptable risk (HI = 1.77). Children show slightly higher average HI (GM = 0.47) than adolescents (GM = 0.41) and *vice versa* at the 95th percentile (HI = 1.71 vs. 1.86) (Table 3). No significant difference in the average level of HI between the age groups (Fig. 3, panel A), nor the sexes (Fig. 3, panel B) is observed in GLM models when controlling for the other three variables.

Fig. 1 shows the analysis of combined exposure of all study participants (children in purple, adolescents in green) with individual HI versus MCR depicted in a scatter plot. One child, depicted to the utmost right, is reaching an HI as high as 119. Analysing the percentage of the population with HI > 1, gives an indication of the population share that exceeds exposures deemed tolerable for the above five phthalates. Our analysis show, that while at population level, no indication of a mixture risk is observed, however, at individual level 17% (n = 708) of all children and adolescents investigated exceeded HI of 1, thus have high

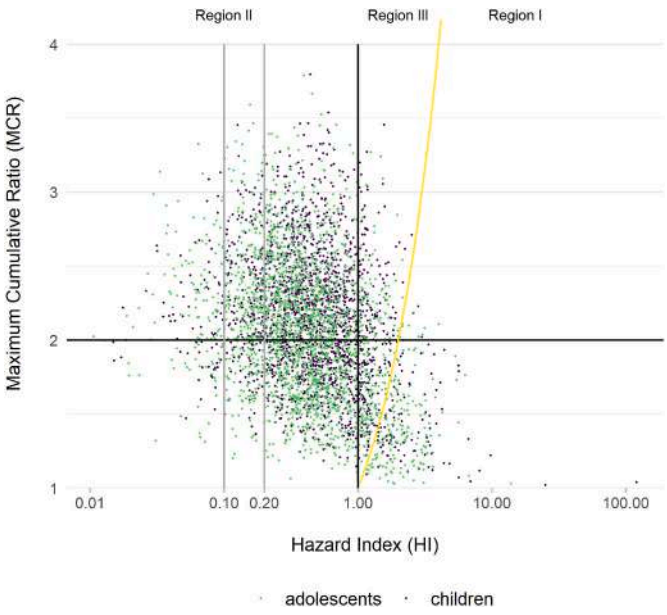

Fig. 1. Analysis of combined exposure to five phthalates in European children and adolescents
Presentation of maximum cumulative ratio (MCR) versus hazard index (HI) as scatter plot according to Apel et al. (2020a) with risk management categories (Region I-III) introduced by Price et al. (2012). Please note, the Region I-III introduced by Price et al. (2012) are not geographical areas as defined in the term "European regions" (referring to East, West, South, North Europe). Dots represent each study participant, with purple dots for children and green dots for adolescents. The horizontal line represents MCR = 2; the black vertical line represents HI = 1; the grey vertical lines represents adapted HI = 0.2 and HI = 0.1; the curved yellow line represents MCR = HI. Definition of regions according to Price: Region I depicts combined exposures of concern as one or more individual chemicals exceed the HBM-GV (area right to yellow line); Region II (area left to black vertical) depicts combined exposures where there is a low concern for both individual chemicals and for their combined effects (HI < 1); Region III (area between black vertical line and yellow curved line) depicts combined exposures with a low concern for individual chemicals, but concern for the combined effects (all RQs < 1, but HI > 1). Dots below the horizontal black line (MCR < 2) represent combined exposures in which one chemical accounts for the majority of combined exposure, whereas dots above the vertical black line (MCR > 2) represent combined exposures driven by multiple chemicals. Individual HIs range from > 0.01 to 119. 17% of the study participants have HIs > 1 of which the majority (~63%) lies in Region III. Thus, they would have gone unnoticed in single substance risk assessment. Approximately 60% of the children and 54% of the adolescents have MCRs above 2, showing that the combined exposure to phthalates is driven by multiple substances instead of only one. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Descriptive statistics for hazard indices (HI) in European children and adolescents.

	N	GM_{HI}	95%th CI	$P95_{HI}$	95%th CI	Min_{HI}	Max_{HI}
Total study sample (6–18 years)	4,198	0.44	0.42–0.45	1.77	1.69–1.86	0.42	1.86
Female	2,098[a]	0.44	0.42–0.46	1.84	1.72–2.07	0.43	2.07
Male	2,097[a]	0.43	0.42–0.45	1.72	1.6–1.84	0.41	1.84
Children (6–11* years)	1,980	0.47	0.45–0.48	1.71	1.58–1.82	0.45	1.82
Adolescents (12–18 years)	2,218	0.41	0.39–0.42	1.86	1.74–1.98	0.38	1.98
Total study by region (6–18 years)							
North	781	0.42	0.4–0.45	1.75	1.48–2.01	0.38	2.01
South	856	0.36	0.34–0.38	1.38	1.19–1.54	0.34	1.54
East	849	0.58	0.55–0.62	2.42	2.16–2.8	0.56	2.8
West	1,712	0.42	0.4–0.44	1.51	1.4–1.67	0.41	1.67

N = number of study participants, GM = geometric mean, CI = confidence interval; Min = minimum value of HI; Max = maximum value of HI. * For ESTEBAN and GerES V-sub (unweighted) 18 and 19 participants, respectively were 12 years old at the time urine samples were collected. [a] For three children, no information on sex is available.

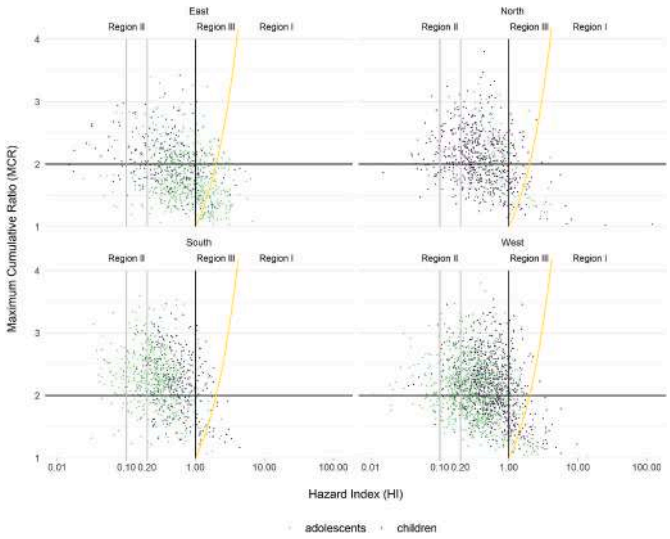

Fig. 2. Analysis of combined exposure to five phthalates per European region

Analysis of combined phthalate exposure for children and adolescents per European region with MCR versus HI scatter plots. The results of the individual study participants are shown as dots (purple = children; green = adolescents). The vertical black line shows HI = 1, the vertical grey lines the adapted HI = 0.2 and HI = 0.1. The horizontal black line depicts an MCR = 2 and the curved yellow line represents MCR = HI. Please note, the Region I-III introduced by Price et al. (2012) are not geographical areas as defined in the term "European regions" (referring to East, West, South, North Europe), but risk management categories (more details can be found in the description of Fig. 2). Highest percentage above HI = 1 is observed for participants from Eastern Europe (31%), whereas the percentages of other European regions with HI > 1 are similar (11–15%). The high percentage in the Eastern region is due to the high percentage of adolescents above HI = 1 (37% versus 17% for children). In Eastern Europe for the majority of children and adolescents, only one phthalate does drive the HI with 64% of the participants having MCR < 2, whereas for Southern Europe the mixture risk is driven by multiple phthalates (with 70% of participants having MCR > 2). For the majority of participants from Northern and Western Europe multiple phthalates contribute to the HI (57% and 60%, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mixture risks from the above five phthalates (Table 4). Depending on age and sex, these exceedances varied between 15 and 19%. The percentage of the two age groups above the hazard indices per study can be found in the supplementary material (Tables S2a and b).

MCRs indicate how many chemicals drive the mixture risk. Slightly more than half of the study population (57%, n = 2,372) have MCR > 2, thus their HI is driven by multiple phthalates rather than by only one compound (MRC < 1) (Fig. 1, MCR = 2 is depicted by the black horizontal line). In comparison, the share of children for which the combined risks are driven by multiple phthalates is higher (60%, n = 1,178) than in adolescents (54%, n = 1,194).

3.2. Drivers of the mixture risk

In the total sample, DnBP and DiBP contribute most to the HIs (Table 5). In about half (52%, n = 2,170) of the study participants the highest risk quotients are observed (RQ_{max}) for DnBP, followed by DiBP in 42% of participants (n = 1,767). The percentage of participants with RQ_{max} for DEHP and DiNP are over ten times lower with 3%. BBzP does not reach RQ_{max} in any of the participants. A similar pattern (DnBP > DiBP) is observed for adolescents (54 and 39%) as for the total sample, while it was slightly different for children. DiBP and DnBP more or less equally contributed to the HI (45% and 49%, respectively, Table 5) of children exposures.

Table 4
Percentage of individuals exceeding respective hazard indices (HIs) per subsample.

	N	% > HI = 1	% > HI = 0.2	% > HI = 0.1
Total study sample (6–18 years)	4,198	17	83	95
Female	2,098[b]	18	82	95
Male	2,097[b]	16	84	96
Children (6–11[a] years)	1,980	17	86	96
Female	974[b]	19	85	96
Male	1,003[b]	16	86	97
Adolescents (12–18 years)	2,218	17	80	94
Female	1,124	18	79	94
Male	1,094	15	81	95
Total study by region (6–18 years)				
North	781	15	83	95
South	856	11	79	94
East	849	31	88	96
West	1,712	14	82	96
Children (6–11[a] years)				
North	600	13	79	93
South	310	18	93	99
East	262	18	78	92
West	808	20	90	99
Adolescents (12–18 years)				
North	181	20	96	100
South	546	7	70	91
East	587	37	92	97
West	904	9	74	93

N = number of study participants; HI = hazard index. All values are rounded.
[a] For ESTEBAN and GerES V-sub (unweighted) 18 and 19 participants, respectively were 12 years old at the time urine samples were collected.
[b] For three children, no information on sex is available.

Table 5
Percentage of study population in which the respective phthalates reached RQ_{max}.

	DnBP	DiBP	DiNP	DEHP
Total study sample (6–18 years)	52	42	3	3
Female	51	43	4	2
Male	52	42	3	3
By region				
North	60	35	4	2
South	43	51	3	4
East	64	30	3	3
West	46	47	4	3
Children *(6–11* years)*	49	45	3	4
Female	47	47	3	2
Male	50	43	2	4
Adolescents *(12–18 years)*	54	39	4	2
Female	55	38	4	2
Male	54	40	3	3

RQ_{max} = maximum risk quotient. The proportion of individuals that reached RQ_{max} are presented for DEHP, DnBP, DiBP and DiNP. BBzP did not reach RQ_{max} in any of the participants. Sums of each row add up to 100%. Deviations are due to rounded values. *For ESTEBAN and GerES V-sub (unweighted) 18 and 19 participants, respectively were 12 years old at the time urine samples were collected.

Follow-up GLM analyses investigating the role of sex and age on HI levels reveal no difference between children and adolescents or between sexes (Fig. 3, panel A and B). Median cumulative risk quotients for all five phthalates and age group can be found in Fig. 4a and b.

Our results are in line with previous findings. In the REACH restriction proposal of phthalates, a combined risk assessment of four phthalates (DEHP, DnBP, DiBP, BBzP) was conducted based on 95th percentile urinary biomonitoring exposure levels of children from DEMOCOPHES data (2011–2012). Whereas BBzP did not at all, DnBP and DiBP contributed most to the risk of combined exposures in European children (ECHA, 2017a; 2017b). Furthermore, in a combined risk

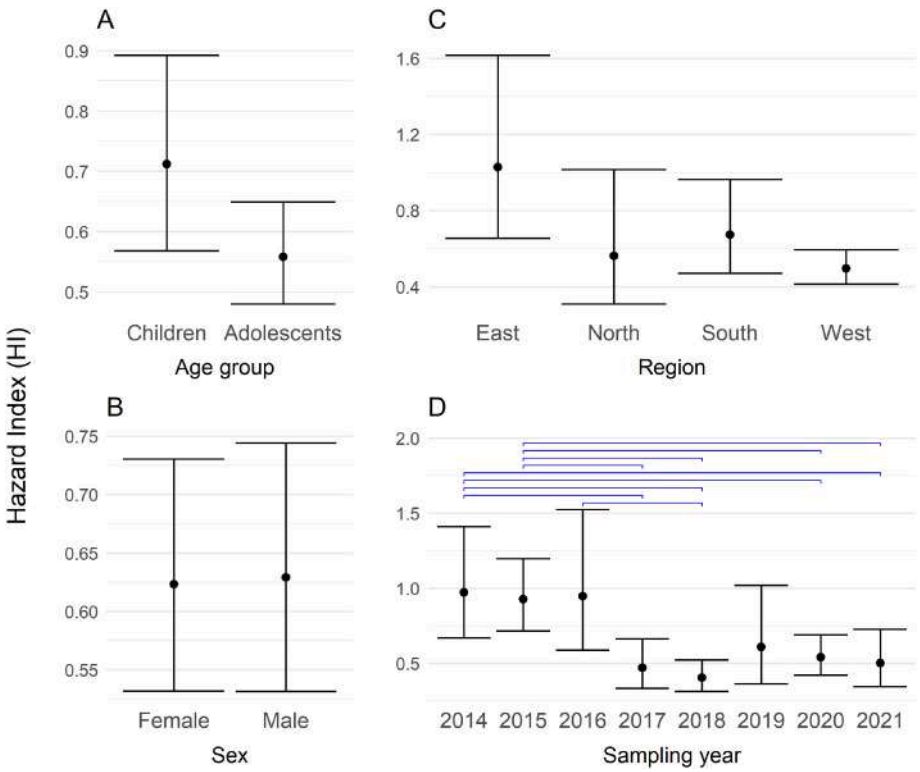

Fig. 3. Results of the multivariate linear regression analysis (GLMs)
Depicted are the results from the multivariate linear regression analysis (GLMs) predicted by age (panel A), sex (panel B), European region (panel C), and sample collection year (panel D) controlling for the other three predictors in each case, respectively. Black dots represent averages of the hazard indices (HI). Black bars represent the corresponding confidence intervals. No statistically significant difference in the level of the HI were obtained for sex or age. Eastern participants have significant higher HI levels than Southern and Western participants. Earlier sample collection years have significant higher HI levels compared to later years (blue brackets). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

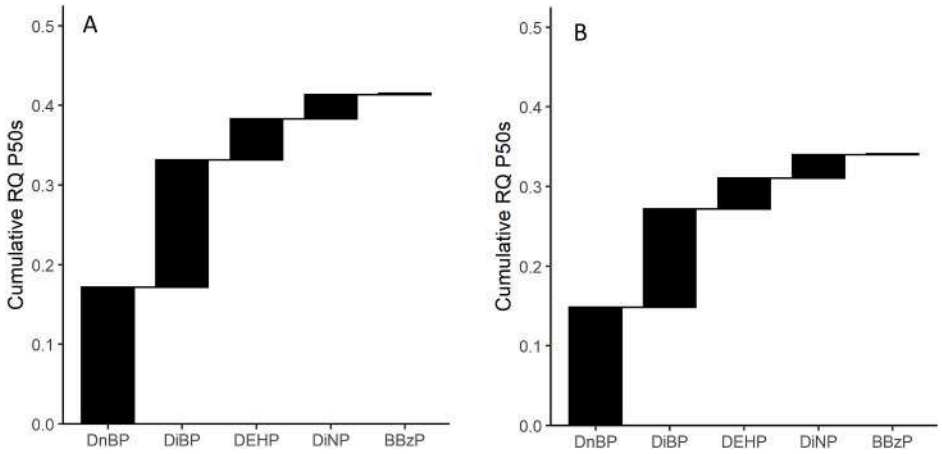

Fig. 4. Median cumulative risk quotients (RQs) for European children and adolescents for five phthalates
The 50th percentiles (P50) of the risk quotients (RQs) per phthalates are displayed (black bars) in a cumulative fashion for the European children (Figure 4Fig. 4a) and adolescent subpopulation (Fig. 4b). DnBP (P50 = 0.17 children; P50 = 0.15 adolescents) and DiBP (P50 = 0.16 in children; P50 = 0.12 in adolescents) are the main drivers for the mixture risk in both subpopulations. Cumulative RQs (corresponds to hazard index) at P50 are for 0.4 children and 0.34 for adolescents.

assessment of the above five phthalates in Finnish adults, also DnBP and DiBP appeared to be the main drivers, whereas in German adults the main risk driver is DEHP, followed by DnBP (Apel et al., 2020a; Porras et al., 2020). As underlying toxicological endpoints and subsequent derived reference doses used as toxic unit values to assess the risk are not the same throughout these risk assessments and these do largely affect which phthalates does drive the mixture risk, comparison need to be done with caution (Kortenkamp and Koch, 2020; Søeborg et al., 2012). Considering only those participants with HI > 1, the two main risk drivers remain DnBP and DiBP. Contributions of phthalates to HIs are very similar: In 51% (n = 364) of the study participants mixture risks are driven by DiBP, followed by DnBP (40%, n = 283), and DEHP and DiNP (4 and 5%). In 6% (n = 260) of the European children and adolescents at least one RQ for any phthalate is > 1, thus having levels exceeding HBM-GVs (Fig. 1, Region I) with more adolescents (7%, n = 161) above respective HBM-GVs, than children (5%, n = 99) (data not shown). Exceedances are mostly observed for DnBP and DiBP with 3% of all participants exceeding the HBM-GV_{GenPop} for DnBP (n = 141) and DiBP (n = 111), and to a lesser extent DiNP and DEHP (≤ 0.5%). Only one child has exposure levels of MBzP above the respective HBM-GV_{GenPop}. Considering only adolescents, the contribution is similar with most exceedances observed for DnBP (5%) > DiBP (2%) > DiNP (0.5%) > DEHP (0.3%). In children, however, most exceedances are observed for DiBP (3%), followed by DnBP (2%) and only few exceeds HBM-GVs for DiNP (0.5%) and DEHP (0.4%). It is noteworthy, that of those individuals who exceeds HBM-GVs, mostly only one phthalate exceeds the respective HBM-GV_{GenPop}. In only few cases an individual exceeds HBM-GV_{GenPop} of two substances at the same time, and only in one case exceeds three substances simultaneously. The pHBM-$GV_{GenPop\text{-}MRA}$ for DiNP is not consolidated within HBM4EU and solely derived for the purpose of the MRA based on a common anti-androgenic endpoint. As for DiNP toxicity the liver is the most sensitive target organ, the HBM-GV is not suitable for single substance risk assessment (EFSA, 2019; Kortenkamp and Koch, 2020). Exceedances of the pHBM-$GV_{GenPop\text{-}MRA}$ were

reported solely for the purpose of reporting the share of DiNP to the mixture risk and do not indicate a risk from adverse effects of DiNP exposure.

Strikingly, of those participants who have HIs >1, the majority (63%, n = 446) would have not been identified as being at risk in traditional single compound risk assessment as all single substances are below HBM-GVs (see Fig. 1, Region III). Comparing the subsamples, this contribution is higher in children with 70% (n = 239), whereas considering only adolescents about half (56%, n = 207) would have gone unnoticed in single substance risk assessments. This highlights the urgent need to include mixture risk assessment approaches in current risk assessment practices on a regular basis. The restriction of four reprotoxic phthalates in consumer articles serves as good example. In the joint proposal by ECHA and the Danish Environmental Agency a cumulative risk assessment based on exposure data for DEHP, DnBP, DiBP and BBzP from the DEMOCOPHES program was conducted and based on its outcome were further restriction in plasticised articles (EC, 2018; European Chemicals Agency ECHA and Danish Environmental Protection Agency, 2016; ECHA 2017a, 2017b).

3.3. Comparison of European regions

Comparison of European regions reveal the Eastern region is markedly different with highest HI at GM of 0.58 (P95 = 2.42). Lowest HI (GM = 0.36, P95 = 1.38) is found in the Southern region, and the Western and Northern region have similar HIs at GM (GM = 0.42, P95 = 1.51 and GM = 0.42, P95 = 1.75, respectively). Results from GLMs indicates that Eastern participants have significant higher average HI levels than Southern and Western participants, when controlling for sex, age group and sampling year (see Fig. 3, panel C). No significances emerged from the pairwise comparison of the Eastern region with the Northern region. Despite the alignment and post-harmonisation of the different studies in the HBM4EU Aligned Studies, some differences remain (Gilles et al., 2021) and consequently, caution must be given in regard to the comparability of results of single data sets. No conclusions can be drawn from single data sets of a country to the whole country itself as data sets used are not nationally representative, even though some subsets used here were based on nationally representative HBM programs (i.e. ESTEBAN, GerES V-sub). Furthermore, the single studies differ in sampling periods, age distribution and study design including urine sampling method (Gilles et al., 2022). Therefore, only European regions comprised of data sets from different countries are compared to one another in this analysis. The influence of differences in study design will decrease to a certain extent by pooling the single data sets into European regions, however, uncertainties remain with regard to the validity of the extrapolation of the results per European region that might lead to over- or underestimation of the real risk for that European region. A major limiting factor for comparability of the HBM4EU Aligned Studies is the large time span of the sample collection period (2014–2021) that might confound the metabolite levels. Indeed, certain phthalates, i.e. DEHP, DnBP, DiBP and BBzP have been shown to decrease considerable over time in European adult populations, whereas a clear trend for DiNP across European adult populations cannot be observed. While DiNP exposures decreased over time in Denmark, no such trend can be observed in recent years in Germany and Sweden (Frederiksen et al., 2020; Gyllenhammar et al., 2017; Koch et al., 2017). However, these time trend studies only included exposures until 2014 (Sweden), 2015 (Germany) and 2017 (Denmark). To investigate the possible impact of temporal changes in exposures to the level of HIs, sampling year as predictor was included in GLM analyses. No linear effect of sampling year on the HI was found, controlling for age, sex and European region, but differences in the pairwise comparison of sampling years emerged as significant (Fig. 3, panel D). Participants whose urine samples were taken in the earlier years (2014–2016) seem to have higher average HI levels than participants from studies with later sampling periods (2017–2021). In detail, the pairwise comparisons suggest that participants whose urine samples were collected in 2014 have, on average, higher HI levels than participants that were sampled in 2017, 2018, 2020 and 2021. The same effects are observed for urine samples from 2015, whereas the only pairwise comparison emerged significant for participants sampled in 2016 is that they have higher average HI levels than participants sampled in 2018. Most of the data from the earlier sampling years (2014–2016) are from studies of the Western region, whereas for later sampling years (2017–2020) pooled data come from all geographical regions. Models also indicate an interaction between European region and sampling year suggesting that for some European regions differences between sampling years are stronger than others. Since the data is based on cross-sectional data from studies with different sampling frames we refrain from going into detail here. The reported effects might be confounded with study characteristics. The sampling years 2014 and 2021 only have data from one study each (ESTEBAN and CROME), not allowing comparisons between data collections. For the sampling years 2016–2020 data from at least three studies per year are pooled, thereby increasing the validity of the observed temporal differences in average HI levels.

Considering the individual level, participants from the Eastern region are most at risk from phthalates mixture exposure with one third (31%) having HIs >1 (Table 4). The other European regions are in the range of 11–15% with the lowest percentage with HI > 1 observed for participants living in Southern Europe (11%). A higher share of children with HI > 1 is observed for children from Southern (18%) and Western Europe (20%) than adolescents from that region (7 and 9%, respectively) and *vice versa* for the Eastern (18 vs. 37%) and Northern (13 vs. 20%) regions (Table 4). Interpretation needs to be drawn with caution since only one study each representing European children in Eastern Europe (InAirQ) and European adolescents in Northern Europe (NEB II). In addition, not all studies provided data on both, children and adolescents. Data on both age groups is only available for GerES V-sub (unweighted), ESTEBAN, SLO-CRP and CROME. Again, apparent differences between the age groups at regional level might be confounded with study characteristics.

Further differences in Eastern Europe are observed as for the majority of Eastern children and adolescents, only one phthalate drives the HI, namely DnBP, with 64% of the participants having MCR < 2, whereas for Southern Europe the mixture risk is driven by multiple phthalates (with 70% of participants having MCR > 2). For the majority of participants from the North and West of Europe multiple phthalates contributes to the HI (57% and 60%, respectively) (see Fig. 2).

In all four European regions, DiBP and DnBP contribute mostly to the HI explaining more than 90% of the HI, whereas the contribution of DEHP and DiNP are negligible (Table 5). In the Eastern and Northern region, the proportion of RQ_{max} for DnBP is twice as high as for DiBP, whereas in the Western region RQ_{max} for DnBP and DiBP are equally distributed. When considering only the subsample of children and adolescents with HI > 1, for most regions DiBP is the main driver of the mixture risk, followed by DnBP, except for the Eastern region (data not shown). Here, DnBP is by far the main driver for which 76% of the children and adolescents reach RQ_{max}. Taken together, this might indicate different usage patterns of phthalates in the European regions, that result in higher exposure levels for Eastern children and adolescents, but this is rather speculative and follow-up studies are needed to investigate possible differences in exposure sources in the European regions. Nevertheless, our results on the identification of risk drivers can serve as basis for setting priorities for further research in terms of regional differences and specifically for risk managers where to start for possible refinement of mitigation measures. DEHP, DnBP, DiBP and BBP are already strictly regulated under REACH. The extension of the REACH restriction to plasticised articles came into force only 2020, thus, data from the HBM4EU Aligned Studies used in our analysis collecting samples in 2014–2021 is not able to show the effectiveness of this restriction in the management of risk. Only one study sampled participants in 2021 (CROME). In addition, these phthalates are still allowed to be used in

food packaging and medicinal products, and with our study it is not possible to inform on the contribution of these (or other) exposure sources to total exposure.

Most exceedances of single guidance values are observed by far in the Eastern region, with 14% (n = 122) of Eastern children and adolescents are above single HBM-GVs. From those, the majority are adolescents (89%) and most exceedances are observed for DnBP (79%). For the other European regions, percentages of exceedance are similar, ranging from 3 to 5% (see Fig. 2, Region I). Here, in contrast to the subsample from Eastern Europe, children have most exceedances (on average 60%) and for all three geographical regions most exceedances are observed for DiBP. More extended information and details on single substance risk assessment of phthalates in European children and adolescents will be found in Vogel et al., (submitted). As can be seen in Fig. 2 in Region III, especially in the Southern and Western region, children and adolescents with high risks from cumulative exposure to the five phthalates would not have been detected in single substances risk assessment (74 and 69%, respectively).

Besides the differences between studies in their sample collection periods, there are other uncertainties that affect the comparability of the studies and consequently the comparability of European regions. In about half of the studies included in our analysis, spot urine samples were collected, and the other half did sample morning urine samples (GerES V-sub, SLO CRP, ESTEBAN, CROME, BEA, 3xG, CELSPAC:TE). Considering the short-half lives of the phthalate metabolites in the human body, both spot and morning urine measurements can only reflect recent exposure and temporary intra-individual variability cannot be assessed using single samples. This may lead to over- or underestimation of exposure depending on the time span between exposure and sampling. Given the likely longer time span between exposure and sample collection with morning urine samples compared to spot urine samples, there might be a difference in dilution of the metabolite concentration between these two sampling methods. However, this is assumed to be negligible as morning urine as well as spot urine sample seem to be as useful as 24h-urine samples for the assessment of phthalate metabolites exposure in population studies (Frederiksen et al., 2013).

3.4. Uncertainties in the health risk assessment

The HBM-GVs are based on the most sensitive effects which are on male reproductive health when the male foetus was exposed gestationally. As the current MRA is conducted for both, the male and (non-pregnant) female subpopulation of children and adolescents and not the foetus, the predicted risk for female participants and for adolescents might be overestimated. However, phthalate exposure at environmental concentrations has been associated with male and female reproductive impairment in humans when exposed as a child or adult (Frederiksen et al., 2012; Jurewicz and Hanke, 2011; Radke et al., 2018, 2019). Despite toxic effects on reproduction and development, epidemiological studies indicate a possible association of phthalate exposure (DEHP, DnBP, DiBP) and obesity, diabetes and insulin resistance (Dales et al., 2018; Jurewicz and Hanke, 2011; Kim et al., 2013; Radke et al., 2018; Zhang et al., 2022). There is also increasing evidence that phthalates have a negative effect on the immune system, in particular an increased risk of developing asthma has been postulated (Bornehag and Nanberg, 2010; Franken et al., 2017; Wu et al., 2020). Likewise, negative effects on cognitive and neurological development are possible (Benjamin et al., 2017; Olesen et al., 2018), but the data on this is not clear (Benjamin et al., 2017; Hyland et al., 2019; Radke et al., 2020). The risk assessment committee (RAC) of the European Chemicals Agency acknowledged that it cannot be excluded that effects on the immune system and/or metabolic system might be equally or even more sensitive than the effects on male reproductive health (ECHA, 2017a; EFSA, 2019). Overall, uncertainties remain, whether the risk from concurrent phthalate exposure is over- or underestimated based on the selected common endpoint on male reproductive development after prenatal exposure.

3.5. Practical considerations in assessing the mixture risk: using precautionary factors

Already without including other anti-androgenic chemicals in this analysis, exceedances are substantial. When comparing the data to the adapted HIs (HI = 0.2 and HI = 0.1) to account for these substances, the percentage exceeding these HIs increase to 83% (HI = 0.2) and 95% (HI = 0.1) (Table 4). Although the amount of these precautionary factors is currently under discussion, they are not implausible (Apel et al., 2020a; Kortenkamp and Koch, 2020; KEMI, 2015; Van Broekhuizen et al., 2016). Structural analysis data suggests that phthalates with a linear ester side length of 4–7 carbon atoms in total, such as DnBP, di-n-pentyl phthalate (DnPeP), di-n-hexyl phthalate (DnHP), di-n-heptyl phthalate (DHP) and phthalates with a branched or non-linear side chain of 4–9 carbon atoms in length, such as DEHP, DiNP, BBzP, DiBP, di-isopentyl phthalate (DiPeP), diisoheptyl phthalate (DiHP), and dicyclohexyl phthalate (DCHP), are toxic to male development (Furr et al., 2014; Kortenkamp and Koch, 2020; Li et al., 2019). Thus, in the group of phthalates alone, six other substances not included here could add to the risk not assessed in the current analysis. DnPeP and DCHP are excluded from our MRA, as metabolites were either not assessed (in 3xG, CELSPAC:TE, PCB cohort follow-up, FLEHS IV), or metabolites were only detected in very few or no samples at all (0–32% > LOQ) and co-exposure is not given for the majority of the study participants (Vogel et al., *submitted*). For those individuals with exposure levels > LOQ these substances could, however, potentially add to the mixture risk. As previously mentioned, beside phthalates, other anti-androgenic substances can contribute to the risk of reproductive malformations, such as pesticides, parabens, pharmaceuticals (Conley et al., 2018, 2021; Kortenkamp, 2020; Rider et al., 2010). Although it can be assumed that a more complex mixture interaction than mere dose addition is present in humans, it has been shown that dose addition best predicts the mixture effects of some of these anti-androgens, although they do not act via the same mechanism of action or even the same pathway (Christen et al., 2012; Howdeshell et al., 2017; Rider et al., 2010). In reality the exact composition of real mixtures within a human body, i.e. individual chemicals that may act together adversely is unknown, nor are sensitive analytical methods in place for the comprehensive analysis of all chemicals. It is therefore not feasible to assess the real risk from anti-androgenic chemical mixtures on reproductive health. To reach the goals of the EU's chemicals strategy for sustainability towards a toxic-free environment (EC, 2020), risks from chemical mixtures need to be assessed and recommendations to risk managers and policy makers need to be formulated to protect the European population. The precautionary factors are a tool to approximate to the real mixture risk to reproductive health from concurrent exposure to multiple chemicals and to get an impression of the level of concern. In the future, more knowledge will become available on real-life mixtures in the human body and their biological interactions that may lead to an adverse outcome. For the time being, lowering the HI can serve as an easy and practical approach to identify priorities for risk managers and policy makers. Our analysis suggest that risks are heavily underestimated given the vast number of anti-androgenic chemicals present in the human body not included in the current MRA. Consequently, our findings highlight the need to adapt current risk assessment practices to truly protect children and adolescents from irreversible effects that might only become apparent later in life.

4. Conclusion

Our results indicate that 17% of the European children and adolescents are at risk from concurrent exposure to five reprotoxic phthalates. We could show that while there was no significant influence of sex (male vs. female) or age (children vs. adolescents), the geographical region

and the sampling year seem important with highest average HI in the Eastern European region and in the earlier sampling year (2014–2016). The two phthalates DnBP and DiBP were the clear drivers of the mixture risk in all cases. Strikingly, for about 63% the risk from combined phthalate exposure would have gone unnoticed in a single substance evaluation. This demonstrates the urgent need to incorporate mixture risk assessment into current regulatory practice at a regular basis. The overall HI of 0.44 at GM of the aligned study population also shows, that the buffer to potential exceedances is rather small. Consequently, if considering likely co-exposures to other anti-androgenic chemicals by adjusting the acceptable HI by a factor of 5 or 10, substantial exceedances of 83% and 95% are observed. While acceptable HIs lowered to 0.1 or 0.2 might be tackled as too conservative, a wealth of data proves dose additivity, especially of anti-androgenic substances (Conley et al., 2021; Howdeshell et al., 2017; Orton et al., 2014). Thus, the more anti-androgens are assessed in human biomonitoring studies, the higher the actual average HI for the population would become. It remains to be seen what HI will be reached in these population samples, if exposures to other chemicals were to be included. Recently derived reference doses for anti-androgenic mixture risk assessments of polybrominated diphenyl ethers (PBDEs) (Ermler and Kortenkamp, 2022) and bisphenol A (Kortenkamp et al., 2022a) in conjunction with known population exposures indicate substantial contributions of these substances to the mixture HI of anti-androgenic substances. Just recently, Kortenkamp et al., 2022a showed that the combined exposures to 29 chemicals including bisphenols, polychlorinated dioxins, paracetamol, and phthalates substantially exceeds HI of 1 to more than 100-fold in an individual and 17-fold at median for 9 chemicals alone. Each of the participants exceeded an HI of 1 for the 9 chemicals jointly measured in urine samples. For the total study population and all 29 chemicals a median HI of 20 was observed (Kortenkamp et al., 2022b). The results observed in the Kortenkamp et al. (2022b) study, supports our practical approach to use a precautionary factor to account for other anti-androgenic chemicals. Further, it strengthens the hypothesis that the actual risk on reproductive health from mixture exposure of anti-androgenic chemicals is higher than indicated in this or previously conducted MRA including only substances from similar chemical classes. Our study underlines the need for follow-up investigations of human internal exposures of the European population to chemical mixtures by continuous HBM studies harmonised at EU level.

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Declaration of competing interest

The authors declare no conflict of interest related to this work.

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Appendix A. Supplementary data

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Exposure errors due to inaccurate residential addresses and their impact on epidemiological associations: Evidence from a national neonate dataset

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ABSTRACT

Background: Studies assessing the associations between prenatal air pollution exposures and birth outcomes commonly use maternal addresses at the time of delivery as a proxy for residency throughout pregnancy. Yet, in large-scale epidemiology studies, maternal addresses commonly originate from an administrative source.
Objective: This study aimed to examine the use of population registry addresses to assign exposure estimations and to evaluate the impact of inaccurate addresses on exposure estimates and association measures of prenatal exposures with congenital hypothyroidism.
Methods: We used morbidity data for congenital hypothyroidism from the national program for neonatal screening for 2009–2015 and address data from two sources: population registry and hospital records. We selected neonates with geocoded addresses from both sources (N = 685,491) and developed a comparison algorithm for these addresses. Next, we assigned neonates with exposures from ambient air pollution of PM and NO_2/NO_X, evaluated exposure assessment differences, and used multivariable logistic regression models to assess the impact that these differences have on association measures.
Results: We found that most of the exposure differences between neonates with addresses from both sources were around zero and had a leptokurtic distribution density, with most values being zero. Additionally, associations between exposure and congenital hypothyroidism were comparable, regardless of address source and when we limited the model to neonates with identical addresses.
Conclusions: We found that ignoring residential inaccuracies results in only a small bias of the associations towards the null. These results strengthen the validity of addresses from population registries for exposure assessment, when detailed residential data during pregnancy are not available.

1. Introduction

Studies of associations between environmental exposures and health outcomes require reliable assessment of the exposures, often assigned based on residential addresses from one data source at a single time point. In large-scale epidemiology studies, residential addresses of study participants commonly originate from administrative data and are used under the assumption that they are up-to-date. Yet, if residential mobility is not reported, inaccurate addresses may introduce exposure estimation errors that might affect the study results.

Studies assessing the associations between prenatal air pollution exposures and birth outcomes commonly use maternal residential addresses at the time of delivery as a proxy for the mothers' residency throughout pregnancy. This is supported by several studies that found that only a small portion of mothers change their address between conception and delivery. These changes are of a relatively short

Abbreviations: CHT, Congenital hypothyroidism; $PM_{2.5}$, Particulate matter with aerodynamic diameter ≤2.5μm; PM_{10}, Particulate matter with aerodynamic diameter ≤10μm; $PM_{10\text{-}2.5}$, Particulate matter with aerodynamic diameter ≥2.5 and≤10μm; NO_2, Nitrogen dioxide; NO_X, Nitrogen oxide; OR, Odds ratio; CI, Confidence of Interval; IQR, Interquartile range.

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distance, and accordingly, they do not represent large differences in exposure estimations (Bell and Belanger, 2012; Chen et al., 2010; Hodgson et al., 2015; Lupo et al., 2010; Pennington et al., 2017; Pereira et al., 2016; Warren et al., 2018). To examine changes in residential addresses during pregnancy, researchers used complete address histories from birth certificates, surveys, phone or in-person interviews, and self-reported addresses. Some of these studies further examined how exposure estimation errors from such changes of address impact the associations between prenatal air pollution and birth outcomes (Pennington et al., 2017; Pereira et al., 2016; Warren et al., 2018).

In large-scale studies, which utilize mandatory data collected nationally for administrative reasons and not for research purposes, examining the mobility of the participants is challenging, as complete address histories are commonly unavailable. In addition, the residential addresses in most of these studies originate from a single administrative source, typically with very large sample size. Therefore, conducting follow-up or in-person interviews is not feasible. Accordingly, the validity of such large-scale single-source addresses and their consequences to epidemiological association measures are sometimes questionable.

This study aimed to validate the use of official addresses originating from population registries and reported by the citizens, and to evaluate the impact of inaccurate addresses on air pollution exposure estimates and epidemiological association measures. For this, we compared the Israeli population registry addresses for a national neonate population with addresses from hospital records, which are reported at the hospital at the time of birth and directly by the parents. Citizens are required to report changes in their official residential address yet this may not always be the case (e.g. students or young couples who move to a temporary address). Thus, official addresses may differ from the addresses reported at the hospital. Consequently, we assume that the address from hospital records is the most updated and represent, with higher validity, the residential addresses during pregnancy. We had no data on residential mobility of the mothers during pregnancy.

We further used data from a recent study on the associations between prenatal air pollution exposure and congenital hypothyroidism (CHT) (Harari Kremer et al., 2021), and examined the effect of the exposure errors on the measures of association.

2. Methods

2.1. Study design and study population

This study is based on data from a recent historical cohort that evaluated the associations between prenatal air pollution exposure and CHT (Harari Kremer et al., 2021). The initial study population included all term neonates born in Israeli hospitals between 1.1.2009 and 28.3.2015, who were not hospitalized in a neonatal intensive care unit (N = 936,601). We excluded neonates with missing gestational age (N = 106,537), yielding a cohort of 830,064 neonates. Each neonate had two maternal addresses: an official address originating from the population registry, reported by the citizens and held by the Israel Ministry of Interior, and an address obtained from the records of the hospital where the delivery took place and reported by the parents. Ethical permission for the study was received from the supreme ethics committee of the Israel Ministry of Health.

2.2. Geocoding maternal addresses

We geocoded all addresses using ArcGIS version 10.6 (ESRI, Redlands, California) and street reference data from ©HERE (HERE technologies, Tel-Aviv, Israel). Geographical coordinates of geocoded addresses were of the Israeli Transverse Mercator projection. Addresses were geocoded to one of the following levels: full address (house level); partial address (street level); neighborhood center (only Jerusalem neighborhoods); locality center; or "not mapped".

We assume most streets, small localities (population <10,000 residents) and neighborhoods have comparable air pollution across their area. Therefore, we considered geocoding to a full address, partial address, neighborhood and small locality centers as high geocoding accuracy for exposure estimation.

To validate the geocoding process, we generated a proportional sample of 100 geocoded population registry addresses, randomly selected after stratification by the level of geocoding. Then, we ran an accuracy assessment by manually comparing point geocoded locations with two base maps: OpenStreetMap and World Street Layer. We found all sample addresses to be geocoded correctly.

2.3. Exposure assessment

We examined two size-fractions of particulate matter (PM): PM with aerodynamic diameter ≤2.5 μm ($PM_{2.5}$) and 2.5–10 μm ($PM_{10\text{-}2.5}$), calculated by subtracting $PM_{2.5}$ estimations from PM with aerodynamic diameter ≤10 μm (PM_{10}). In addition, we examined two markers of traffic-related pollution: nitrogen dioxide (NO_2) and nitrogen oxide (NO_X). Half-hourly resolved air pollution monitoring and meteorological records were obtained from the Technion Center of Excellence in Exposure Science and Environmental Health air pollution monitoring database. Daily (24-h) and intra-daily (around the overpass time of Terra and Aqua) mean PM concentrations were calculated as the mean of the half-hourly PM concentrations. Satellite aerosol optical depth data were obtained from the Moderate Resolution Imaging Spectroradiometer satellite (onboard both the Terra and Aqua platforms) using the Multi-Angle Implementation of Atmospheric Correction retrieval algorithm. We then generated $PM_{2.5}$ and PM_{10} exposure estimates from hybrid spatiotemporal models, which use the satellite and meteorological data and spatial PM predictors, with a daily 1 km resolution (Kloog et al., 2015; Shtein et al., 2018). The PM models were validated using standard methods, with out-of-sample cross-validation R^2 values of 0.92 and 0.87 for PM_{10} and $PM_{2.5}$, respectively.

We estimated exposure to NO_2 and NO_X from the half-hourly 500m-resolution output of the optimized dispersion model, incorporating emissions input from various source types. Specifically, the model disperses traffic and industrial proxies of emissions to optimally fit high temporal resolution pollutant observations, using simultaneously measured wind field (Chen, Yuval, and Broday, 2020; Yuval et al., 2013). The traffic volumes and speed data were purchased from Decell Ltd. The wind data were obtained from observations in the monitoring stations. The half-hourly output was then lumped as mean daily exposure data. Leave-one-out cross-validated performance was used for estimating the spatiotemporal NO_2 concentrations on a weekly scale. For example, for 2015 $R^2 = 0.75$.

We aggregated mean daily exposure estimations for each air pollutant at each grid point into mean weekly exposure estimates based on calendar weeks. Then, we assigned neonates with the grid area within which it resides, based on residential addresses. As the grids for PM and NO_2/NO_X models are different, we gave each neonate two grid identifiers, one for each grid. Next, we set neonates with their gestation weeks based on date of birth and gestational age and merged neonate data and mean weekly exposure estimations, based on grid identifier and gestation calendar weeks. Finally, we aggregated, for each neonate, the weekly exposure estimates for each air pollutant at each grid point into mean pregnancy and mean trimester exposures, based on gestational weeks: 1st trimester: 1–13, 2nd trimester: 14–27, and 3rd trimester: week 28 until birth. For addresses geocoded to locality or neighborhood center, we assigned the mean pollutant concentrations over the entire locality or neighborhood.

2.4. Validation of official residential address

We received two addresses for each neonate from the Israel Ministry of Health: an official address from the population registry and an address from the hospital records. Each neonate has a national ID, which was

used when we linked the two geocoded addresses to ensure a correct link for each neonate. We assumed that the hospital address was the most updated one since it was obtained at the hospital at the time of birth and directly from the parents. Accordingly, the exposure estimates of the neonates for whom the addresses from both sources were identical are expected to be more accurate than the official address-based exposure estimates of all the neonates.

To validate the official addresses, we selected neonates with geocoded addresses from both sources and examined if both have the same street and locality. Initially, we used a text-wise comparison. However, we noticed that occasionally pairs of addresses were incorrectly identified as discordant due to spelling differences such as spelling mistakes, address abbreviations (e.g., "street" versus "st."), different word order in street names, or due to errors in locality codes that resulted with a wrong locality. We calculated the aerial distances between pairs of geographical coordinates given to each neonate during the geocoding to improve the address comparison efficiency. We assumed that the larger the aerial distance, the more likely the addresses are different.

We developed a comparison algorithm that used four data elements: geocoding category, same locality (yes/no), same street and locality (yes/no), and aerial distance. The algorithm sorts pairs of geocoded addresses into four categories: 1) *identical addresses*; 2) the *same locality, different address*; 3) *same locality, insufficient street data*; and 4) *different locality* (see Web Fig. 1 for a detailed flowchart of the comparison algorithm.). Each neonate was assigned one of the comparison categories.

2.5. Differences in exposure

We calculated the difference between exposures estimates for neonates with two geocoded addresses by subtracting the exposure estimate assigned based on the population registry address, from the exposure assigned based on the hospital records address. We standardized the exposure differences mean by the standard deviation of the exposure estimations based on original addresses from the population registry that were used in a previous study on associations between prenatal exposure to air pollution and CHT (Harari Kremer et al., 2021). Additionally, we calculated the coefficient of variance of the original exposure estimations to compare the dispersion of each pollutant around the mean. We then described the distribution density of these exposure estimate differences for the entire study population, and by aerial distance categories for a subpopulation of neonates with both addresses geocoded to either full or partial addresses. In addition, we calculated the kurtosis values of the distribution densities by aerial distance to examine the distribution tails. Lastly, as a case study, we assessed the different exposure estimates' impact on association measures of air pollution exposure with CHT.

2.6. Statistical analyses

In a previous study, we found that third-trimester traffic-related air pollution exposures (NO_X and NO_2) were positively associated with CHT (Harari Kremer et al., 2021). We repeated the statistical analysis using the population registry addresses, and used multivariable logistic regression models to assess odds ratios (OR) and 95% confidence interval (CI) of CHT and trimester-specific exposures to NO_2. The ORs were reported per one interquartile range (IQR) increase in each pollutant. We adjusted the models for the following covariates: ethnicity, socioeconomic status, geographical area, conception season, conception year, gestational age, birth weight, and child sex. We defined four geographical areas: north, central, Tel-Aviv, and south, by aggregating the sub-district areas. We repeated these statistical analyses with hospital records addresses, for a subpopulation limited to neonates with identical addresses from both sources, and for a subpopulation limited to neonates with different addresses.

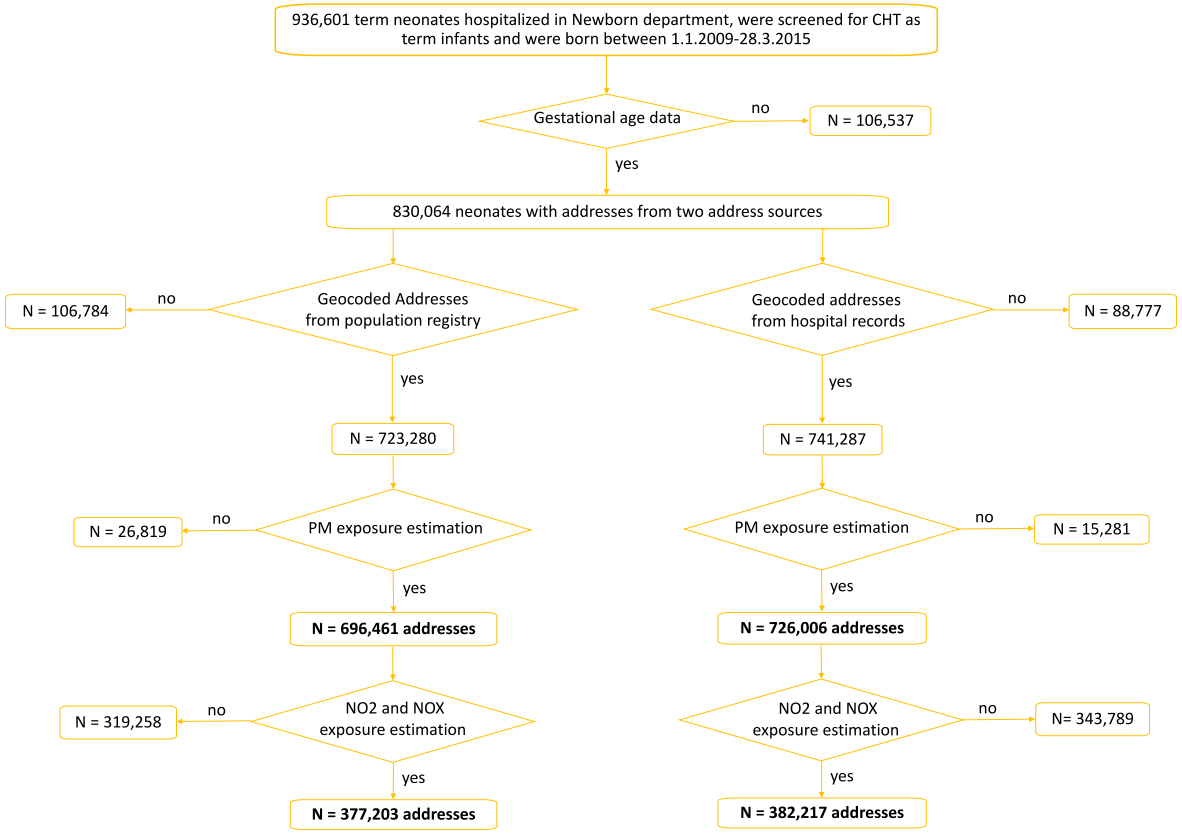

Fig. 1. A flowchart of the study population of each of the two address sources.

3. Results

Our study population included 830,064 neonates with gestational age data born at term (gestation weeks 38–42) between 1.1.2009 and 28.3.2015. Of the population registry addresses, 133,603 neonates resided in the Judea and Samaria region (West Bank), were outside the PM exposure model area, or could not be geocoded, resulting in a population of 696,461 neonates for analysis with PM exposure models. As NO_2 and NO_X exposure models cover only the central coastal area of Israel, where 54% of the study population reside, population registry addresses of 377,203 neonates were available for analysis with these pollutants. Similarly, we found 104,058 neonates with hospital records addresses that could not be geocoded, reside in the Judea and Samaria region (West Bank), or had no PM exposure estimates. This resulted in a population of 726,006 neonates for analysis with the PM exposure models and 382,217 neonates available for analysis with NO_2 or NO_X (Fig. 1).

We have successfully geocoded 62% of addresses originating from the population registry to their exact residential location (full address) and an additional 9% to their street address (partial address). Other addresses were geocoded with a decreased accuracy to neighborhood and locality centers. Overall, 83% of the population registry addresses were geocoded with high accuracy, and only 2% of the addresses were geocoded with low accuracy (Table 1). Geocoding results of addresses originating from the hospital records were slightly different, yet they showed a similar distribution of geocoding accuracy (Table 1).

Comparing geocoding results between the two address sources revealed that 685,491 neonates (98.5% of neonates with geocoded population registry addresses) had geocoded addresses from both sources. Among these, 53% had identical addresses in both sources, 10% had a different address within the same locality, and about 7% had a different locality (Table 2). The remaining 30% were from the same locality, but with incomplete addresses (missing street name) in at least one of the sources. In the subpopulation of neonates with both addresses geocoded with high accuracy (street and locality names; N = 458,560), 80% of the neonates had identical addresses, 15% had different addresses within the same locality, and only 5% had different localities. Hence, a comparison of both addresses for each neonate revealed that 93–95% of the addresses were within the same locality (Table 2).

When comparing the aerial distances between addresses, pairs of addresses with a different locality were on average 27 km apart (SD = 33 km), with a median of 13 km and a range of 0.25–253 km. The distance between two different addresses within the same locality was on average 1.3 km (SD = 1.4 km), with a median of 0.74 km and a range of 0.25–13 km (see Web Fig. 2).

When we examined the differences between the exposure estimates of neonates with addresses from both sources, we found that their mean difference values, as well as the standardized mean differences, were around zero and had a leptokurtic distribution density, with most of the values being zero (Table 3, Fig. 2). Additionally, examining the coefficient of variance of the original exposure estimations we found that NO_2 and NO_X had higher dispersion around the mean than PM (Table 3). Examining the distribution density of exposure differences by four aerial distance categories (<500, 500-1,000, 1000–5,000, >5,000m) for neonates with full and partial addresses from both sources revealed a more dispersed distribution as distance increased (Fig. 3). In pairs of addresses with distances up to 500 m, the differences between the exposure estimates had leptokurtic distribution, with most values being zero along with long distribution tails and very high kurtosis values (Web Table 1). As aerial distance increased, the distribution density of all pollutants widened and distribution tails became shorter and fatter. Distribution density had a leptokurtic distribution until it approached normal distribution at aerial distances above 5000 m (Fig. 3, Web Table 1).

When comparing trimester-exposures between the two address sources, we found comparable associations between exposure estimates and CHT, regardless of address source. For example, when we limited the model to neonates with identical addresses, we found an OR per NO_2 IQR of 1.30 (95% CI: 1.07, 1.58) for the third trimester. We found similar results for neonates with addresses originating from the hospital records addresses, with an OR per NO_2 IQR of 1.28 (95% CI: 1.09, 1.50) and an OR per NO_2 IQR of 1.25 (95% CI: 1.06, 1.47 for neonates with addresses originating from the population registry (Fig. 4). On the contrary, limiting the model to a subpopulation of neonates with different addresses resulted in null associations between exposure assessment and CHT, regardless of address source. For example, OR per NO_2 IQR of 1.16 (95% CI: 0.88, 1.54) for the third trimester epidemiological model with exposure estimations based on addresses originating from the population registry.

Table 2
Differences in geocoded addresses from both sources.

Comparison category	Neonates with both addresses geocoded to all geocoding types		Neonates with both addresses geocoded to full or partial address[a]	
	N	% of total	N	% of total
Identical address[b]	366,323	53	367,537	80
Same locality, different address	66,929	10	67,362	15
Same locality, insufficient street data[c]	204,584	30	—	—
Different locality	48,105	7	23,661	5
Total	685,941	100	458,560	100

Numbers may not add to 100% due to rounding.
[a] Partial addresses refer to addresses with street and locality names with no building/house number.
[b] Identical address category can also be when the street name is the same and building number is not the same or is missing for at least one of the addresses, and aerial distance is up to 250m.
[c] Missing street data for at least one of the addresses.

Table 1
Geocoding results of maternal addresses from two sources: population registry and hospital records.

Geocoding level[a]	locality size[b]	Population registry		Hospital records		Geocoding accuracy	Population registry	Hospital records
		N	% of total	N	% of total		% of total	% of total
Full address		432,686	62.1	423,027	58.3	**High**	83.4	82.1
Partial address		59,758	8.6	82,853	11.4			
Neighborhood center		22,910	3.3	17,103	2.4			
Locality center	Small	65,346	9.4	72,771	10.0			
	Medium	100,916	14.5	101,923	14.0	**Medium**	14.5	14.0
	Large	14,845	2.1	28,329	3.9	**Low**	2.1	3.9
Total		696,461	100.0	726,006	100.0		100.0	100.0

[a] Geocoding levels include: full address (house level); partial address; neighborhood center (only Jerusalem neighborhoods); or locality center.
[b] Locality size: small: population<10,000; medium: population 10,000–100,000; large: population >100,000.

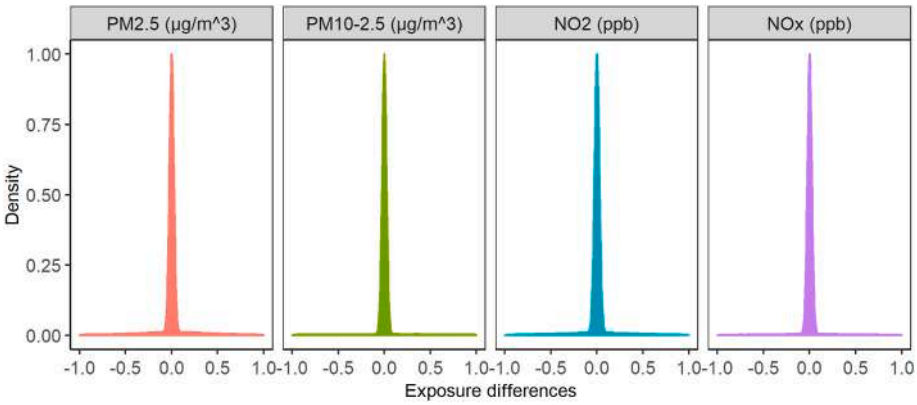

Fig. 2. The distribution density of differences in exposure assessments between two address sources among neonates with two geocoded addresses: hospital records vs. population registry addresses (N = 685,941).
$PM_{2.5}$ and $PM_{10\text{-}2.5}$ units are $\mu g/m^3$; NO_2 and NO_X units are ppb. The x-axis was limited to (−1,1).

Table 3
Statistics summary of prenatal exposure assessment differences between addresses from two sources.

Pollutant	Exposure assessment difference		ExposureOriginal[a]			Mean difference/ SDOriginal[a]
	Mean	SD	Mean	SD	Coefficient of Variance	
$PM_{2.5}$ (µg/m^3)	0.01	0.57	21.10	2.01	0.095	0.005
$PM_{10\text{-}2.5}$ (µg/m^3)	0.05	1.28	30.07	4.99	0.166	0.010
NO_2 (ppb)	−0.02	1.21	13.33	3.64	0.273	−0.005
NO_X (ppb)	−0.06	2.41	20.90	7.16	0.343	−0.008

N = 685,941 neonates with PM exposure differences; N = 369,358 neonates with NO_2/NO_X exposures differences.

[a] Original addresses refer to addresses from the population registry that were used in a previous study of associations between prenatal exposure to air pollution and congenital hypothyroidism. N = 696,461 neonates with PM exposures; N = 377,203 neonates with NO_2/NO_X exposures.

4. Discussion

In this historical cohort study of 685,941 term Israeli neonates with two geocoded addresses, we found that two-thirds had two complete location data, and a third had missing street data for at least one of the addresses. Most of the neonates with two complete addresses had identical locations and discordant addresses were mainly within the same locality. In addition, we found that differences in the exposure estimates were higher with the increase in aerial distance between the two subject addresses, and for the exposures with higher spatial variability. However, we demonstrated that these differences did not affect associations between NO_2 and CHT.

Our results showed that the spatial extent of address inaccuracies and the spatial variability of the exposures may be important factors when estimating exposure, in particular when differences in the residential addresses of study subjects exist between different databases. The greater the aerial distance (i.e., uncertainty) between the reported home addresses, the more likely the exposure estimates will also differ. This demonstrates the extent of exposure errors introduced by assigning exposure estimates to inaccurate residential locations.

In Israel, ambient PM concentration originate to a large extent from sources outside of the country, with the PM passing long-range transport. Hence, a smoother spatial variability of PM is expected compared to the higher spatial variability of NOX and NO2, whose origins are local. Accordingly, we found that NO_2 and NO_X concentrations had higher coefficient of variance and kurtosis values. Our findings that exposure errors were larger as aerial distance increased demonstrate the effect of spatial variability of the exposure on the extent of such errors. Yet, pollutants with high spatial variability may also introduce high exposure errors even when small errors in addresses exist (e.g. distances <1,000m); this is evident by long distribution tails along with high kurtosis values. Accordingly, NO_2 and NO_X concentrations, which have higher spatial variability, have higher kurtosis values in small distance categories, compared to the PM concentrations. Our results extend the findings of previous studies that indicated the importance of considering the exposure spatial variability when mobility data are unavailable (Hodgson et al., 2015; Pennington et al., 2017).

For our study population and based on the exposure estimation models we used, we found that ignoring residential inaccuracies results in only a small bias of the associations towards the null. These results are in line with the results of recent studies that reported a small bias of prenatal epidemiological associations towards the null when ignoring residential mobility during pregnancy (Pennington et al., 2017; Pereira et al., 2016; Warren et al., 2018). While two of these studies have used simulations to examine associations, we used a very large cohort with addresses from two different sources. Comparing the associations of subpopulations of neonates that differ in their extent of address similarity and distances, we found that the larger the extent of discordant addresses, the higher the extent of exposure errors along with their impact on the associations, which are biased towards the null. These findings may indicate that the pattern of misclassification due to inaccurate addresses is probably non-differential.

It is important to note that a larger ratio of inaccurate to correct addresses in our study population, might have led to larger proportions of neonates with exposure misclassification and to smaller association measures. Thus, our study results may not be generalized beyond our study population, as these exposure misclassifications may vary across regions and populations due to different patterns of residential mobility and address quality or accuracy.

Our study has several limitations. We used residential data from hospital records to validate official addresses from the Ministry of Interior. We assume that these residential data provide the correct (more updated) address since they were obtained from the parents at the hospital right after birth. Although we cannot confirm this assumption, we can regard hospital data as a second address source and assume a high address accuracy if neonates have identical addresses from both sources.

Another limitation is that one-third of the neonates had at least one missing street address. This limited our ability to compare their addresses and exposure assessments, as neonates with pairs of addresses geocoded at different levels had their exposure assigned differently. Address quality or the incompleteness of the reported residential

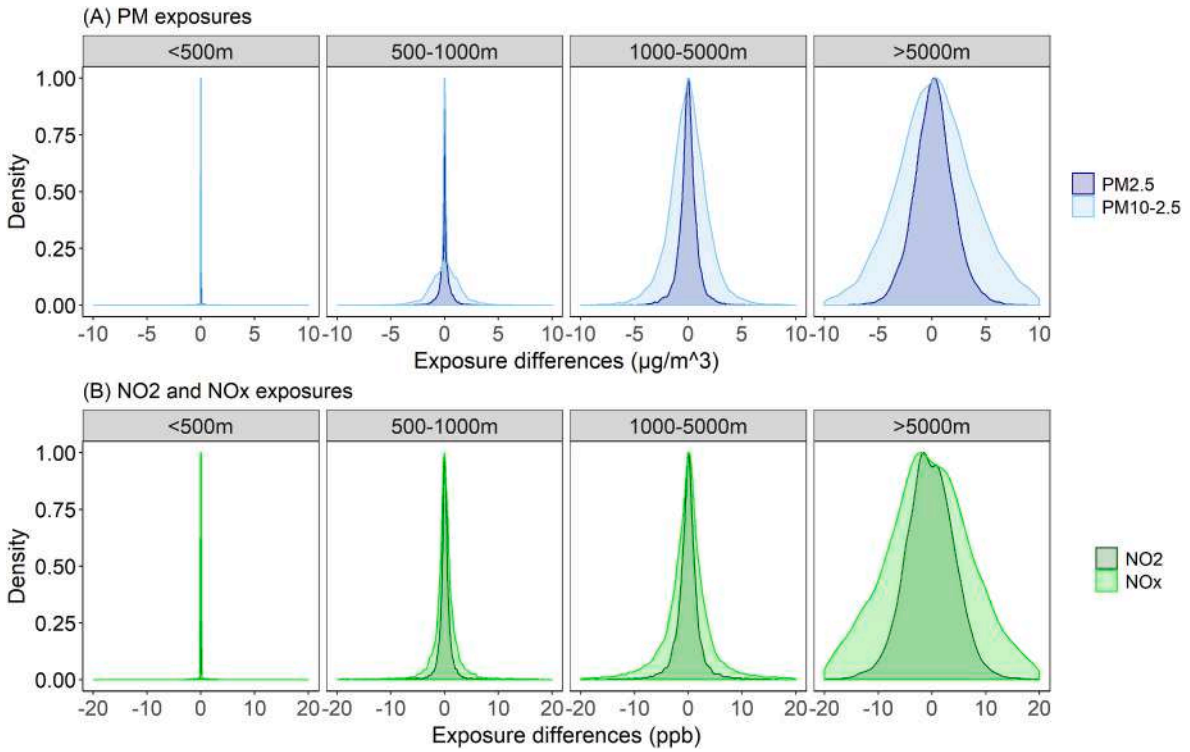

Fig. 3. The distribution density of exposure assessment differences between hospital records and population registry addresses by four aerial distance categories for neonates with full and partial addresses (street and locality).
(A) PM exposures; N < 500m = 388,660 (85%) neonates; N500-1000m: 19,336 (4%) neonates; N1000-5000m: 27,442 (6%) neonates; N > 5000m: 20,076 (4%) neonates. Percentage may not add to 100% due to rounding. **(B)** NO_2 and NO_X exposures; N < 500m = 259,199 (87%) neonates; N500-1000m: 11,820 (4%) neonates; N1000-5000m: 17,116 (6%) neonates; N > 5000m: 10,650 (4%) neonates. Percentage may not add to 100% due to rounding. The x-axis was limited to (−10,10) for A and (−20,20) for B. Note the different scales of the two parts of the figure.

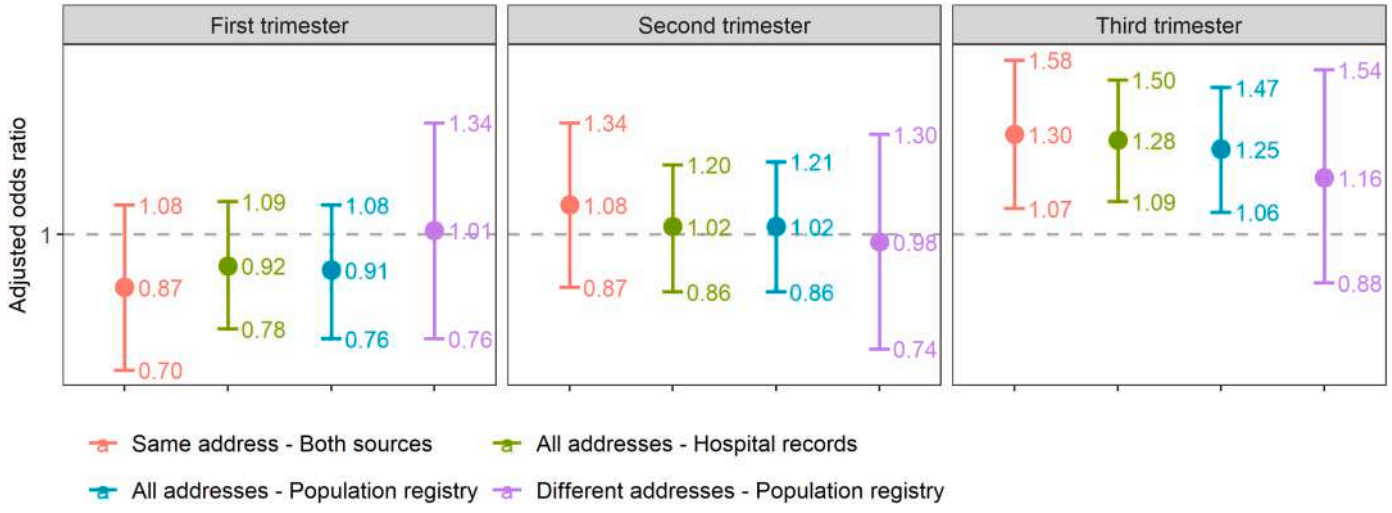

Fig. 4. Adjusted logistic regression models with NO_2 air pollution trimester exposure and congenital hypothyroidism from four epidemiological models based on addresses originating from neonates with same addresses from both sources (N = 245,626), all neonates with addresses from the population registry (N = 377,203), or from hospital records (N = 382,217), and neonates with different addresses from both sources (N = 128,684).
Models were adjusted for ethnicity, socioeconomic status, geographical area, conception season, conception year, gestational age, birth weight, and child sex. NO_2 IQR = 5.2 ppb.

addresses affect the positional accuracy of the geocoded addresses and their geocoding levels (house, street, neighborhood, or locality level). The differences in the geocoding levels can be a source of exposure misclassification as exposure estimation assignment is address-based.

In our study population, most pairs of incomplete addresses were within the same locality, mainly from small and medium-size Arab localities or very small Jewish communities. In these localities, a comprehensive naming of streets has not always been launched and there are no, or few, street names. In such cases, the exposure estimate errors are smaller than errors between discordant addresses from different localities or large cities. Consequently, although the incompleteness of these addresses limited our ability to evaluate their

similarities, its effect on exposure errors was relatively small. Furthermore, comparable association measures between the three epidemiological models, including the model with a subpopulation of neonates with complete addresses, indicated that these exposure errors had a negligible effect on epidemiological association measures.

Lastly, our study results are limited to exposure estimation models with spatial resolutions of 500–1000 m^2. Exposure estimations and their consequences on epidemiological associations may differ for models with smaller grid sizes.

Our study has several unique strengths as well. Our dataset covered almost all births in Israel over a long period, thus minimizing selection bias and allowing us to adjust for geographical, social, and seasonal factors. In addition, most neonates had two address sources, which allowed us to compare many addresses, providing important insights on the use of administrative, residential addresses in epidemiological studies. Furthermore, we examined associations between air pollution exposure and clinical CHT diagnoses, which allowed us to examine the consequences of address inaccuracies on epidemiological association measures. Finally, using four exposures with different spatial distributions allowed us to demonstrate how exposure errors may differ by pollutant.

5. Conclusions

We found that ignoring residential inaccuracies in our cohort results in only a small bias of the associations towards the null. This strengthens the validity of residential addresses from the population registries for exposure assessment in studies of PM, NO_2, and NO_X exposures, when more detailed residential data during pregnancy are not available.

Even though our study findings cannot be generalized to other study regions and populations, they highlight the importance of evaluating exposure misclassification due to inaccurate addresses in studies of air pollution and birth outcomes whenever data is available.

We encourage governments and research teams to work together and to provide this important information, which will allow better estimations of the impact of exposure to air pollution on neonates. Additionally, studies are needed to examine these questions in models with a higher spatial resolution and for other pollutants.

Declaration of competing interest

The authors declared no conflict of interest.

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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.2022.114032.

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Exposure to multiple trace elements and thyroid cancer risk in Chinese adults: A case-control study

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ABSTRACT

The incidence of thyroid cancer (TC) has increased rapidly in last decades. Multiple trace elements in the external environment have important effects with thyroid function. However, the evidence for these on TC risk were rarely reported. A total of 585 newly diagnosed TC patients and 585 healthy controls were included in this study, and 14 urinary elements were measured to explain the fixed-exposure effect on TC risk. Conditional logistic regressions were used to reflect the multi-element associations, and Bayesian kernel machine regression (BKMR) was applied to show the tendency of mixed effects. Furthermore, the interaction effects were examined by Generalized linear model (GLM). The levels of lithium (Li), cobalt (Co), strontium (Sr), zinc (Zn) and copper (Cu) had negative effects with TC risk, nevertheless lead (Pb), arsenic (As) and chromide (Cr) showed positive effects. The BKMR and GLM models reflected the effect fluctuations of different elements, and there was a slight interaction effects between Li and Cr, Co, Zn and Pb. Further study is required to confirm these results in the future.

1. Introduction

The endocrine system is one of the most important part of metabolism, and it plays a vital role for growth of humans (Mullur et al., 2014). However, when the endocrine is disordered, our bodies will suffer from many kinds of diseases consequently, even malignant tumors (Wright et al., 2021). As the largest endocrine gland, thyroid has multiple functions such as synthesizing thyroid hormones, regulating growth and development and metabolism in the body, and its lesions will inevitably cause serious damage to the body (Cabanillas et al., 2016). Thyroid cancer (TC) is the most common endocrine cancer nowadays, which causes a particularly serious burden. The latest cancer statistics of the World Health Organization (WHO) in 2020 had showed that new TC cases were more than 580 thousand worldwide, accounting for nearly 3.0% of the total cases of all cancers (Siegel et al., 2021). The age-standardized rate (ASR) of TC increased nearly by 20% from 1990 to 2013 (Lim et al., 2017); it is estimated that TC will become the fourth most common cancer in 2030. More seriously, TC incidence among females was higher than males, and the ratio of males to women was approximately 1:3 (Siegel et al., 2020; Sung et al., 2021). In 2016, the incidence of TC in China ranked eighth for all malignant tumors, and the ASR had risen from 2.89 in 2003 to 10.37 in 2015 (per 100,000); moreover, this number in females were higher (15.81). Therefore, TC has become one of main tumors endangering the health of Chinese (Wang et al., 2021).

According to the pathological classification, TC can generally be divided into several categories, in which papillary thyroid cancer (PTC) accounted for more than 80% of the total (Stojsavljević et al., 2021). Currently, the most widely known factors of TC were the exposure to ionizing radiation, and excessive or deficiency intake of iodine

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(Zimmermann and Boelaert, 2015). Besides, some countries like South Korea, has carried out many large-scale health examinations frequently, and these results revealed that some daily behaviors (e.g. smoking, alcohol, and dietary habits, etc.) were related to PTC risk (Kang et al., 2022; Kim et al., 2022). Additionally, other factors, such as reproductive factors for females, and abnormal levels of estrogen, which were used to explain the increasing risk (He et al., 2021b). Of course, the personal and family history of thyroid-related diseases was always the key issued in previous researches to explore the etiology of thyroid cancer (Banik et al., 2021). In addition, some people believed that the increasing incidence of TC is closely related to the development of medical diagnostic technology, which made it easier to detect TC with a diameter of <1 cm (Nabhan et al., 2021). However, a study from Shanghai, China pointed out that the incidence of PTC of 1.1–2.0 cm increased by 30%, and the tumor diameters >2.0 cm increased by 20% (Wang and Wang, 2015). Therefore, over-diagnosis cannot fully explain the high incidence. Therefore, we wanted to start with the actual level of metabolites in the human body, and tried to further explain the potential factors.

Nowadays, a large quantity of researches has confirmed that there is an inseparable relationship between metabolic disorders and cancers (Gyamfi et al., 2022). The various systems of our bodies are coupled with each other, so the disorder or disease of other systems may also affect the endocrine system. There could be a question existed: why is normal metabolism destroyed? We should admit that genetic mutations must exist; but it is not advisable to attribute so many cancer patients to their own genetic material (Abdul-Maksoud et al., 2022; Martínez-Camberos et al., 2022). Human beings live in nature and exchange materials from environment all the time. By this way, external substances can enter in our bodies through various ways. Therefore, it is urgent to pay attention to the effects of external environmental exposure on endocrine, especially thyroid function.

With the rapid development of population growth, industrialization, urbanization and agricultural modernization, the chances of Chinese people being exposed to external substances were also increasing (Wu et al., 2019). Trace elements are ubiquitous in the natural environment; humans are often exposed to them in many ways, such as ingesting food, drinking water, air inhalation, and skin contact (Cannas et al., 2020). In terms of human needs, these trace elements in the environment are generally divided into essential elements and non-essential elements. As an important component of body functions, the change of these elements is always subtle. Some of them are absorbed and utilized by related tissues; others are preserved in specific tissues for future use; the remaining part is excreted with urine and feces (Martinez-Finley et al., 2012; Potocki et al., 2012). But when there were significant changes in their levels, even beyond the threshold, it will cause compensatory negative effects in homeostasis. Undoubtedly, abnormal changes in the concentrations of these elements will inevitably lead to disorders in the endocrine system, also including thyroid gland, definitely.

As we all know, long-term exposure to non-essential metals may have adverse effects on the immune, neurological and endocrine systems, as well as increase the risk of liver and kidney dysfunction, cardiovascular diseases and malignant tumors (Bjorklund et al., 2018; Zhang et al., 2020; Zhou et al., 2021). Especially, heavy metals (including metalloid elements) can also cause severe damages to thyroid. For example, arsenic (As) and cadmium (Cd) had been confirmed as common carcinogens by the International Agency for Research on Cancer (IARC) (Stojsavljević et al., 2019b); coincidentally, mercury (Hg) and lead (Pb) were also be listed as possible carcinogens (Zhang et al., 2021). In particular, Cd can accumulate in follicular cells actively, and further cause thyroid tissue damages. The result from a previous study in Serbia showed TC patients who had higher Cd than healthy people. Besides, Cd can significantly decrease triiodothyronine (T3) and thyroxine (T4) secretion in rats (Stojsavljević et al., 2019a). Conversely, the higher level of selenium (Se) was be reported to have a protective effect on thyroid cells (Duntas, 2006). Interestingly, Se was seemed to protect serum T4 level which induced by Cd, indicated that Se has antagonistic effect with Cd. Zhang et al. had showed a significant dose-response effect for Hg exposure and PTC risk (Zhang et al., 2019). Other elements, such as copper (Cu) or iron (Fe), have also been reported to be positively associated with TC risk (Kosova et al., 2012).

However, the relationships between mixed-exposure of multiple elements and TC had not been determined. On the other hand, previous studies had failed to vividly reflect the mixed effects of different elements. Considering the severe situation of thyroid cancer in China, we designed a case-control study in Anhui Province, to explore the combined effects of multiple trace elements, and evaluated the fixed-exposure effects on TC risk, for explaining its etiology among Chinese people.

2. Materials and methods

2.1. Study population

The selection process of participants was shown in Fig. S1. All TC cases were selected from Anhui Provincial Cancer Hospital during May 2017 to February 2019. All cases were over 18 years old, and newly diagnosed with TC at the time being collected from the surgery department of head and neck. Meanwhile, we also collected pathological types for all cases. None of them have undergone TC-related surgery or medication (including iodine supplements or treatment). In this process, we excluded others with serious diseases like liver, kidney, digestive system, mental diseases, and malignant tumors. Pregnant and breast-feeding females were also excluded for the extreme changes in estrogen levels.

The controls were matched by 1:1 according to their gender and age (±3 years). They were from other departments in the First Affiliated Hospital of Anhui Medical University. They were from non-endocrinology departments who were admitted to the hospitals at the same time as cases, and all had a general examination on admission. They were not suffering from thyroid diseases, and other diseases of liver, kidney, digestive tract, mental disorder, and cancers. Besides, we performed head-neck ultrasound of each control person, to ensure that they did not suffer from thyroid diseases. Similarly, they were not also treated with iodine drugs. This study was approved by the Project for Anhui Medical University Biomedical Ethics Committee (Approval No. 20170305).

2.2. Questionnaire and variable definition

All people were invited to complete a structured face-to-face questionnaire after agreeing to join in this study. The questionnaire mainly included demographic characteristics (e.g. sex, age, household annual income, education level, etc.), personal behaviors (smoking, passive smoking, sleeping quality, alcohol drinking, physical activity frequency), radiation-exposure history, personal diseases history, family history (thyroid diseases and cancers), etc. On the other part, we collected participants' clinical information, just like height, weight, and pathological information of TC by the guideline of National Comprehensive Cancer Network (NCCN, version 2018) (Haddad et al., 2018). In this process, all sensitive information of patients was strictly protected. Informed consent was obtained from all participants included in this study.

We also accurately defined some variables of the questionnaire. Smokers were defined as people who were smoking currently, or have quitted smoking <6 months. Passive smokers were prescribed as participants who lived or worked around smokers, and could feel the smoke exhaled by smokers. Alcohol consumption was defined as when the investigation was conducted, the participants drank at least 1 time per month on average for more than 1 year.

2.3. Measurement of urinary elements

In this study, we measured 14 urinary trace elements to assess their exposure levels. Among them, 13 elements [i.e. molybdenum (Mo), manganese (Mn), selenium (Se), cobalt (Co), lead (Pb), chromium (Cr), lithium (Li), iron (Fe), arsenic (As), strontium (Sr), barium (Ba), cooper (Cu) and zinc (Zn)] were measured simultaneously by inductively coupled plasma optical emission spectrometry (ICP-OES, PerkinElmer Optima 7000DV, USA). Each sample was participant's middle-segment urine in the early morning and stored at −80 °C immediately after collection. We took 3 mL from each sample, then diluted with 1:3 with 5% nitric acid. After 90 °C digestion and centrifugation, the supernatant was extracted for automatic sampling.

In addition, the level of cadmium (Cd) was measured by using graphite furnace atomic absorption spectrometry (GFAAS, Analytik Jena, ZEEnit700P, Germany). We used 1% nitric acid as the solvent, and took 1.5 mL of supernatant after centrifugation for automatic sample injection. Furthermore, considering the important effect of iodine (I) with thyroid, we also measured urinary I by using As^{3+}-Ce^{4+} catalytic spectrophotometry (Chinese Health Industry Standard, WS/T 107.1–2016) (Zhang et al., 2019). The result of urinary creatinine was used to adjust the concentration of all elements. We used Jaffe-compensated assay to measure the urinary creatinine detected by UV spectrophotometry analyzer (Beckman, DXC-800, USA).

2.4. Measurement of thyroid function index

The blood samples were also collected at the same time as urine samples. Participants were required to fast for at least 8 h. After collecting samples, the serum was separated and stored at −80 °C immediately. The concentrations of serum free triiodothyronine (FT3), free thyroxine (FT4) and thyroid-stimulating hormone (TSH) were measured by electrochemiluminescence immunoassay, and we used the automatic biochemical analyzer (Roche Cobas e−411 analyzer, Germany). The samples were measured in batches at the same time.

2.5. Quality assurance and quality control

For testing by ICP-OES, we applied mixed standard solution to calibrate standard curve. The interference between elements was controlled by the mixed standard solution. We had set up the spectral lines corresponding to various elements in advance, and recorded the detected peak area. Each time for recording was 12s, and every sample were measured twice. The blank sample were accompanied after each group (20 samples).

When detecting Cd, we applied 1% diammonium hydrogen phosphate as matrix modifier. One sample in every 10 samples was selected for spike recovery detection, and the range of spike-and-recovery was 94%–107%. Moreover, we use standard kits for iodine, thyroid hormones and creatinine for testing.

For the detected elements, the detection rate of each one was required to exceed 70%, and the results were < LODs should be replaced as LODs/ $\sqrt{2}$.

2.6. Statistical analysis

Paired -test and χ^2 test were used to describe the general characteristics of all participants. The levels of elements were adjusted by creatinine before analysis, and then converted by natural logarithm (μg/g). Spearman correlation was applied to calculate the correlations between elements. Subsequently, we established conditional logistic regression models (meant single-element models) to evaluate odds ratio (OR) and 95% confidential interval (95% CI) on TC risk. The concentrations of all elements are categorized into quartiles, and lowest quartiles were defined as reference. Model 1 was only adjusted by gender and age, and Model 2 was adjusted for other demographic factors additionally. In the Model 3, considering the significant role of thyroid hormone and iodine, we also added FT3, FT4, TSH, and urinary I as covariates into the equation.

In order to estimate the mixed effect of multi-elements on TC risk, we used the multi-element logistic model to show the associations. For maintaining the robustness of the model, we further selected these trace elements through Least Absolute Shrinkage and Selection Operator (Lasso) regression, to choose "representative elements" for subsequent analysis. It obtains a more refined model by constructing a penalty function, and it sets some regression coefficients to 0, so it can retain the advantage of subset shrinkage, thereby avoiding overfitting (McEligot et al., 2020; Yin et al., 2020; Zheng et al., 2020).

On the other hand, since the environmental exposure was always non-linear, we further evaluated the nonlinear trends of these trace elements. In this case, we used Bayesian Kernel Machine Regression (BKMR) to vividly demonstrate the effect of varying concentrations of different elements on the risk of TC, and the effects between each other (He et al., 2021a; Yin et al., 2020). We bright these elements which were selected by Lasso regression, and included the covariates for the Monte Carlo algorithm through 10,000 iterations. At first, we fitted the univariate cross-section model of each element, which others were fixed at a specific value (e.g.P25, P50, P75). These can be used as cross-sectional plots to reflect the exposure effects of individual elements. We further simulated the exposure effect of a single element when the concentrations of other elements were changed. Moreover, we built a "double--element" model based on the univariate model. to estimate the exposure effect of one element, and make the concentration of another element located at its P25, P50 and P75, while the other elements remain localized at their P50 concentrations.

Obviously, there must be interactions with each other in the mixed-exposure model of multiple pollutants. For example, we wished to compare the effect when one element was at its P75, and then compared to when it was at its P25 (ΔP75-P25), where others were fixed at particular percentiles (P25, P50, P75). Finally, we used a cumulative-effect plot to reflect the overall effect, that is, all elements were fixed at the same quantile, arranged in order from lower to higher (e.g. P25–P75). For the possible interactions, generalized linear model (GLM) can reflect the statistical multiplicative interaction (INTM) between exposure factors (Jünemann et al., 2013; Tang et al., 2013). Thus, we set a multiplicative interaction coefficient and calculated its OR. OR<1 indicated antagonism effect, and OR>1 indicated synergy effect. In addition, we also explored the additive interactions (INTA), for calculating 3 indexes [i.e. the relative excess risk due to interaction (RERI), attributable proportion due to interaction (AP) and the synergy index (S)]. In detail, if the two factors have additive interactions, the 95% CIs for RERI and AP should not include 0, and the 95% CIs for S should not include 1. In the same way, we further explored the interaction between thyroid hormones and elements.

All analyses were analyzed by using R software (version 4.1.0). Overall, the p values were two-sided, and $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Characteristics of participants

This study included 585 TC newly diagnosed cases and 585 controls. The age of all participants ranged from 18 to 74, and the difference between both groups was not significant ($p = 0.405$). The number of females were 372 (63.6%), which were extremely higher than males (36.4%). Among all cases, PTC had the highest proportion (60.2%). In terms of the distribution of factors, such as household annual income, frequency of activity, smoking status, passive smoking, family history of thyroid disease and cancer, there was a statistical significance between both groups (all $p < 0.05$). The mean concentrations of FT3, FT4, TSH of

TC cases were higher than controls (Table 1).

3.2. Levels of urinary trace elements

All of the original data of urinary elements were showed in Table S1, for the detection rates were >70%. After adjusting by urinary creatinine, we also summarized the adjusted values of all elements by Wilcoxon signed-rank test. As showed in Table S2, the levels of Cr, As, Pb, and I of cases were generally higher than controls; on the contrary, the concentrations of Zn, Co, Cu, Li, Ba and Sr were lower, exactly. However, there were not any significance in Cd, Se, Mn, Fe and Mo (all $p > 0.05$).

Table 1
The demographic and pathologic characteristics of participants.

Characteristics	Case (n = 585)	Control (n = 585)	*p* value
Age, year, mean (SD)	48.3 (13.6)	48.8 (14.3)	0.405
BMI, kg/m^2, n (%)			0.010
<18.5	255 (43.6)	312 (53.3)	
18.5~	21 (3.6)	31 (5.3)	
24.0~	231 (39.5)	188 (32.1)	
≥28.0	78 (13.3)	54 (9.1)	
Women, n (%)	372 (63.6)	372 (63.6)	/
Household annual income, RMB, n (%)			<0.001
<30000	20 (3.4)	42 (7.2)	
30000~	317 (54.2)	347 (59.3)	
≥60000	248 (42.4)	196 (33.5)	
Physical activity, per week, n (%)			<0.001
0	330 (56.4)	397 (67.9)	
1–2	87 (14.9)	46 (7.9)	
3–4	55 (9.4)	33 (5.6)	
≥5	113 (19.3)	109 (48.6)	
Smoking status, n (%)			0.028
Never	482 (82.4)	509 (87.0)	
Ever	103 (17.6)	76 (13.0)	
Passive smoking, per week, n (%)			0.022
0	374 (63.9)	423 (72.3)	
1–3	57 (9.7)	46 (7.9)	
4–6	32 (5.5)	22 (3.8)	
7	122 (20.9)	94 (16.0)	
Alcohol drinking, days per week, n (%)			0.309
<1	217 (37.1)	232 (39.7)	
1–4	259 (44.3)	263 (44.9)	
5–7	109 (18.6)	90 (15.4)	
Sleeping quality, n (%)			0.040
Poor	127 (18.8)	100 (18.6)	
General	144 (25.5)	130 (22.2)	
Good	314 (55.7)	355 (59.1)	
History exposure of X-ray, n (%)			0.006
Never	327 (55.9)	373 (63.8)	
Ever	258 (44.1)	212 (36.2)	
Family history of thyroid diseases, n (%)			<0.001
No	502 (85.8)	567 (96.9)	
Yes	83 (14.2)	18 (3.1)	
Family history of malignant tumor, n (%)			0.005
No	463 (79.1)	500 (85.5)	
Yes	122 (20.9)	85 (14.5)	
[a]Pathological types of cases, n (%)			/
PTC	352 (60.2)	/	
PTMC	136 (23.2)	/	
FTC	55 (9.4)	/	
MTC	42 (7.2)	/	
FT3 (pmol/L), median (P25–P75)	4.70 (4.16–5.25)	4.42 (3.55–5.07)	<0.001
FT4 (pmol/L), median (P25–P75)	16.46 (14.68–18.63)	15.27 (13.25–17.26)	<0.001
TSH (mIU/L), median (P25–P75)	2.63 (1.60–4.53)	2.46 (1.53–3.65)	0.002
I (μg/L), median (P25–P75)	248.28 (131.12–406.69)	166.44 (105.76–267.86)	0.010

[a] Abbreviations of each pathological types. PTC: papillary thyroid cancer; PTMC: papillary thyroid microcarcinoma; FTC: follicular thyroid cancer; MTC: medullary thyroid cancer.

3.3. Exposure of elements and TC risk

According to logistic regression, in Model 1, the levels of Cr, As and Pb were significantly positively associated with the risk of TC, but the opposite trend was found in Zn, Sr, Co, Se, Cu, Ba and Li. After adjusting some demographic factors, these associations still existed (Model 2). Subsequently, thyroid hormones and urinary iodine were further included into the Model 3 as adjusted factors (Table 2).

Specifically, Cr, As and Pb were shown to be increasingly associated with TC risk at their highest quartiles (Q4 vs. Q1: OR = 1.71, 95%CI = 1.12–2.81; OR = 1.84, 95%CI = 1.21–2.79; OR = 2.36, 95%CI = 1.51–3.67). Nevertheless, decreased associations were found in Zn, Ba, Cu, Li, Sr, Co and Se. In particular, significant declining differences were found at each quartile in Ba (Q2 vs. Q1: OR = 0.42, 95%CI = 0.28–0.64; Q3 vs. Q1: OR = 0.29, 95%CI = 0.15–0.48; Q4vs. Q1: OR = 0.30, 95%CI = 0.19–0.47), and the extremely similar tendency was also found in Co, Li and Sr. As for Cu, the negative association was also found from Q3 (OR = 0.55, 95%CI = 0.36–0.84) and Q4 (OR = 0.30, 95%CI = 0.18–0.49).

3.4. Multiple-element model for TC risk

Firstly, we drew a Spearman correlation heatmap between these elements. As Fig. S2 showed, there was an extreme correlation between Pb, Se and Mn ($r \approx 0.70$). The coefficient between Se and Co also reached 0.60, but there were not obvious correlations between others. Since there were many elements with statistical significance in the single-metal model, we further selected them by Lasso regression, to achieve dimensionality reduction of the multi-element model, and substituted covariables (including iodine) to select these elements. By Lasso regression (Fig. 1), we selected Cr, As, Zn, Co, Cu, Pb, Sr and Li. For these 8 elements, obviously, the maximum correlation coefficient was 0.59, while Mn and Se, which had the strongest correlation with Pb, have been excluded (Fig. 2).

According to the multivariate analysis, these eight trace elements were included into function together, and adjusted by demographic factors, thyroid hormones (FT3, FT4, TSH) and urinary I (Table 3). The higher level of Pb, the more significant the effect with the risk of thyroid cancer (Q4 vs. Q1: OR = 9.09, 95%CI = 4.34–15.49); the same phenomenon was also found in As (p-trend < 0.001). Co and Sr seemed to get protective effects, owing to their ORs <1 compared with Q1. In addition, high levels of Li and Cu were also found to be negatively associated with TC; for Zn, we only found negative association in highest quartile (Q4 vs. Q1: OR = 0.40, 95%CI = 0.19–0.83).

3.5. BKMR for nonlinear effects

Based on the results of Lasso regression, we included the "representative" elements into BKMR analyses. Included covariates were consistent with logistic regression. Due to the principle of Bayesian regression, we firstly checked the posterior inclusion probability (PIP) for these 8 independent variables (Table S3A). In this study, the importance of the eight elements is not much different. Subsequently, we fitted the nonlinear effect curves for single elements, for analyzing only one element's effect on TC risk, while others were all fixed at their medians. In the univariate cross-section plots (Fig. 3A), it can be clearly seen that with the increase of As concentration, the risk of thyroid cancer presents a monotonically increasing trend. Basically, Pb also showed a positive correlation, but when the concentration was higher, the change trend of its effect gradually flattened (the interval was large). The effect curve of Cu does not show obvious increase or decrease; interestingly, however, both Sr and Zn showed a trend of first increase and then decrease. On the contrary, Co and Li showed a change of first decrease and then increase, and the change curve of Li was more asymmetric. The trends of these changes were basically consistent with the changes in ORs displayed in Model 3.

Table 2
Odds ratio (OR) and 95% confidence interval (95% CI) for each element in single-element model.

Elements[a]	Q1	Q2	Q3	Q4	*p*-trend
Cr					
Model 1	1.00 (ref)	0.74 (0.53–1.05)	0.88 (0.62–1.25)	1.68 (1.22–2.32)	<0.001
Model 2	1.00 (ref)	0.71 (0.48–1.05)	0.94 (0.63–1.40)	1.87 (1.29–2.71)	<0.001
Model 3	1.00 (ref)	0.63 (0.41–0.98)	0.84 (0.54–1.32)	1.71 (1.12–2.81)	<0.001
Mo					
Model 1	1.00 (ref)	0.84 (0.60–1.16)	0.90 (0.64–1.25)	1.00 (0.72–1.39)	0.656
Model 2	1.00 (ref)	0.86 (0.59–1.25)	1.00 (0.68–1.46)	1.23 (0.84–1.80)	0.352
Model3	1.00 (ref)	0.62 (0.40–0.96)	0.66 (0.42–1.04)	0.90 (0.58–1.41)	0.092
Zn					
Model 1	1.00 (ref)	0.58 (0.42–0.79)	0.73 (0.54–0.99)	0.38 (0.26–0.54)	<0.001
Model 2	1.00 (ref)	0.59 (0.42–0.85)	0.74 (0.52–1.05)	0.37 (0.25–0.56)	<0.001
Model 3	1.00 (ref)	0.64 (0.40–1.03)	0.70 (0.47–1.05)	0.31 (0.20–0.50)	<0.001
Fe					
Model 1	1.00 (ref)	1.07 (0.77–1.49)	1.35 (0.97–1.87)	1.08 (0.77–1.52)	0.283
Model 2	1.00 (ref)	1.03 (0.71–1.50)	1.50 (1.03–2.18)	1.13 (0.77–1.66)	0.121
Model 3	1.00 (ref)	0.86 (0.56–1.31)	1.23 (0.81–1.88)	0.84 (0.54–1.30)	0.214
As					
Model 1	1.00 (ref)	0.74 (0.52–1.05)	0.95 (0.68–1.33)	1.68 (1.22–2.31)	<0.001
Model 2	1.00 (ref)	0.87 (0.59–1.30)	0.91 (0.62–1.34)	1.94 (1.35–2.79)	<0.001
Model 3	1.00 (ref)	0.90 (0.57–1.41)	0.83 (0.54–1.28)	1.84 (1.21–2.79)	<0.001
Ba					
Model 1	1.00 (ref)	0.54 (0.39–0.74)	0.35 (0.24–0.49)	0.38 (0.27–0.54)	<0.001
Model 2	1.00 (ref)	0.49 (0.34–0.71)	0.33 (0.22–0.49)	0.40 (0.27–0.59)	0.013
Model 3	1.00 (ref)	0.42 (0.28–0.64)	0.29 (0.15–0.48)	0.30 (0.19–0.47)	<0.001
Co					
Model 1	1.00 (ref)	0.56 (0.41–0.77)	0.29 (0.20–0.42)	0.55 (0.40–0.76)	<0.001
Model 2	1.00 (ref)	0.52 (0.36–0.74)	0.29 (0.19–0.44)	0.55 (0.39–0.79)	<0.001
Model 3	1.00 (ref)	0.43 (0.28–0.65)	0.20 (0.12–0.33)	0.41 (0.27–0.64)	0.014
Cu					
Model 1	1.00 (ref)	0.70 (0.50–0.96)	0.65 (0.46–0.90)	0.36 (0.24–0.52)	<0.001
Model 2	1.00 (ref)	0.80 (0.55–1.16)	0.73 (0.50–1.06)	0.41 (0.26–0.63)	0.031
Model 3	1.00 (ref)	0.68 (0.44–1.03)	0.55 (0.36–0.84)	0.30 (0.18–0.49)	0.024
Li					
Model 1	1.00 (ref)	0.43 (0.31–0.59)	0.33 (0.23–0.46)	0.31 (0.22–0.42)	<0.001
Model 2	1.00 (ref)	0.38 (0.27–0.55)	0.31 (0.21–0.46)	0.29 (0.20–0.44)	<0.001
Model 3	1.00 (ref)	0.37 (0.25–0.56)	0.27 (0.17–0.43)	0.26 (0.17–0.41)	<0.001
Mn					
Model 1	1.00 (ref)	1.36 (0.99–1.87)	1.08 (0.78–1.51)	1.29 (0.92–1.82)	0.216
Model 2	1.00 (ref)	1.45 (1.01–2.66)	1.24 (0.85–1.81)	1.68 (1.13–2.51)	0.059
Model 3	1.00 (ref)	1.24 (0.83–1.86)	1.04 (0.68–1.61)	1.25 (0.79–1.96)	0.644
Pb					
Model 1	1.00 (ref)	0.98 (0.69–1.40)	1.20 (0.85–1.68)	2.04 (1.46–2.84)	<0.001
Model 2	1.00 (ref)	1.02 (0.68–1.51)	1.22 (0.83–1.79)	2.65 (1.79–3.91)	<0.001
Model 3	1.00 (ref)	0.91 (0.58–1.42)	1.12 (0.73–1.73)	2.36 (1.51–3.67)	<0.001
Se					
Model 1	1.00 (ref)	0.65 (0.47–0.92)	0.57 (0.41–0.81)	1.04 (0.76–1.43)	0.024
Model 2	1.00 (ref)	0.65 (0.43–0.97)	0.59 (0.40–0.88)	1.21 (0.84–1.74)	0.019
Model 3	1.00 (ref)	0.48 (0.31–0.74)	0.48 (0.30–0.75)	1.05 (0.69–1.62)	<0.001
Sr					
Model 1	1.00 (ref)	0.39 (0.28–0.55)	0.32 (0.23–0.45)	0.34 (0.24–0.48)	0.015
Model 2	1.00 (ref)	0.43 (0.30–0.63)	0.37 (0.25–0.54)	0.36 (0.25–0.53)	<0.001
Model 3	1.00 (ref)	0.38 (0.24–0.58)	0.28 (0.18–0.45)	0.26 (0.17–0.41)	<0.001
Cd					
Model 1	1.00 (ref)	0.68 (0.49–0.93)	0.78 (0.55–1.06)	0.77 (0.55–1.07)	0.096
Model 2	1.00 (ref)	0.77 (0.54–1.11)	0.74 (0.52–1.09)	0.85 (0.59–1.24)	0.423
Model 3	1.00 (ref)	0.82 (0.56–1.25)	0..85 (0.35–1.23)	0.69 (0.41–1.03)	0.012

Model 1: Only adjusted by age (continuous variable) and gender.
Model 2: Additionally adjusted for covariates: BMI, household annual income, physical activity, smoking status, passive smoking, sleeping quality, history exposure of X-ray, family history of thyroid diseases, family history of malignant tumor.
Model 3: Additionally adjusted for FT3, FT4, TSH, and lnI (continuous variables).
[a] The levels of trace elements were processed through natural logarithm (ln).

Fig. 3B showed the difference between these elements when they were at their median levels and when they were at other percentiles, such as P25 to P75. When the concentration of each element was lower than P50, the overall effect was statistically significant compared with it at their P50s, but showed a decreasing trend. However, when they were higher than P50s, the significant effect disappeared (Table S3B).

Another method that could be interest was estimating the contribution of single exposure on overall effect. As showed in Fig. 3C and Table S3C, when other elements were fixed at the concentrations of P25, P50, and P75, the differences in their respective concentrations (ΔP75-P25) of CO, Cu, Pb, Sr, and As are not much different. On the contrary, as the concentration of other elements increased, the value of Li (P25 vs. P25) increased significantly. Additionally, ΔP75-P25 of Pb also increased as others' levels increased.

In Fig. 3D, where all of the other exposures are fixed at medians. In this plot, the three effect curves in each grid in the figure reflected the effect curves of "element A" when "element B″ was at the concentration of its P25, P50, and P75, and other 6 elements were at the medians. Through the three curves, it can be seen whether there is an interaction between "element A″ and "element B". if these three curves were basically parallel, there was no obvious interaction between the two; if the curves showed a clear trend change or intersect, there maybe was an interaction between A and B. So, we found that there were obvious interactions between Co, Cr, Zn, Pb and Li.

3.6. Interaction effects

We used the GLM function to fit the interaction effects between Li and Co, Cr, Zn, Pb. As showed in Table S4A, the ORs of the interaction between Li and the other four elements in the GLM model were all statistically significant (all ORs >1.00); however, when we added Li and the other four elements sequentially, the results of the indicators (RERI, AP, S) showed that the additive interaction was not significant.

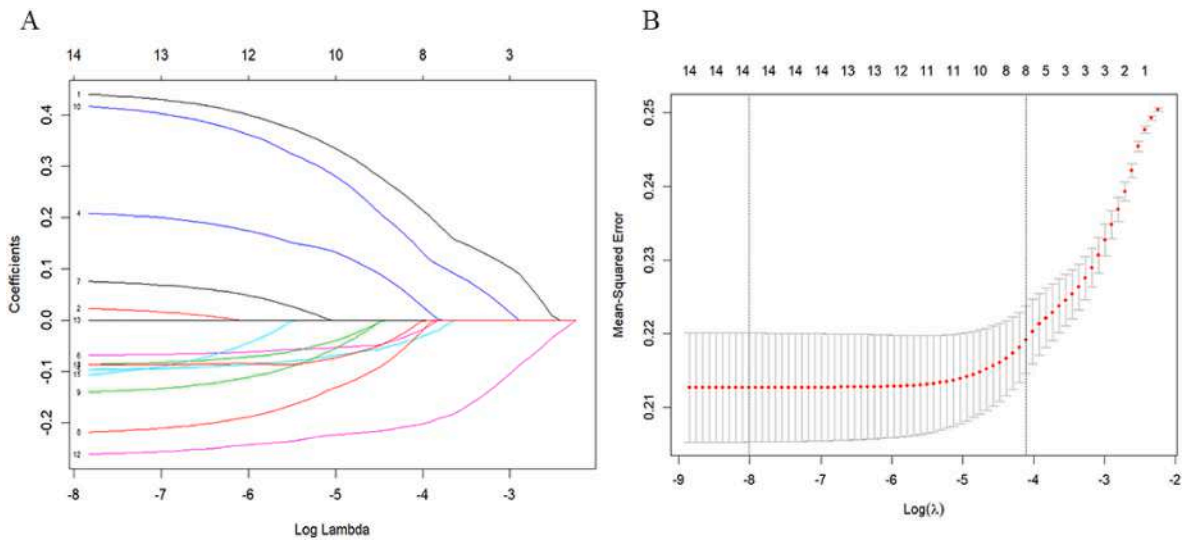

Fig. 1. The elements selected into the multi-element model by Lasso regression. (A) The prediction error of Lasso regression in function of penalty parameter (log λ). (B) The solution path with the coefficient profiles for 14 elements.

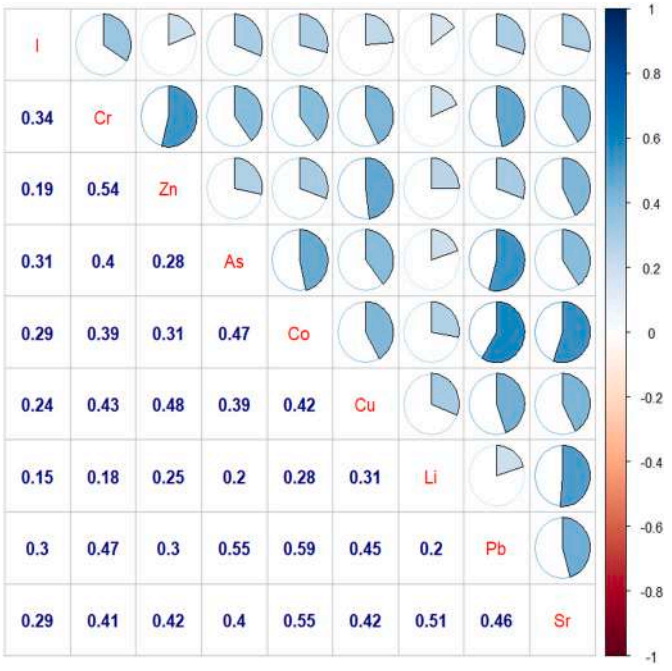

Fig. 2. The Spearman correlation heatmap of 8 urinary elements selected by Lasso regression.

Furthermore, we also showed the interaction 3D stereogram in Fig. 4. Therefore, we could think that there was a synergy between Li and the other 4 elements, respectively.

On the other hand, we also analyzed the potential interactions between thyroid hormones and elements by GLM. As listed in Table S5, there was a little bit of interaction between Sr and T3, Cu, Li and TSH, respectively (all ORs <1.00), for speculating that there may be antagonism.

4. Discussion

In the past decades, thyroid cancer has shown an upward trend worldwide. It was generally believed that TC was caused by various internal and external factors such as environment and heredity. Because

Table 3
Multi-elements model for TC with multiple elements' mixed exposure[a].

Elements	Q1	Q2	Q3	Q4	*p*-trend
Cr	1.00 (ref)	0.76 (0.37–1.55)	1.88 (0.85–4.14)	4.66 (2.10–7.34)	<0.001
As	1.00 (ref)	2.14 (1.07–4.30)	2.18 (1.08–4.37)	4.40 (2.11–9.18)	0.007
Zn	1.00 (ref)	0.88 (0.48–1.64)	1.15 (0.61–2.16)	0.40 (0.19–0.83)	0.015
Li	1.00 (ref)	0.41 (0.22–0.76)	0.61 (0.31–1.19)	0.39 (0.21–0.75)	0.019
Co	1.00 (ref)	0.44 (0.23–0.83)	0.11 (0.07–0.25)	0.18 (0.07–0.36)	<0.001
Pb	1.00 (ref)	2.39 (1.16–4.95)	3.37 (1.53–7.53)	9.09 (4.34–15.49)	<0.001
Sr	1.00 (ref)	0.29 (0.14–0.61)	0.33 (0.15–0.69)	0.23 (0.11–0.51)	0.002
Cu	1.00 (ref)	0.70 (0.35–1.39)	0.40 (0.19–0.83)	0.32 (0.14–0.71)	0.036

Q1-Q4: These quartiles were consistent with those in Table 2.
[a] Adjusted by age, gender, BMI, household annual income, physical activity, smoking status, passive smoking, sleeping quality, history exposure of X-ray, family history of thyroid diseases, family history of malignant tumor, FT3, FT4, TSH and lnI.

of the important role of thyroid in the endocrine system, people have tried to establish a link between external environmental pollutant's exposure and TC. Metals and metalloids are persistent and irreversible in external environment (Guo and Yang, 2016), have a magnifying effect potential long-term risks on people (Bing et al., 2016). Previous researches had shown that As was one of main pollutants in lower reached of Yangtze River, China (Wang et al., 2016). In India, similarly, the levels of Pb, Cd and Zn in surface water were exceeded the standard (Singh and Kumar, 2017). These trace elements can enter the human circulation through various environmental media and through various ways (such as food intake, drinking water, skin contact, breathing, etc.), and cause damage to the body through accumulation. However, the current evidence for inorganic elements and the risk of thyroid cancer was lacking.

Our results reflected the different associations with TC. We realized that the ORs of the highest concentration group of Cr and Pb were amplified, but the overall trends of these eight elements did not change too much. With BKMR model, we further delineated the exposure curves of these eight elements when other exposures were fixed, and discovered

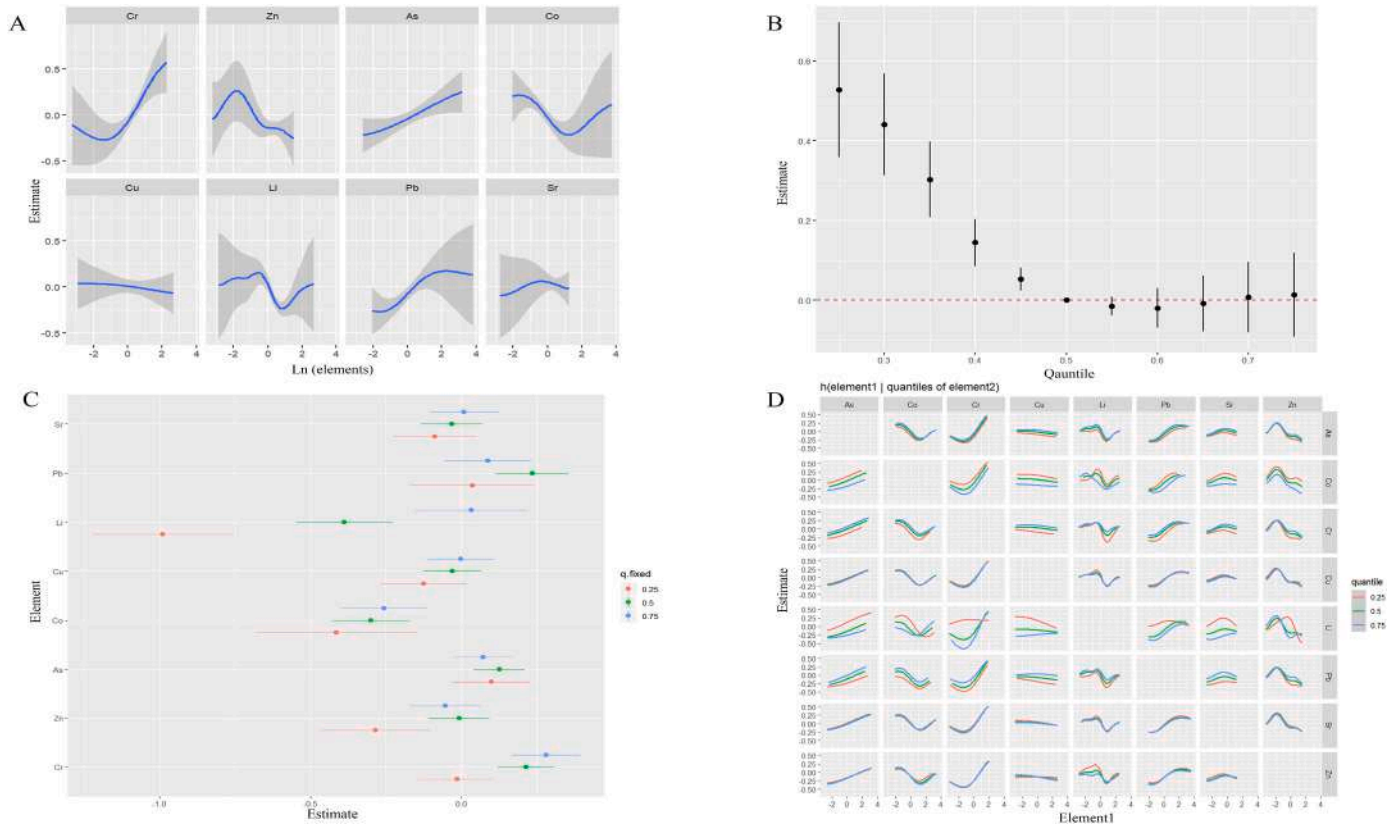

Fig. 3. The BKMR model for each element's exposure effect and tendency for the changes. (A) Univariate cross-section of each element (95%CI) with others fixed at P50s. (B) Cumulate effects of mixed-exposure in elements fixed to different percentiles as compared when they were at their medians (P50). (C) The effects of single-exposure when an individual element was at its 75th percentile as compared to when that exposure was at its 25th percentile, while other exposures were fixed at P25, P50 and P75. (D) The bivariate cross-section effects of the exposure-response function of a single element where the second element was fixed at P25, P50 and P75.

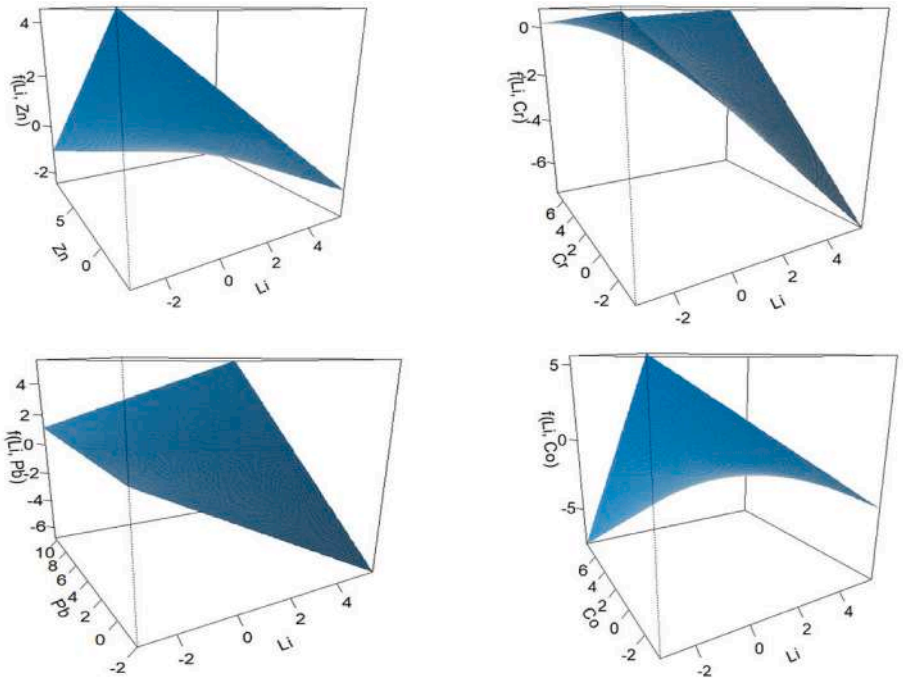

Fig. 4. The 3D stereogram for interactions between Li and Zn, Cr, Pb and Co by GLM model.

potential interactions through bivariate-section plots. Through the GLM model, we concluded that there is a certain synergy between Li and Co, Cr, Zn, and Pb.

In this study, we confirmed a significant difference in Cr levels between both groups, several studies form other countries seemed to support this point. Rezaei et al. conducted a comparative analysis of TC patients in Iranian, and found that serum Cr in patients with abnormal thyroid function was significantly higher than healthy people (Rezaei et al., 2019). Another meta-analysis of heavy metals showed that hexavalent Cr [Cr (VI)] could cause thyroid cancer from multiple cohort studies (Deng et al., 2019). There was some evidence could clarify of carcinogenesis Cr (VI). For example, long-term exposure to low level of Cr (VI) could induce DNA damage and silencing of tumor suppressor gene expression (Husain and Mahmood, 2020; Nascimento et al., 2018). This was exactly what we should confirm in the future.

We found that Pb was significantly associated with the risk of TC. Previous research had found a distinct dose-response curve between Pb exposure and the onset of PTC, suggesting that it played an important role in the pathogenesis of TC (Zhang et al., 2019). An epidemiological survey of occupational workers exposed to Pb found that in people exposed to Pb for a long time, the level of TSH was significantly increased, while the level of FT3 was significantly lower than that of healthy people (López et al., 2000). Pb also accumulated in the thyroid and disturbed the feedback regulation level of the hypothalamic-pituitary-thyroid (HPT) axis, which could be the main mechanism of Pb for thyroid cancer (Rana, 2014).

We also estimated As levels' change with TC risk, and the similar phenomenon was also reported in China (Liu et al., 2021). Another case-control study supported our view (Zhang et al., 2021), and the OR of the highest group of As was 5.35 (95%CI = 2.39–11.96). On the contrary, our previous study also found that there was a certain protective effect on goiter at lower concentrations of As (He et al., 2021a); thus, the effect of As on thyroid disease is still uncertain, and further clinical judgment is needed.

After adjusting for confounding factors, logistic regression suggested that Co might have a protective effect on the occurrence of TC. An American birth cohort study showed that increased Co levels were associated with thyroid hormone levels, including changes in FT3 and TSH levels (Rivera-Núñez et al., 2021). Animal experiments have shown that long-term Co exposure could reduce TSH levels, and then leaded to thyroid dysfunction (Falfushynska et al., 2016). Hu et al. also confirmed that in patients with confirmed PTC, those with higher Co levels have a significantly lower risk of lymph node metastasis (Hu et al., 2021a,b).

We found that the negative associations of Li and TC were also very obvious. Li is often used as a drug component in the treatment of thyroid cancer in clinic. It can change the structure of thyroglobulin, which are essential for the development of hypothyroidism and TC (Lazarus, 2009). Li can also affect the glycogen synthase kinase 3β (GSK-3β) signaling pathway, and its overactivity is directly associated with a variety of tumors (Theeuwes et al., 2018).

In Model 3, we found protective effects of Sr and Cu. Currently, a few of researches have been reported about Sr and TC risk. A former case-control study showed that the content of Sr in the blood of TC patients was significantly lower than that of healthy people. However, A previous meta-analysis showed that Chinese TC patients had higher serum Cu level (Shen et al., 2015). Other observational studies have found no significant association between Cu and thyroid cancer (Zhang et al., 2021). The opposite results might be explained by differences in the distribution of study subjects. In 2017, a case-control study of MTC found that serum Zn levels in cases were lower than in healthy controls (Emami et al., 2017). However, in the NIH-AARP diet and health study, the content of Zn in food did not differ significantly between TC and controls (O'Grady et al., 2014). Coincidentally, the lack of Zn is related to the level of thyroid hormone, which is an essential substance for the synthesis of thyrotropin and thyrotropin-releasing hormone, and Zn had an antagonistic effect with Cr, which had a protective effect on the thyroid of rats (Fedala et al., 2021; Przybylik-Mazurek et al., 2011).

Through graphical visualization of BKMR, we found interactions in mixed exposures of trace elements, which at a certain concentration changed the trend of the exposure curve of the other element. However, the interaction we were referring to is only through graphical and statistical modeling that Li has a significant impact on the exposure effects of other elements at lower concentrations. According to the results of the multiplicative interaction effect, the interaction effect is indeed small (OR< 2.00). However, this also suggested that we need to further explore the mechanism of interaction and the specific concentration range through animal experiments when we continue to deal with mixed exposures.

From the above evidence, we could know that there were significant relationships between trace elements and thyroid hormone levels in this study. In this study, we measured the levels of thyroid hormones in all subjects and included them as correction variables in the multivariate models. In our previous similar study, we focused on the relationship between population differences in thyroid hormone levels and metal exposure within a similar area. For example, FT3 had a negative correlation with urinary As, but a significant positive effect with Se. However, the interaction of thyroid hormones and elements as factors leading to TC is rarely mentioned (Hu et al., 2021a,b). We analyzed potential multiplicative interactions using the GLM model, but the effect was small (ORs close to 1). This still needs to be demonstrated through extensive mechanistic studies. In this study, we achieved dimensionality reduction of multiple element exposure models through Lasso regression, and visualized nonlinear exposure curves. Besides, we have earlier linked multiple environmental exposures to thyroid cancer development and explored the effects of mixed exposures in detail. Third, the effects between these trace elements and TC has guiding significance for the prevention of TC in Chinese population, as well as the governance and protection of the environment. In addition, trace elements generally have a longer half-life in the body, so they can reflect the long-term exposure of the human body. However, this study still has several limitations. First, as a case-control study, we could not explain the real causality, and recall bias was unavoidable. Moreover, the single measuring of urinary elements might not be accurately exposure status. Second, all participants were from hospitals, although marched by sex and age, the extrapolation of the results was limited. Third, although we adjusted for some confounding factors in our model, some potential factors were not considered. In addition, the mechanism by which exposure causes TC was not investigated in this study, and the adjustment factors included in the model may still be incomplete. Therefore, more large-scale prospective studies and laboratory trails are needed to further explore the mechanism of its occurrence.

5. Conclusions

In this case-control study, we estimated the fixed exposure of multiple elements on thyroid cancer. High levels of Cr, As and Pb are risk factors for the development of TC, while Li, Sr, Cu, Zn and Co may be protective factors. Li had interaction effects with Cr, Co, Zn and Pb. The substantive effect on it still needs to continue to be explored.

Informed consent

Informed consent was provided from all participants in this study.

Declaration of competing interest

The authors declared that they have no conflict of interest.

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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.2022.114049.

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Health information for human biomonitoring studies

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Administrative register
Record linkage

Numerous studies have shown that human exposures to contaminants present in our environment are associated with adverse health outcomes (Tolonen et al., 2021; Pruss-Ustun et al., 2019; Fuller et al., 2018) and therefore, contribute to the overall burden of non-communicable diseases (Pruss-Ustun et al., 2011). It has been estimated that environmental factors globally, can be attributed to 5.2% of disability-adjusted life years (DALYs) (Global Burden of Disease Risk Factors Collaborators Forouzanfar et al., 2015).

Furthermore, it has been estimated that almost 2% of the total annual health care costs in high-income countries and 7% in middle-income countries are caused by pollution-related diseases (Landrigan et al., 2018). The associated worldwide health costs of human exposure to environmental chemicals have been estimated to possibly exceed 10% of the global domestic product (Grandjean and Bellanger, 2017). Theoretically, this disease burden and the associated costs are avoidable through preventive initiatives such as legal restrictions related to production and use of such substances, and guidelines on how to minimize the exposure to harmful substances. It is, however, necessary to obtain information on chemical exposure burden as well as health of the population to support evidence-informed policy making.

People are part of their living environment which includes their personal characteristics such as age, sex and other constitutional factors, e.g. genetic markup, individual lifestyle factors, social and community networks, and living and working conditions (Dahlgren and Whitehead, 2007). Human biomonitoring (HBM) provides information on individual level exposure to environmental chemicals by measuring the substance itself, or its metabolites, in biological matrices such as urine or blood. To be able to evaluate the impact of environmental exposures on health, extensive data on socio-demographic characteristics, lifestyles, and health itself is also needed. Thus, a holistic approach when evaluating the impact of environmental exposures on health is warranted.

In the framework of the HBM4EU project (HBM4EU, 2021; Ganzleben et al., 2017), possible ways to add more extensive health information to HBM studies were evaluated. Two main sources of health information were identified: health examination surveys and linkage to administrative health registers.

1. Different study settings provide different possibilities

Data comprising both chemical exposure information as well as health outcomes, can be used in many ways to provide evidence to support policy decision making. Depending on the level of granularity of available data, either analysis using aggregated data or individual level data can be utilised (Coggon et al., 2003).

Ecological analysis allows investigation of geographical correlation of exposure level and disease incidence, prevalence, or mortality. For ecological analysis where the level of observation is country, region or other similar entity, required health information can usually be obtained

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from official, national level statistics, such as mortality statistics, without any additional data collection. Ecological analysis can however become biased if exposure or disease outcomes are not uniformly distributed across the unit/area of interest e.g. country, region. Further, both exposures as well as diseases may have time dependent variations, i.e. some diseases may be more prevalent during the hot or cold seasons. For time trend analysis, it is important also to consider factors such as improvement of diagnoses and health care services, differences in disease registration and procedures over time, changes in population structure in different geographical areas due to aging, migration, etc.

Etiological studies, on the other hand, allow examination into associations between exposures and health outcomes on an individual level. Cross-sectional studies provide a snapshot of the situation at a given time point within a defined target population. Because information on exposure and outcomes are collected at the same time point, it does not allow estimation of causal relationships between exposures and health outcomes, only observed associations. To determine a causal relationship, either a longitudinal approach following a cohort or case-control studies would be required although these study designs also have their limitations.

Longitudinal studies, refer to studies characterised by repeated measurements of exposure and/or health outcomes. However, re-examinations of study participants is time consuming and expensive and is often challenged by attrition bias (Nunan et al., 2018). For many health outcomes such as cardiovascular diseases (CVD), cancer or diabetes, large cohorts are needed, and they must be followed up for a long time before any results can be seen as these diseases take a long time to develop. If we, however, have the possibility to link previously collected cohorts/surveys to administrative registers such as electronic health records and mortality registers, we could facilitate retrospective information as well. This would allow us to study if those exposed at baseline in comparison to those non-exposed at baseline differ in relation to the incidence or prevalence of disease of interest during the follow-up.

2. Health data obtained through a health examination module

Combining a HBM study with a health examination survey (HES) where information is collected through questionnaires, objective health measurements and by analysing biological samples, is one approach to add more extensive health information to the exposure data. This can be done either by adding a HES module to an ongoing HBM study, adding an HBM module to an ongoing HES or planning a new combined study. A combined HBM and HES approach allows collection of both chemical exposure information as well as health information on the same individuals.

In HBM and health surveys, self-administered questionnaires or interviews are often used to collect information on lifestyle factors such as smoking dietary habits and physical activity, but also on diagnosed diseases and use of medications. Self-reported questionnaire data may suffer from social desirability, recall or awareness bias (Althubaiti, 2016). When an activity, for example smoking among adolescents or pregnant women, is considered an undesirable behaviour, there will be a risk of experiencing social desirability bias in the survey resulting in an underestimation of the true situation. For recall bias, participants may for example have trouble remembering which medications they are using, or they are not sure of their consumption of specific foods within the asked time frame. Awareness bias may occur when we for example ask if a person has a specific disease which may be asymptomatic for a long time before actual diagnoses. A good example of this is hypertension.

Therefore, whenever possible, direct health measures, such as weight and height measurement, blood pressure measurement, spirometry to determine lung function, or analysis of biomarkers from blood, urine or other biological samples, should be used as they provide more reliable information (Maukonen et al., 2018; Prince et al., 2020; Taylor et al., 2010; Paalanen et al., 2019). It should also be noted that, health measurements are prone to bias through device and measurement procedures (Tolonen et al., 2015), and laboratory analysis may also suffer from bias due to pre-analytical procedures (Tolonen et al., 2005), but also during the actual laboratory analysis due to variability between reagents, devices and laboratory procedures (Alfthan et al., 2018). However, with detailed, standardised measurement protocols and adequate training of qualified personnel, many of the sources of bias for objective health measurements and analysis of biomarkers can be minimised. Especially when cross-country comparisons are done, or time trends are analysed, a special attention should be paid for the selection of laboratories to ensure that they have passed accreditation, but also external quality assessment.

2.1. Potential for adding HBM module to ongoing or planned HES in Europe

From the evaluations conducted within the framework of HBM4EU, we know that the vast majority (90%) of the health surveys conducted in Europe collect and store biological samples for future use or have the potential to include collection of such samples in their future surveys. The most frequently collected samples are blood (plasma or serum) (71%) and urine (64%), the latter either as first morning void or spot samples. Obtained ethics approvals for these surveys often (83%) cover the possibility to analyse environmental markers from collected and/or stored samples (Tolonen et al., 2021).

The feasibility of combining HBM studies with health surveys has been demonstrated in several studies around the world (Balicco et al., 2017; Berman et al., 2017; Kolossa-Gehring et al., 2007; St-Amand et al., 2014; Centers for Disease Control and Prevention (CDC), 2021) and currently guidelines for doing this exists (Tolonen et al., 2022). Nevertheless, many study principal investigators still avoid this combination, due to lack of required knowledge and resources. The added value of extended data with both chemical exposure and health outcomes is not always seen to fit the focus of the survey (Tolonen et al., 2021).

3. Health data obtained through record linkage

Since self-reported information on diagnosed diseases may suffer from recall and awareness bias, but also social acceptability may affect the reporting behaviours (Althubaiti, 2016), administrative health records could provide a more reliable and cost-effective way to obtain required health information. Electronic health records, information on medical prescriptions, as well as birth and mortality data can be used to obtain both information on medical history, but also follow-up of morbidity and mortality if linkage to survey data is technically possible, legally allowed and required practices in the country are in place. Usually, these kinds of administrative registers have a good coverage of the population, and they tend to accumulate automatically as a result of health care services and recording of vital statistics. Therefore, there is no additional data collection costs if they also can be used for research.

For linkage of HBM survey data to administrative registers, the key requirement is that national legislation allowing the secondary use of such administrative data sources for research are available. After that, more technical details come to play a role, i.e. different data sources which are to be linked together should have common identifier(s) to allow linkage. Ideally, there is one common identifier, such as a national identification code, which is used systematically across different data sources. Then deterministic data linkage can be done. When a national identification code is not available, but all data sources to be linked have enough common elements, such as age, name, address and data of birth, linkage can be done using probabilistic methods (Harron et al., 2015).

To perform record linkage between HBM survey data and data from administrative registers, in most of the countries, informed consent from the survey participants is required together with approval from the ethics committee and data owners. It should also be noted that administrative registers are primarily generated for other purposes than

research. Even though data is accumulating continuously and tend to cover the entire population of the country/region, the quality of data may vary over time and regions due to differences in recording practices, changes in personnel, etc.

3.1. Possibilities for record linkage in Europe

The availability of different types of health-related administrative registers is good in Europe (Tolonen et al., 2021; Meltzer et al., 2022). All countries have vital statistics on births and deaths as well as either national or regional cancer registers. Availability of in-patient and out-patient hospital records (electronic health records) or medical prescriptions are, on the other hand, not systematically available in all countries. Based on this, at least mortality follow-up should, in theory, be possible in all European countries.

However, findings from the evaluation within the HBM4EU frame showed that only about half of the countries use a national identification code systematically in their administrative registers as well as in survey samples to allow simple deterministic record linkage. A few countries in Europe do not have a national identification code in use. Also, in some countries strict data protection regulations related to sensitive health data are preventing the record linkage (Tolonen et al., 2021; Meltzer et al., 2022).

4. Summary

Many environmental contaminants have been shown to be associated with adverse health effects. Up-to-date and high-quality scientific research results are needed to guide and support evidence-informed policy decision making. Data on both exposures to environmental substances as well as health outcomes in the same individuals are essential. To this end, there is a great potential to combine HBM and health surveys in Europe.

Human biomonitoring studies can be used to obtain information on exposure levels. Information on health outcomes can be obtained through health examination surveys or record linkage to administrative health registers. The availability and feasibility to use different data sources varies by country.

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Author contributions

HT, AMA, SAH and HMM wrote this commentary.

Declaration of competing interest

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Higher proportion of agricultural land use around the residence is associated with higher urinary concentrations of AMPA, a glyphosate metabolite

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ABSTRACT

Introduction: Pesticides, including herbicides, are widely used for agricultural and sanitary reasons and concerns have been raised about their various health effects. Little research has been done into the extent to which agricultural land use in the residential surroundings contributes to (internal) exposure of pesticides.
Objectives: We investigated the associations between the proportion of agricultural land use around the residence and the exposure to pesticides in adolescents in Flanders (Belgium).
Material and methods: We included 424 adolescents participating in the fourth Flemish Environment and Health Study (FLEHS IV) between 2016 and 2020. The residential address of all participants was geocoded and the proportion of agricultural land use around the residence was estimated in several buffers (300 m, 500 m, 1000 m and 2000 m). The concentrations of the following biomarkers of pesticides were measured in urine and adjusted for the specific gravity: glyphosate and its metabolite, aminomethyl-phosphonic acid (AMPA); 3-phenoxybenzoic acid (3-PBA); 3,5,6-trichloro-2-pyridinol (TCPy) and 2,4-dichlophenoxy-acetic acid (2,4-D). We categorized the pesticide biomarkers in three categories according to the exposure levels and used ordinal logistic regression models adjusted for sex, season and household education to estimate the odds ratio for an increase in an interquartile range (IQR) of proportion of agricultural land use. We also used binary logistic regression models in which the category of highest exposure was compared to the category of lowest exposure. In addition, we explored potential effect modification by sex and season.
Results: We found a significant association between the proportion of agricultural land use in a buffer of 2000 m around the residence and the levels of urinary AMPA divided into three categories (OR = 1.35 for an IQR increase in the proportion of agricultural land use around residence; 95% CI: 1.00–1.83). This association was less pronounced and not statistically significant for the other studied pesticides (OR ranging between 0.95 and 1.16). Stratified analysis showed the strongest association of the proportion of agricultural land use within 2000 m buffers for AMPA among boys (OR = 1.89; 95% CI: 1.19–3.04). Results using smaller buffers were comparable, but did not reach statistical significance.

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Conclusion: Our findings suggest that a higher proportion of agricultural land use around the residence might increase exposure to AMPA.

1. Introduction

Globally, pesticides, including herbicides, are widely used for agricultural and sanitary purposes and exposure to these products is associated with various health outcomes, such as cancer, diabetes, respiratory diseases and neurological disorders (Kim et al., 2017). At present, the determinants of exposure to specific pesticides are not fully understood. To date, the majority of pesticide studies that have been conducted are among farmers and their families; however, there are few studies of exposure levels among the general population. In particular, it is unclear to what extent living near agricultural fields where pesticides are applied determines and influences exposure.

The results of studies among farmers and their families show that pesticide exposure can occur by inhalation, dermal contact or ingestion, through direct exposure on the agricultural field or through residues tracked home from the field (Lopez-Galvez et al., 2019; US EPAa). However, pesticides could also be released into the environment via spray drift or volatilization, potentially affecting the population living around agricultural fields (Kubiak et al., 2008). Studies measuring pesticides in the environment have shown increased residual levels of glyphosate and its metabolite, aminomethyl-phosphonic acid (AMPA) in water, precipitation, and soil in the vicinity of crops where glyphosate is applied (Battaglin et al., 2014; Silva et al., 2018). In addition, pesticide and herbicide residues were also higher in indoor dust from houses in the vicinity of pesticide-treated agricultural fields for different insecticides and herbicides, including chlorpyrifos and glyphosate (Deziel et al., 2017).

Biomarkers of several pesticides have also been measured in biological samples (e.g. urine, blood) of the general population (Chevrier et al., 2014; Doganlar et al., 2018; Galea et al., 2015; Mercadante et al., 2018; Munoz-Quezada et al., 2012; Pirard et al., 2020). These studies mainly include children and pregnant women, and show generally higher concentrations among participants living close to agricultural fields (Dereumeaux et al., 2020). However, this association is not consistent across studies for all studied pesticide metabolites. In Chili, Muñoz-Quezada et al. found an association between the distance of housing to a farm and the urinary dialkylphosphate (DAP) organophosphate (OP) marker dimethyl alkyl phosphate (DMAP), but no clear association with diethyl alkyl phosphate (DEAP) levels, another DAP OP metabolite (Munoz-Quezada et al., 2012). In France, Chevrier et al. found an association between residential distance to corn fields and urinary concentrations of dealkylated triazine metabolites, but not with the other urinary herbicide metabolites investigated (acetochlor, alachlor, metolachlor, atrazine, hydroxylated triazine metabolites) (Chevrier et al., 2014). One of the reasons hypothesized for these inconclusive results is the fact that only the distance to the agricultural area is taken into account, but rarely the surface of agricultural land (Dereumeaux et al., 2020). In Wallonia (Belgium), Pirard et al. (2020) (Pirard et al., 2020) found significant associations between the agricultural surface around the residence and biomarker levels of different currently used pesticides (dimethyldithiophosphate (DMDTP), 3-phenoxybenzoic acid (3-PBA) and trans-3-(2,2-dichlorovinyl)-2,2-dimethylcylopropane (t-DCCA)) in children.

Building on these findings, we aimed to further investigate these associations between the agricultural land use around the residence and the concentrations of several pesticides and herbicides that are currently used in Flanders (Belgium). We used data from the fourth Flemish Environment and Health study (FLEHS IV), the fourth cycle of the human biomonitoring programme FLEHS in Flanders, Belgium. In this study, adolescents (14–15 years) were recruited through twenty schools spread across all five provinces and proportional to the population size of the province. More information can be found elsewhere (Schoeters et al., 2022). Previous studies conducted on the same study population reported the distribution of internal exposure to various pollutants, including urinary pesticide levels (Buekers et al., 2021; Schoeters et al., 2022). Here, we focussed on the associations of proportion of agricultural land use in different buffers around the residence and the concentrations of urinary levels of glyphosate and its metabolite AMPA, 3-PBA, 3,5,6-trichloro-2-pyridinol (TCPy), and 2,4-dichlophenoxy-acetic acid (2,4-D).

2. Material and methods

2.1. Study population and design

Flanders is the northern part of Belgium and is a densely populated region with almost half of the land area being classified as agricultural land. In 2017, most of the cultivated land consisted of meadows and pastures (36%), followed by wheat fields (21%), maize fields (20%) and potato fields (8%) (Departement Landbouw en Visserij, 2020). Pesticides and herbicides are extensively used in Flanders, mainly in fruit, potato, ornamental, cereal and beet cultivation (Departement Landbouw en Visserij, 2020). It is estimated that about 7.5 kg of pesticides and herbicides are used per hectare cropland every year, making Belgium the second pesticide user per cropland area in Europe (Food and Agriculture Organization of the United Nations, 2019).

In FLESH IV, human samples were taken to measure biomarkers of internal exposure to environmental pollutants and health effects. Several samples for biomonitoring were taken from 428 adolescents during a physical examination at the schools between September 2017 and June 2018, including urine samples for pesticide exposure. These biomarker data was enriched with information from questionnaires distributed to both parents and the adolescents. As such, information on, among others, socio-demographics, eating habits, the use of pesticides around the house and medical history was collected.

To be eligible for the study, participants had to have lived in Flanders for at least 5 years, the parents and the adolescent had to have given their informed consent and the participants and their parents had to speak the language sufficiently to fill in the Dutch questionnaire. Exclusion criteria were: staying in boarding school, more than one missing questionnaire, missing blood and urine sample and having repeated more than one year of schooling. One participant was excluded from the study because of pregnancy.

2.2. Agricultural land use around the residence

The agricultural land use around the residence was calculated as the proportion land classified as agricultural in circular buffers of 300, 500, 1000 and 2000 m around the residence. The residential addresses of the participants were geocoded and the agricultural land use around the residential address in these buffers was calculated using the Geographic Information System (GIS) ArcGIS 10 software. We based the classification of agricultural land on “Groenkaart Vlaanderen 2013”. This is a segmented grid classification with a resolution of 1 m^2. Every pixel on the map is classified as “not green”, “agriculture”, “low green” (<3 m) or “high green” (>3 m). First, a classification is made into three classes (“not green”, “low green” and “high green”) based on summer flight photographs of 2012. Consequently, some “not green” and “low green” areas are classified as “agriculture” based on the 2012 agricultural land use map of the department of Agriculture and Fisheries which shows the

parcels of land with an application to the department. In addition, the geocoded address was also used to classify the residential area into an urban, suburban or rural area based on the DEGURBA classification of the Statistical Office of the European Union (Eurostat, 2011). For statistical analysis, this variable was dichotomized. Cities (DEGURBA 1) were classified as urban and towns and suburbs (DEGURBA 2) and rural areas (DEGURBA 3) were classified as non-urban. In a sensitivity analysis, we also used land use map 2013 to classify agricultural land in the same buffers. This map has a spatial resolution of 10 × 10 m and contains information about the actual use of the land cover. In addition, we used the information of this map, to subdivide the proportion of agricultural land. Accordingly, we calculated the proportion of 'arable farming', 'cultivated grassland', 'high stem orchard', 'low stem orchard' and 'unregistrered agriculture' in buffers around the residence.

2.3. Biomarkers of exposure to pesticides and herbicides

During the physical examination, the adolescents were asked to provide a urine sample. Spot urine samples were collected in clean polyethylene (PE) containers and were processed immediately. To determine the specific gravity of the urine samples, 2 mL was aliquoted in a polypropylene tube, kept at 4 °C and analyzed within 48 h after collection by refractometry at the accredited medical laboratory A.M.L. (Algemeen Medisch Laboratorium, Antwerp, Belgium). The samples for pesticide analysis were sent cooled to the Flemish Institute of Technological Research (VITO) at a temperature of 4 °C. At latest the day after sample collection, the urine samples were frozen at −20 °C.

The internal exposure to pesticides was determined by quantifying the following biomarkers in urine: glyphosate and AMPA, 3-PBA, TCPy, and 2,4-D. Glyphosate and AMPA are biomarkers for the exposure to the herbicide glyphosate and were measured by means of gas chromatography - tandem mass spectrometry (Conrad et al., 2017). 3-PBA is a biomarker for exposure to 6 different pyrethroid insecticides (Cypermethrin, Deltamethrin, Permethrin, Cyhalothrin, Fenpropathrin and Tralomethrin) and was measured by high performance liquid chromatography - tandem mass spectrometry (Dalsager et al., 2018). TCPy is a biomarker of exposure to chlorpyrifos, one of the most used organophosphate pesticide and 2,4-D is a biomarker of exposure to the phenoxyherbicide 2,4-D. The latter two were also measured by means of high-performance liquid chromatography-tandem mass spectrometry (Dalsager et al., 2018). For the analyses, the concentrations of all biomarkers were adjusted for the specific gravity of the urine by the following formula:

$$Concentration\ biomarker \left[\frac{1.024-1}{(specific\ gravity-1)}\right]$$

Data on specific gravity was missing for nine samples and these samples were excluded from the analysis. All biomarkers are expressed in μg/L.

2.4. Potential confounders and effect modifiers

Potential confounders considered were household education (as a proxy for socio-economic status), sex and season of measurement. Information about the household education (i.e. the highest level of education reported by the parents) was obtained from the parents and the answers were classified into "high", "medium" or "low" education according to the International Classification of Education (ISCED) (UNESCO Institute for Statistics, 2012). Sex and season of measurement were also considered as potential effect modifier. In sensitivity analyses, consumption of local food as well as private use of insecticide/herbicide were additionally considered as potential confounders. This information was available through the questionnaire distributed to the parents. Average weekly use of vegetables and fruits was reported by the parents on a 5-point scale (never, less than 1x/week, 1–3x/week, 4–6x/week or daily).The private use of insecticides was reported both indoor as outdoor. Outdoor use was a binary variable (yes/no), while indoor use was reported as a 5-point frequency scale (never, sometimes, weekly in some seasons, daily in some seasons, weekly the whole year, daily the whole year). The private use of herbicides was reported as outdoor use on a binary scale (yes, no).

2.5. Statistical analysis

The biomarkers of pesticides were left censored because several samples had values below the limit of quantification (LOQ). The percentage of samples with values < LOQ varied across biomarker. For 2,4-D, TCPy and 3-PBA, less than 15% had values < LOQ. For the descriptive statistics, we imputed these values < LOQ with a single imputation. First, a truncated lognormal distribution was fitted based on the observed values (i.e. values > LOQ). Then, values < LOQ were randomly imputed based on this fitted distribution (Lubin et al., 2004). For AMPA and glyphosate, more than 40% had values < LOQ. Because of this large number, values < LOQ were not imputed but reported as such. As a consequence, geometric means could not be calculated. We computed descriptive statistics both for the total study population and stratified for the participants in non-urban and urban areas, as well as stratified for season.

For further statistical analysis, we used the non-imputed data for all pesticide biomarkers and transformed the continuous variables into categorical variables with three categories. When the percentage of samples < LOQ was above 33% (i.e. AMPA and glyphosate), we used the LOQ and the median of the values above LOQ as cut-offs. We categorized these values because of the high number of values < LOQ and therefore otherwise a considerable amount of missing data. When the percentage of samples with values < LOQ was less than 33% (i.e. 2,4-D, TCPy and 3-PBA), we used tertiles. Although not strictly necessary for the analysis, we also categorized these variables to be able to use the same regression models as for AMPA and glyphosate.

The association between proportion of agricultural land use around the residence and biomarkers of pesticides was estimated by means of an ordinal logistic regression. We obtained OR and 95% CI for an interquartile range (IQR) increase in the proportion of agricultural land use around the residence. Additionally, we used a binary logistic regression in which the category of highest exposure was compared to the category of lowest exposure. All models were adjusted for all potential confounders. We also explored potential effect modification by stratifying the analysis and by including interaction terms. The linearity of the association was explored using generalized additive models (GAMs) with smooth terms of the proportion of agricultural land use.

We performed various sensitivity analyses. First, we repeated the analysis using tertiles of proportion of agricultural land use if GAMs showed non-linearity. Second, analysis was also repeated with urinary biomarker values adjusted for creatinine instead of specific gravity, as those values did not contain missing data. We also repeated the ordinal regression models excluding adolescents with at least one of the parents working in the agricultural sector and repeated the analysis with the consumption of locally consumed food as well as private use of insecticide/herbicide as additional confounding variables in the models. Finally, we classified agricultural land in GIS based on the land use map 2013 and examined associations between the proportions of different subcategories of agricultural land use around the residence (arable farming, cultivated grassland, high stem orchard, low stem orchard, unregistered agriculture) and urinary pesticide levels. All statistical analyses were performed using codes written in the statistical software R, version 4.1.2 (R Core Team, 2022).

3. Results

Of the 1655 participants initially approached, 428 were included (Schoeters et al., 2022). The main reason for non-participation was lack

of informed consent. No urine was collected from two participants and from two other participants the amount of urine was not sufficient for analysis. This eventually resulted in 424 participants. The characteristics of the study population are summarized in Table 1. The age of the adolescents ranged from 13 to 16 years. In 25 adolescents (6%), the highest education of the household was low. For the largest part of the study participants (60%), the household education was high. Urine was mostly sampled during spring (45% of the samples).

The LOQ and distributions of the measured pesticides adjusted for specific gravity are described in Table 2. For AMPA and glyphosate, a high proportion of samples had values below the LOQ (44% and 58%, respectively). The median value adjusted for specific gravity was 0.11 μg/L for AMPA and < LOQ for glyphosate. For 2,4-D, TCPy and 3-PBA, the percentage of samples < LOQ was lower than for AMPA and glyphosate, ranging from 3% (TCPy and 3-PBA) to 14% (2,4-D). After single imputation of the values < LOQ based on a fitted truncated lognormal distribution, the geometric mean adjusted for specific gravity was 0.26 μg/L, 4.25 μg/L and 0.95 μg/L, for 2,4-D, TCPy and 3-PBA, respectively. The concentrations of the pesticide biomarkers in urine were generally slightly lower among urban participants than among non-urban participants for AMPA, 2,4-D and 3-PBA. This was not the case for glyphosate and TCPy. Urinary AMPA levels and glyphosate levels were moderately correlated (Spearman rank coefficient 0.39, $p < 0.001$). By season, the strongest correlations were observed in fall (Spearman rank coefficient 0.43, $p < 0.001$) and the weakest in winter (Spearman rank coefficient 0.33, $p < 0.001$).

Both before and after adjustment for sex, season and education, urinary AMPA levels were more likely to be higher and > LOQ in function of higher residential agricultural land use. We observed these associations for every buffer and in both models, although only statistically significant for the proportion of agricultural land use within a 2000 m buffer in the ordinal regression models (comparing the lowest level of biomarkers with the medium and the highest level) (aOR = 1.35; 95% CI: 1.00–1.83) (Table 3). For the other pesticide biomarkers measured in this study, the associations were close to null and not statistically significant.

After stratification by sex, the association was stronger in boys for every pesticide, except for 2,4-D (Table 4). However, the interaction terms between the proportion of agricultural land use and sex and season were not significant. The association between the proportion of agricultural land use and urinary AMPA levels were statistically significant for every buffer in the ordinal regression models. No significant association was found after stratification by season (data not shown).

Table 1
Characteristics of the study population (n = 424).

	Number (%)	Median (IQR)
Age		14.8 (0.64)
Girls	227 (54%)	
Household education		
Low	25 (6%)	
Medium	138 (33%)	
High	253 (60%)	
Missing	8 (2%)	
Season of measurement		
Winter	137 (32%)	
Spring	189 (45%)	
Summer	0 (0%)	
Autumn	98 (23%)	
Proportion of agricultural land use		
300 m buffer		0.14 (0.33)
500 m buffer		0.22 (0.37)
1000 m buffer		0.29 (0.40)
2000 m buffer		0.38 (0.36)
Urban residents*	58 (14%)	

Urban residents are defined as adolescents living in an area classified as DEGURBA 1 and non-urban residents as those living in an area classified as DEGURBA 2 or DEGURBA 3.

We applied GAMs to check for the linearity of the association. We obtained significant smooth terms in the association of agricultural land use in a 1000 m buffer and urinary AMPA and in the association between agricultural land use in a 2000 m buffer and glyphosate. The other smooth terms were not statistically significant. In sensitivity analyses, we repeated our models for AMPA and glyphosate including the exposure to residential agricultural land as 3-category variable (tertiles). The results were similar to the ones reported in Table 3. For an increase from the first to the third tertile in residential agricultural land use within 2000 m of residence, the odds ratio for urinary AMPA values was 1.73 (95% CI: 1.10–2.73) in the ordinal regression models and 2.20 (95% CI: 1.18–4.16) in the binary logistic regression models (supplementary material, Table S2). The odds ratio for urinary glyphosate levels for an increase from the first to the third tertile in residential agricultural land use within 2000 m of residence, was 1.50 (95% CI: 0.93–2.44) in the ordinal regression models and 1.99 (95% CI: 1.01–4.00) in the binary logistic regression models (supplementary material, Table S2).

Sensitivity analyses using urinary biomarker values adjusted for creatinine instead of specific gravity showed similar association estimates (data not shown). Also, we obtained similar results when we conducted analyses excluding 7 adolescents whose parents had a job in the agricultural sector although associations between the proportion of agricultural land use and urinary AMPA levels were slightly stronger and became significant in all buffers (supplementary material, Table S3). After additional adjustment for local consumed food (supplementary material, Table S4) and private use of insecticides/herbicides, associations were comparable (supplementary material, Table S5). Finally, when we used land use map 2013 to calculate the proportion of agricultural land use, results were comparable (supplementary material, Table S7). When agricultural land was subdivided, the association between the proportion of agricultural land and urinary AMPA was only comparable for arable farming (supplementary material, Table S8). We did not find any clear association between the proportion of cultivated grassland, high stem orchard, low stem orchard or unregistered agriculture and urinary AMPA or other pesticide biomarker levels (supplementary material, Table S8).

4. Discussion

We found that, in adolescents, a higher proportion of agricultural land use within 2000 m around the residence was associated with higher urinary levels of AMPA, a degradation product of glyphosate. No significant associations were found between the proportion of agricultural land use and the other studied pesticide biomarkers, including glyphosate, 3-PBA, TCPy and 2,4-D. To date, little research has been carried out on determinants of glyphosate exposure in the general population (Connolly et al., 2020). In contrast to our results, a study of Stajnko et al. (2020) in children and adolescents in Slovenia (Stajnko et al., 2020) found no difference in the urinary AMPA levels depending on the distance between homes and agriculture, both in winter and in late spring/early summer. However, they did find a difference in urinary glyphosate levels depending on the agriculture distance, although this difference was only significant in winter. Differences in climatological conditions between Belgium and Slovenia as well as difference in defining agriculture around the residence and correction for potential confounders might explain these contrary results. We did not find any other study that investigated the association between the presence of agriculture around the residence and glyphosate or AMPA levels.

Previous research has demonstrated that pesticides can be released into the environment through primary spray drift during application or as secondary spray drift through volatilization of pesticide residues from crops or soil (Kubiak et al., 2008). Elevated levels of glyphosate and AMPA have been found in water, precipitation, soil and house dust in the vicinity of crops where glyphosate is applied (Battaglin et al., 2014; Silva et al., 2018). The association we found between the proportion of agricultural land use and urinary AMPA levels could potentially be

Table 2
Concentrations of pesticide biomarkers measured in the urine and adjusted for specific gravity, expressed in μg/L (n = 415).

Parent pesticide	Biomarker	LOD	LOQ	Region	<LOQ (n (%))[a]	GM	P25	P33	P50	P66	P75	P95	Max
Glyphosate	AMPA	0.03	0.10	Total	187 (44%)	.	<LOQ	<LOQ	0.11	0.17	0.19	0.37	2.82
				Urban	27 (47%)	.	<LOQ	<LOQ	0.09	0.14	0.15	0.41	0.75
				Non-urban	160 (44%)	.	<LOQ	<LOQ	0.12	0.17	0.20	0.38	2.82
				Autumn	51 (52%)	.	<LOQ	<LOQ	<LOQ	0.15	0.18	0.38	2.82
				Winter	70 (51%)	.	<LOQ	<LOQ	<LOQ	0.14	0.20	0.30	0.48
				Spring	66 (35%)	.	<LOQ	<LOQ	0.14	0.18	0.21	0.37	1.43
	Glyphosate	0.03	0.10	Total	248 (58%)	.	<LOQ	<LOQ	<LOQ	0.11	0.15	0.39	3.75
				Urban	33 (57%)	.	<LOQ	<LOQ	<LOQ	0.13	0.16	0.58	3.75
				Non-urban	215 (59%)	.	<LOQ	<LOQ	<LOQ	0.11	0.15	0.36	1.41
				Autumn	55 (56%)	.	<LOQ	<LOQ	<LOQ	<LOQ	0.11	0.35	0.78
				Winter	92 (67%)	.	<LOQ	<LOQ	<LOQ	0.12	0.17	0.34	0.95
				Spring	101 (53%)	.	<LOQ	<LOQ	<LOQ	0.14	0.18	0.40	3.75
2,4-D	2,4-D	0.03	0.09	Total	60 (14%)	0.26	0.16	0.19	0.27	0.38	0.45	0.99	2.38
				Urban	12 (21%)	0.20	0.11	0.13	0.19	0.32	0.41	1.05	2.18
				Non-urban	48 (13%)	0.27	0.17	0.21	0.28	0.40	0.46	0.95	2.38
				Autumn	17 (17%)	0.24	0.15	0.18	0.24	0.38	0.43	0.82	2.29
				Winter	27 (20%)	0.22	0.16	0.19	0.27	0.38	0.45	1.07	1.53
				Spring	16 (9%)	0.30	0.19	0.23	0.33	0.41	0.47	1.00	2.38
Chlorpyrifos	TCPy	0.30	0.50	Total	14 (3%)	4.25	2.82	3.27	4.45	5.86	6.46	12.1	50.0
				Urban	1 (2%)	4.68	3.02	3.33	4.53	6.29	7.16	13.2	18.6
				Non-urban	13 (4%)	4.19	2.73	3.31	4.45	5.68	6.34	11.3	50.0
				Autumn	3 (3%)	4.11	2.65	3.04	4.48	5.78	6.48	12.0	28.6
				Winter	6 (4%)	4.04	2.85	3.18	4.11	5.09	6.04	12.2	18.3
				Spring	5 (3%)	4.47	2.97	3.62	4.74	6.04	6.84	11.7	50.0
Cypermethrin, Deltamethrin, Permethrin, Cyhalothrin, Fenpropathrin and Tralomethrin	3-PBA	0.03	0.09	Total	11 (3%)	0.95	0.58	0.66	0.87	1.30	1.58	4.04	44.7
				Urban	1 (2%)	0.85	0.55	0.64	0.85	1.11	1.45	3.01	23.9
				Non-urban	10 (0.3%)	0.97	0.59	0.67	0.92	1.32	1.62	3.99	44.7
				Autumn	4 (4%)	0.98	0.60	0.67	0.94	1.43	1.91	3.87	23.9
				Winter	6 (4%)	0.81	0.51	0.59	0.77	1.16	1.35	2.89	32.7
				Spring	1 (0.5%)	1.02	0.61	0.70	0.93	1.31	1.61	2.72	44.7

LOD = Limit of Detection; LOQ = Limit of Quantification; AMPA = aminomethyl-phosphonic acid; 2,4-D = 2,4-dichlophenoxy-acetic acid; TCPY = 3,5,6-trichloro-2-pyridinol; 3-PBA = 3-phenoxybenzoic acid; all values expressed in μg/L. GM = geometric mean, the geometric mean was calculated after imputation of the values below LOQ with a single imputation based on the fitted truncated lognormal distribution. P25 = 25th percentile, P33 = 33rd percentile, P66 = 66th percentile, P75 = 75th percentile, P95 = 95th percentile, Max = maximum.

[a] The number < LOQ is calculated based on the data not adjusted for specific gravity (n = 424).

confounded by differences in food consumption or private use of herbicides. However, the association between the proportion of agricultural land use around the residence and urinary AMPA levels remained after additional correction for local consumed food or private use of herbicides. In addition, since July 2017 it is no longer allowed in Flanders to use glyphosate in non-professional setting (Vlaamse Regering, 2017).

We only found a clear association between the proportion of agricultural land use around the residence and urinary AMPA levels, but not for urinary glyphosate. However, for boys, the association between the proportion of agricultural land use around the residence and glyphosate levels was stronger, although not statistically significant (aOR = 1.31 (95% CI: 0.82–2.11) for the proportion of agricultural land use within 2000m around the residence). A previous study in Germany also has shown that AMPA and glyphosate levels are higher in boys than in girls (Conrad et al., 2017), but no explanation could be found. In line with this study, we observe higher AMPA and glyphosate levels among boys. Our results suggest that residential exposure could partly explain the increased exposure in boys, possibly because boys spend more time outdoors (Klinker et al., 2014). However, this would imply that exposure mainly occurs outdoors. Nevertheless, pesticides have also been found in indoor dust samples from homes located near agricultural fields (Deziel et al., 2017). We found a moderate correlation between urinary levels of AMPA and glyphosate, in line with other published studies in Europe (Conrad et al., 2017; Lemke et al., 2021; Stajnko et al., 2020). Possible explanations given for these moderate correlations include alternative sources of AMPA exposure, differences in AMPA/glyphosate absorption rate in gut and the AMPA/glyphosate ratios in diet (Stajnko et al., 2020). AMPA is mainly formed in the environment after degradation of glyphosate. In the human body, metabolism of glyphosate to AMPA is probably very limited (Connolly et al., 2020). Alternatively, AMPA may be formed in the environment following degradation of phosphonic acids found in household and industrial detergents and cleaning products (Nowack, 2003). However, it is unlikely that these products are used more frequently in homes located in areas with a high proportion of agricultural land use than, for example, in urban homes. In addition, we found differences in the correlations across seasons, similar to the findings of Stajnko et al. (2020) (Stajnko et al., 2020). The potential explanations given for the moderate correlation between glyphosate and AMPA could not explain these differences in seasons, except possibly the differences in AMPA/glyphosate ratios in diet. The fact that we found the strongest association in fall is probably related to the recruitment strategy through school, which resulted in no sampling in late spring/-summer. The sampling period closest to the spraying season is probably fall (Quaghebeur et al., 2004). A likely explanation for the differences in the results between glyphosate and AMPA and the differences in correlation across different seasons is the fast degradation of glyphosate, and the longer persistence of AMPA in the environment (Bento et al., 2016). This would lead to higher AMPA concentrations in the

Table 3
Estimated odds ratios and their 95% confidence intervals (aOR and 95% CI) of the level of pesticide biomarkers (three and two categories) per IQR increase in the proportion of agricultural land use in different buffers around the residence.

Radius (m)	Three categories[a]	Two categories[b]
	aOR (95% CI)	aOR (95% CI)
AMPA		
300	1.27 (0.97–1.65)	1.35 (0.93–1.95)
500	1.32 (0.99–1.75)	1.38 (0.94–2.05)
1000	1.35 (0.99–1.85)	1.45 (0.94–2.23)
2000	**1.35 (1.00–1.83)**	1.51 (1.00–2.30)
Glyphosate		
300	0.95 (0.71–1.26)	0.92 (0.62–1.34)
500	0.98 (0.72–1.32)	0.99 (0.65–1.49)
1000	1.00 (0.72–1.39)	1.08 (0.69–1.69)
2000	1.09 (0.80–1.49)	1.25 (0.81–1.92)
2,4-D		
300	1.04 (0.80–1.35)	1.03 (0.72–1.49)
500	1.06 (0.80–1.40)	1.05 (0.71–1.55)
1000	1.07 (0.79–1.45)	1.06 (0.69–1.63)
2000	1.07 (0.80–1.43)	1.06 (0.70–1.59)
TCPy		
300	1.15 (0.89–1.49)	1.22 (0.84–1.76)
500	1.16 (0.88–1.54)	1.22 (0.83–1.80)
1000	1.07 (0.79–1.46)	1.08 (0.71–1.64)
2000	1.07 (0.80–1.44)	1.05 (0.71–1.56)
3-PBA		
300	1.07 (0.83–1.38)	1.09 (0.75–1.60)
500	1.10 (0.83–1.44)	1.13 (0.76–1.69)
1000	1.06 (0.78–1.44)	1.06 (0.69–1.63)
2000	1.07 (0.80–1.44)	1.07 (0.72–1.59)

Models adjusted for sex, season, and household education. Bold indicates p-value<0.05.

[a] Three categories: For AMPA and glyphosate: <LOQ (low), <P50 of values above LOQ (medium) and >P50 above LOQ (high); For 2,4-D, TCPy and 3-PBA: first (low), second (medium) and third tertiles (high).

[b] Two categories: For AMPA and glyphosate: <LOQ (low) and >P50 above LOQ (high); For 2,4-D, TCPy and 3-PBA: first (low) and third tertile (high).

Table 4
Estimated odds ratios and their 95% confidence intervals (aOR and 95% CI) of the level of pesticide biomarkers (three categories) per IQR increase in the proportion of agricultural land use in different buffers around the residence; stratified by sex.

	Three categories[a]	
	aOR (95% CI)	
Radius (m)	Boys	Girls
AMPA		
300	**1.53 (1.01–2.34)**	1.13 (0.79–1.60)
500	**1.64 (1.05–2.59)**	1.16 (0.80–1.70)
1000	**1.80 (1.12–2.93)**	1.14 (0.75–1.75)
2000	**1.89 (1.19–3.04)**	1.13 (0.75–1.70)
Glyphosate		
300	1.32 (0.86–2.02)	0.71 (0.46–1.05)
500	1.30 (0.82–2.04)	0.77 (0.50–1.16)
1000	1.28 (0.79–2.09)	0.80 (0.50–1.27)
2000	1.31 (0.82–2.11)	0.95 (0.62–1.46)
2,4-D		
300	0.90 (0.60–1.35)	1.12 (0.79–1.6)
500	0.89 (0.57–1.37)	1.18 (0.81–1.72)
1000	0.88 (0.55–1.39)	1.24 (0.82–1.91)
2000	1.04 (0.66–1.62)	1.06 (0.70–1.60)
TCPy		
300	1.25 (0.84–1.87)	1.16 (0.82–1.63)
500	1.33 (0.86–2.05)	1.13 (0.78–1.64)
1000	1.23 (0.78–1.96)	1.02 (0.67–1.54)
2000	1.24 (0.78–1.96)	0.98 (0.66–1.46)
3-PBA		
300	1.38 (0.93–2.07)	0.92 (0.65–1.29)
500	1.45 (0.94–2.23)	0.94 (0.65–1.35)
1000	1.36 (0.85–2.16)	0.92 (0.61–1.40)
2000	1.38 (0.88–2.17)	0.93 (0.62–1.38)

Models adjusted for sex, season, and household education. Bold indicates p-value<0.05.

[a] Three categories: For AMPA and glyphosate: <LOQ (low), <P50 of values above LOQ (medium) and >P50 above LOQ (high); For 2,4-D, TCPy and 3-PBA: first (low), second (medium) and third tertiles (high).

environment across seasons compared to glyphosate, and thus higher probability of human exposure. This is also reflected in the high number of samples < LOQ for glyphosate in winter (67%). Therefore, people living in the surrounding of agricultural fields could be exposed to AMPA across seasons through, for example, erosion of contaminated soils. Additionally, this erosion could explain why the associations become stronger with increasing buffers.

Our results are important given the various health effects attributed to exposure to glyphosate and AMPA. In 2015, the International Agency for Research on Cancer (IARC) classified glyphosate as probably carcinogenic compound (Guyton et al., 2015) and a meta-analysis of Zhang et al. (2019) (Zhang et al., 2019) confirmed an increased risk of non-Hodgkin lymphoma at higher exposure to glyphosate-based formulations. However, the carcinogenicity of glyphosate remains under discussion (Meftaul et al., 2020). For AMPA, few studies are available, but there is moderate evidence for its genotoxic potential and strong evidence for induction of oxidative stress (IARC working group, 2015). A recent study in Flanders using AMPA data from an earlier FLEHS programme (FLEHS III), also found an association between urinary AMPA levels and telomere length (Cosemans et al., 2022).

We found no statistically significant association between the proportion of agricultural land use around the residence and the levels of 3-PBA, TCPy and 2,4-D. In contrast, in the southern and more rural part of Belgium (Wallonia), Pirard et al. (2020) (Pirard et al., 2020) found a statistical significant association between the agricultural surface around the residence and urinary 3-PBA levels. However, our results are in line with previous research of Fiedler et al. (2015) (Fiedler et al., 2015) in Thailand where they did not find any difference in urinary 3-PBA levels between children living in the vicinity of rice fields (high pesticide use, geometric mean in high pesticide use season: 1.74 μg/g creatinine (95% CI: 1.13–2.67)), compared to children living close to aquaculture (control group, geometric mean in high pesticide use season: 1.80 μg/g creatinine (95% CI: 1.29–2.51)). Similar to our results, for TCPy, Pirard et al. (2020) could not find an association between the surface of agricultural land and urinary pesticide levels. Fiedler et al. (2015) (Fiedler et al., 2015), on the contrary, did find high urinary TCPy levels in children living in areas of high pesticide use (geometric mean in rice field population in high pesticide use season 6.6 μg/g creatinine (95% CI: 4.29–8.55) vs 2.84 μg/g creatinine(95% CI: 2.06–3.90) in the control group). In addition, in the USA, Fenske et al. (2002) reported high house dust concentrations in the homes of children living close to agricultural land. The parent insecticides/herbicide of 3-PBA and 2,4-D (pyrethroids and 2,4-D) are not only used in agriculture, but also privately (National pesticide information center, 2011; US EPAb). In addition, pyrethroids are also used indoors in insecticide sprays. Therefore, it is possible that the private use of these insecticides/herbicide explains the very weak and not statistically significant associations with the proportion of agricultural land use that we observe in our study. However, additional adjustment for private use of insecticides/herbicides in general, as responded to in the questionnaire, did not change our results. Unfortunately, we did not have information about the private use of the specific parent pesticides by our study participants. Another potential explanation for our results regarding these three biomarkers is the use of the parent insecticide of TCPy (chlorpyrifos) in agricultural surface. In Belgium and at the time the study, the use of this insecticide in agriculture was limited to specific types of crops (cabbages, asparagus, leeks, ornamentals, horticulture and topsoil). In our study, we did not have information on the type of agriculture present around the home which, in this case, may have led to misclassification and bias our results towards the null. Nevertheless, since 2020, the use

of this insecticide is no longer allowed in Europe (European Commision, 2019).

The lack of information about type of crops or pesticide use was a considerable limitation of our study. However, the presence of animal feed crops (grassland, maize) and cereals constitute of a large part of agriculture in Flanders (around 75%) and these crops are well distributed (VITO, 2020). Moreover, the herbicide glyphosate is used in all these cultivations (Antier et al., 2020). In contrast, in a sensitivity analysis using land use map 2013 with agricultural subcategories, we could not find an association between the proportion of cultivated grassland around the residence and urinary AMPA levels. This could possibly be explained by the lack of variability in the proportion of cultivated grassland. Our study had also some additional limitations. We used only one measurement point for urinary biomarker levels. The pesticide biomarkers measured in urine have a short half-life (less than 3 days for all the pesticides measured) (Agency for toxic substances and disease registry (ATSD), 2020; Agency for toxic substances and disease registry (ATSDR), 2003; Connolly et al., 2019; Nolan et al., 1984) and therefore reflect only the exposure in the preceding days. In addition, within-person variability exist for TCPy and 3-PBA (Meeker et al., 2005; Morgan et al., 2016). Potentially, this measurement error may have led to a misclassification bias in our study. As this bias is probably not differential, it would only have been a bias towards the null. Finally, because of the recruitment strategy through schools, we did not have any sampling in the summer months. The fact that pesticides/herbicides are also used in other seasons could mean that we missed an important exposure window.

Notwithstanding these limitations, our study has also some major strengths. We use measurements of the internal exposure to various pesticides for 424 adolescents from all over Flanders and used both glyphosate and AMPA measurements to determine the exposure to glyphosate. The advantage of including this age group is that they are not professionally exposed to pesticides, as could be the case with adults, and do not have increased hand-mouth activity, as is the case with children. Therefore, their exposure reflects the exposure in the general population. In addition, the sampling strategy increased the external validity of our study by sampling a general population sample of adolescents from different geographical areas and with different educational levels. However, with regard to the highest educational attainment of the adolescents' household, low educational attainment was underrepresented (6% of households with low educational attainment in this study population compared to 19% in the general Flemish population) (Schoeters et al., 2022). This may be reflected in differences in household habits (e.g. different hygienic behaviors). Furthermore, rather than just using distance to agriculture as a determining factor for pesticide exposure, we also included information on the amount of agriculture by using the proportion of agricultural land use in various buffers around the residence.

5. Conclusion

We found evidence that more agricultural land use around the residence is associated with an increase in the levels of urinary AMPA, a biomarker for exposure to the herbicide glyphosate. This association is higher in boys than in girls. These findings, examined here for the first time in the general population, suggest that spray drift or volatilization of fields treated with glyphosate may contribute to the herbicide's internal exposure. This is important given the reported health effects of glyphosate and AMPA. Therefore, we suggest that for the people living around agricultural fields, this route of exposure should be taken into account in the health impact assessment of glyphosate. We did not find any significant association between the proportion of agricultural land use around the residence and the other studied pesticide biomarkers, including glyphosate, 3-PBA, TCPy and 2,4-D.

Ethics approval and consent to participate

The FLEHS IV study protocol was approved in June 2017 by the Antwerp University Hospital Ethics committee (Belgian registration number B300201732753).

Author contributions

Katrien De Troeyer Methodology, Formal analysis, Writing – original draft, **Lidia Casas** Methodology, Writing – review & editing, **Esmée M Bijnens** Data curation, Writing – review & editing, **Liesbeth Bruckers** Conceptualisation, Project administration, Writing – review & editing, **Adrian Covaci** Conceptualisation, Project administration, Writing – review & editing, **Stefaan De Henauw** Conceptualisation, Project administration, Writing – review & editing, **Elly Den Hond** Conceptualisation, Project administration**,** Writing – review & editing, **Ilse Loots** Conceptualisation, Project administration, Writing – review & editing, **Vera Nelen** Conceptualisation, Project administration Writing – review & editing, **Veerle J Verheyen** Conceptualisation, Project administration, Writing – review & editing, **Stijn Vos** Data curation, Writing – review & editing, **Greet Schoeters** Conceptualisation, Project administration**,** Writing – review & editing, **Hans-Wolfgang Hoppe** Resources**,** Writing – review & editing, **Helmut Dietrich Köste** Resources**,** Writing – review & editing, **Tim S Nawrot** Conceptualisation, Project administration, Supervision, Writing – review & editing,. All authors read and approved the final manuscript. The authors had full access to all data in the study and take responsibility for the data integrity and the accuracy of the data analysis.

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Consent for publication

Not applicable.

Availability of data and materials

Data are available upon reasonable request (tim.nawrot@uhasselt.be).

Declaration of competing interest

All authors declare they have no competing interest.

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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.2022.114039.

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Increased self-reported sensitivity to environmental stimuli and its effects on perception of air quality and well-being

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ABSTRACT

Background: In previous studies, negative associations were found between increased environmental sensitivity and general well-being as well as positive perception of air quality. However, only a few studies with partly inconsistent results examined this relation under exposure. They tried to determine whether people with increased environmental sensitivity react to real environmental conditions with changes in current well-being and perception of air quality.

Methods: Pooled data from two single-blinded randomized controlled trials with different exposure levels were analyzed. Participants were exposed to different levels of volatile organic compounds (VOC) and carbon dioxide (CO_2) in the front part of a former in-service wide-body airplane inserted in a low-pressure chamber. Three exposure groups were created depending on the VOC/CO_2 levels: low, medium and high. Subjects repeatedly answered questions about their current mental well-being and about perception of air quality and odor intensity. Based on self-reported data the participants were classified into groups with low and higher environmental sensitivity. Data were evaluated using a 2 (environmental sensitivity) x 3 (exposure) ANCOVA with repeated measures.

Results: 503 individuals (221 females) participated (mean age: 42.8 ± 14.5 years). Thereof, 166 individuals were assigned to the group with higher environmental sensitivity; they reported poorer psychological well-being regarding vitality (F (1,466) = 16.42, $p < .001$***, partial $\eta^2 = 0.034$) and vigilance (F (1,467) = 7.82, $p = .005$**, partial $\eta^2 = 0.016$) and rated the pleasantness of air quality (F (1,476) = 7.55, $p = .006$**, partial $\eta^2 = 0.016$) and air movement (F (1,474) = 5.11, $p = .024$*, partial $\eta^2 = 0.011$) worse than people in the low sensitivity group. Exposure levels showed no effects. No systematic differences between men and women were found. Increased environmental sensitivity shared common variance with negative affectivity, another person-related variable. Its explanatory power was higher for evaluations of the environment whereas no differences between the concepts in explaining current psychological well-being were found.

Conclusions: Even a slightly elevated level of environmental sensitivity led to worse ratings of the environment with no clear relation to the real environment. Consequently, environmental sensitivity should be considered as a confounding factor in environmental exposure studies. The independency from real exposure levels is in line with the results from previous studies showing that the differences in environmental ratings are probably also driven by psychological factors.

1. Introduction

Individuals spend a large proportion of their time indoors (residence, factory, etc.) with some studies reporting up to 87% on average (Klepeis et al., 2001). The shift to an indoor lifestyle and the accompanying decreased outdoor exposure during the last decades has increased the prevalence of many allergies (Platts-Mills, 2015). For example, children growing up on farms developed fewer allergies due to exposure to germs (von Mutius and Vercelli, 2010). Moreover, people are more exposed to indoor air pollutants such as VOC (Lundberg, 1996) or (ultra)fine

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particulate matter (e.g., from traffic that can be found both outdoors and indoors, Christian et al., 2022). Poor indoor air quality can negatively affect physical and mental health and thus lead to poor quality of life, what is manifested in terms like idiopathic environmental intolerance (IEI), and multiple chemical sensitivity (MCS; Nordin, 2020; Viljoen and Thomas Neé Negrao, 2021), or just a slight, clinically not relevant increase of environmental sensitivity.

The evaluation of indoor air quality depends on different factors, for example sociodemographic and psychosocial (work) factors. Brauer and Mikkelsen (2010) found that psychosocial work factors were related to the perception of the indoor environment at individual level, but building characteristics were not associated with complaints about indoor environment. Workplace-level psychosocial risk factors could not explain this response heterogeneity whereas type of organization (e.g., office, hospital, school) explained some of the differences in perception. Cheung et al. (2022) showed for the work and private context that people with higher job and life satisfaction rated the indoor environmental quality as more satisfying than people with less job and life satisfaction. Furthermore, in the same study, Cheung et al. (2022) investigated the relationship between the Big Five personality traits (extraversion, agreeableness, conscientiousness, emotional stability, and openness to experience) and indoor environment. Agreeableness was associated with satisfaction with overall workspace environment, but otherwise the Big Five were not associated with evaluations of temperature, humidity, air movement, freshness of the air (stuffiness), or odors. Another study in the work context (Budie et al., 2019) included the Big Five in a path model to examine employee satisfaction with the work environment. Among these traits only agreeableness was positively associated with satisfaction with indoor climate. Individual factors like demographic variables also seem to influence the perception of indoor air environment: Women were found to be less satisfied with the thermal environment at times when ventilating and air conditioning is necessary (Choi et al., 2010); and Karjalainen (2007) found that women were less satisfied with indoor temperature, prefer warmer rooms and rated rooms as more uncomfortably hot or cold than men. In addition, subjects over 40 years rated thermal environment at times when ventilating and air conditioning is necessary as more satisfying than subjects under 40 years (Choi et al., 2010). In sum, individual factors seem to influence the perception of environment. However, up to now environmental sensitivity has not been researched as intensely as MCS or IEI. To approach the phenomenon of increased environmental sensitivity, we therefore first present findings on these clinically discussed phenomena.

MCS belongs to the broader definition of IEI, which additionally includes other environmental sensitivities such as electro (magnetic) sensitivity (Rossi and Pitidis, 2018). Present etiological explanations are not unanimous. On the one hand, there are biological theories, including toxicological or immunological theories that suggest an initially higher or longer exposure that is normally tolerated, as a cause (Bauer et al., 2008; Genius, 2010; Terr, 1987). According to consensus criteria (Bartha et al., 1999), symptoms are subsequently triggered and manifested by low doses and the appearance of symptoms depends on the presence of chemicals. Thus, MCS is defined as a chronic condition with reproducible recurring symptoms in response to different chemical substances in low doses (Bartha et al., 1999; Rossi and Pitidis, 2018). On the other hand, there are psychological theories, which assume a misattribution of symptoms to environmental stimuli leading to associative learning/-conditioning (Siegel and Kreutzer, 1997). These theories "tend [s] to report the source of such disturbances to the psyche, as an endogenous self-induced cause and not as a consequence of excessive and abnormal reaction to an albeit reduced chemical exposure" (Rossi and Pitidis, 2018, p. 139). Other theories are based on a model in which both approaches are relevant. The development of MCS is seen as a multistage and very individual process with different factors: Exposure and vulnerability lead to a sensitive individual (Bauer et al., 2008). A learning approach is used by Van den Bergh et al. (2017) who proposed a biopsychosocial mechanism to explain IEI/MCS. The nocebo effect occurs when subjects associate an odor with symptoms by learning effect. When they perceive the harmless odor again, they expect symptoms independent und dissociated of the current exposure level. According to Van den Bergh et al. (2017) this nocebo/learning effect is stronger if subjects present a high negative affectivity (trait), that is, negative affectivity might act as a moderator. The authors reported several reasons for this relationship, for example, subjects with high negative affectivity are more attentive to the affective elements of a somatic experience and perceive sensory-perceptual elements as less intensive.

After developing environmental sensitivity, individuals exhibit symptoms for many years as shown in some long-term studies. One-, 5-, and 9-year follow up studies about IEI, MCS, and chemical intolerance confirmed their chronic condition, with some individuals reporting improvement in symptoms such as headache, pain, and fatigue, e.g., due to hospitalization, books, support groups (e.g.,Bailer et al., 2007; Black et al., 2000; Skovbjerg et al., 2015). In addition, it was found that negative affect such as anxiety or neuroticism increases the development and persistence of IEI (Bailer et al., 2004; Skovbjerg et al., 2015).

In general, there is significantly more data on people with independently observed/diagnosed MCS according to various criteria (e.g., from Cullen (1987) or Bartha et al. (1999)) than on people who self-report it in questionnaires. Prevalence of these "diagnosed" MCS cases increased from approximately 2.5%–12.8% during the last 10 years until 2018, whereas self-reported MCS increased from approximately 11.1%–25.9% (Driesen et al., 2020; Steinemann, 2018). It can be assumed that even more people show increased sensitivity to environmental stimuli below any clinical thresholds and that these numbers will continue to rise. Also, Van den Bergh et al. (2017) assumed that MCS is "the tip of an iceberg of highly prevalent self-reported chemical hypersensitivity among the general population" (p. 552). Almost all studies examining gender differences reported women to be more likely to have self-reported or observed MCS than men (e.g., Andersson et al., 2008; Berg et al., 2010; Hausteiner et al., 2005).

Besides physical symptoms such as headaches and fatigue, many affected people reported an increase in irritability, anxiety, depressed mood, as well as negative affectivity (Azuma et al., 2019; Bailer et al., 2007; Hausteiner et al., 2005; Papo et al., 2006). A few studies have determined that individuals with MCS showed poorer mental well-being than healthy control groups (Georgellis et al., 2003; Johnson and Colman, 2017). Johnson and Colman (2017) found this result only in men, whereas there was no difference in positive mental well-being between women with and without MCS. Georgellis et al. (2003) found no differences in this relation between men and women. However, these studies investigated only well-being in general and not current well-being in an actual exposure situation. Overall, studies about the relationship between environmental sensitivity and current well-being during exposure are lacking. Changes in current well-being during exposure could help to separate general psychological reactions from reactions to the actual environment.

Another factor that could be influenced by environmental sensitivity is the perception of air quality, but studies show contradictory results. Alobid et al. (2014) showed that individuals with MCS were less able to identify odors compared to a control group. Moreover, affected people reported "more odours as being intense and irritating and less fresh and pleasant" (Alobid et al., 2014, p. 3203). In other studies, individuals with MCS expressed stronger odor perceptions (van Thriel et al., 2008) or had unpleasant sensations in response to more odors (Ojima et al., 2002). In contrast, other studies found that affected individuals were just as capable of identifying odors and perceiving exposures (Georgellis et al., 2003; Ojima et al., 2002) as well as perceiving the intensity of odors as the control group (Andersson et al., 2014; Georgellis et al., 2003). In one study, no difference regarding reported unpleasantness of smell was found (Georgellis et al., 2003).

To sum up, few studies investigated the relationship between environmental sensitivity and perception of air quality and odor intensity

under exposure. However, the results are contradictory. Furthermore, no studies explored the relationship between environmental sensitivity and current mental well-being under exposure. Studies that determined effects of environmental sensitivity in the normal population, that is, beyond clinical phenomena, in their everyday environment such as office buildings or aircraft are missing.

This study explores the effect of increased sensitivity to environmental stimuli on perception of air quality and well-being in different exposure conditions. For this purpose, people of a non-clinical population were exposed to different levels of CO_2 and VOC. The following hypotheses were tested:

H1) Regardless of the level of exposure, individuals with higher environmental sensitivity show worse current well-being than individuals with lower environmental sensitivity.

H2) Environmental sensitivity moderates the relationship between exposure and current well-being: individuals with higher environmental sensitivity show worse well-being at higher exposures than individuals with lower environmental sensitivity.

H3) Regardless of the level of exposure, individuals with higher environmental sensitivity perceive air quality worse than individuals with lower environmental sensitivity.

H4) Environmental sensitivity moderates the relationship between exposure and air quality perception: individuals with higher environmental sensitivity respond more strongly to higher exposures than individuals with lower environmental sensitivity.

Since the described clinical phenomena showed different prevalence in men and women, differential effects are considered. In addition, the increased negative affect found in some studies could also be explanatory. Therefore, it is also considered as a predictor and the findings are contrasted with the results of the analyses with increased environmental sensitivity.

2. Material and methods

2.1. Design and procedure

The presented analyses were conducted in the context of the European Union CleanSky 2 project ComAir ("Investigation of cabin ventilation strategies impact on aircraft cabin air quality and passengers' comfort and well-being through subject study in realistic aircraft environment") and the project CognitAir ("CO_2 and VOCs requirements for aircraft cabins based on cognitive performance, comfort responses and physiological changes depending on pressure level") which took place between November 2019 and January 2020 before the Sars-CoV-2 pandemic in the Flight Test Facility (FTF) at the Fraunhofer-Institute for Building Physics IBP in Holzkirchen, Germany. Both studies were single-blinded, randomized (stratified for age and sex of (business) flight passengers) controlled trials that achieved different cabin air qualities (see below) by different technical means (ventilation rates and CO_2/VOC dosing). Therefore, this is a reanalysis of two different studies. For the analyses presented in this paper, subjects from similar (in terms of air quality and study setting) exposure conditions of both studies were divided into three exposure groups (low, medium, and high exposure, see below). In the pooled data set, participants were also divided into two groups regarding low and higher environmental sensitivity, resulting in a 2×3 design. Each of the two projects showed sufficient power and sample size. Moreover, for the pooled data from both projects a sample size estimation for the 2×3 design was carried out. To detect small to medium sized effects ($f = 0.15$, $\eta^2 = 0.022$), a sample size of 489 subjects was required (based on ANCOVA, alpha-error of 5%, power of .85 ($1 - \beta$), 7 covariates).

Subjects in both studies were healthy adults recruited by a casting agency according to a set age/gender scheme to ensure that participants were representative of (business) air travelers. They received information about the project, procedure, and contact data for further inquiries in advance. People with pre-existing conditions (such as chronic respiratory, heart conditions, or severe anemia), potentially at risk, or who might cause problems during experiments (e.g., because of claustrophobia) were excluded. In addition, pregnant women, and potential outliers in health- and experiment-related measures, such as people who cannot sit for a while, were excluded. People with increased environmental sensitivity were not excluded, but also not specifically invited. We assumed that people with very high environmental sensitivity do not want to participate in an air quality study. Furthermore, the objective was to examine a normal population. A few days before the experiment, people reporting ongoing infectious conditions (e.g., seasonal cold) were excluded.

After welcoming the subjects at the Flight Test Facility, participants had the chance to ask questions about the study and exposure during a Q&A session before giving their signed informed consent. In addition, a final medical check was carried out by the study physicians to ensure that the participants could safely take part in the trials. After that, the participants were placed in the cabin in such a way that there was always an unoccupied seat or aisle between them to avoid disturbances. After boarding was completed, participants answered control questions as well as questions about environmental sensitivity during ascend. It took approximately 25 min to reach cruising altitude in terms of pressure condition and additional 30 min in CognitAir and – due to the slower generation of the different air qualities – 40 min in ComAir to reach fully controlled exposure. Subjects spent on average 4 h in total in the mock-up (about 4:00 in CognitAir and 4:04 in ComAir) and completed a number of questionnaires during this time. Finally, participants deboarded and were debriefed; more details are described elsewhere (Norrefeldt et al., 2021). Both studies were approved by the Ethics Committee at the Faculty of Medicine, Ludwig-Maximilians-University, Munich (ComAir #19–256; CognitAir #19–350).

2.2. Exposure

The exposure took place in the FTF consisting of the front part of a former in-service wide-body airplane inserted in a low-pressure chamber, which can accommodate up to 80 passengers and generate different pressure levels. In the present study, the cabin was half occupied by 30–40 participants and pressure levels of 755 hPa equivalent to 8000 ft cabin altitude were used. In the ComAir project, different air qualities were generated by different outdoor/recirculation airflow ratios. The higher the proportion of recirculation air rate, the higher the levels of CO_2 and VOC as participants' emissions became less diluted by outdoor air; more details are described elsewhere (Norrefeldt et al., 2021). The levels of air quality parameters follow mandatory requirements from the Federal Aviation Administration (FAA, 2019). In contrast, in the CognitAir project different air qualities were generated by dosing VOC and pure CO_2 into the cabin. To achieve different total volatile organic compound (TVOC) levels, a fixed mixture of 12 compounds such as Ethanol, Toluene, and Acetonitrile based on VOCs commonly found in aircrafts (Chen et al., 2021) were dosed in different amounts. For the present study, these different air qualities were allocated to low, medium, and high exposure based on TVOC and CO_2 (see Table 1) following this procedure: Firstly, all relevant experimental sessions were sorted according to measured TVOC level and subsequently by CO_2 level. Since allocation was based on TVOC, standard deviations for CO_2 are rather large. Secondly, differences between the ranked successive sessions were considered along with number of participants to achieve an equal distribution of subjects among the low, medium, and high exposure groups. Thus, the adjacent sessions for low and medium exposure differ only by 94 $\mu g/m^3$ TVOC, the ones for medium and high by 226 $\mu g/m^3$ TVOC (see Appendix B). The resulting three exposure levels show clear average differences in TVOC and CO_2. These clear differences are also shown by the three VOCs with the highest proportions across all exposure groups included as examples in Table 1.

All other factors, such as pressure, temperature, lighting, or non-

Table 1
Characteristics of exposure levels.

	Low exposure	Medium exposure	High exposure
TVOC (µg/m^3), among others	598 (267)	1041 (149)	1616 (289)
Ethanol (µg/m^3)	100 (92)	169 (99)	557 (410)
Total propanol[a] (µg/m^3)	106 (80)	134 (35)	186 (147)
Acetone (µg/m^3)	30 (16)	45 (17)	67 (10)
CO_2 (ppm)	1958 (473)	2324 (660)	3363 (1206)
Pressure (hPa)	755 (0)	755 (0)	755 (0)
Temperature (°C)	22.7 (0.2)	23.0 (0.8)	22.6 (1.0)
Relative humidity (%)	14 (3)	15 (6)	14 (7)

Mean, standard deviation in brackets.
[a] Sum of 1-popanol and 2-propanol.

human noise, were kept as constant as possible. For more details about the measurement devices see appendix C.

2.3. Measures

Sensitivity to environmental stimuli was measured by the chemical odor sensitivity scale by Kiesswetter et al. (1997). The questionnaire consists of eight items (e.g., *Strong smell of paint and smoke makes me dizzy*) on a five-point Likert scale (0 = *not applicable*, 4 = *highly applicable*) to measure how people subjectively react to environmental stimuli. The sum scale shows a good overall internal consistency (Cronbach's Alpha = .904). To examine the effects of low and higher sensitivity to environmental stimuli, sum values on the chemical odor sensitivity scale had to be separated into two groups. Due to the generally rather low level of environmental sensitivity in the sample, the normally used cut-off could not be used, because otherwise most of the subjects would be assigned to the low environmental sensitivity group. To obtain a better cut-off point and a good ratio of numbers of subjects in both groups, tertiles of the sum value were calculated and the cut-off value was set after the first two tertiles. The lower two tertiles (range 0–7) are considered to present low environmental sensitivity, whereas the upper tertile (range 8–32) presents higher environmental sensitivity. Despite the overall very low values on the sum scale, we will use the terms low and higher environmental sensitivity due to better readability in the remainder of this paper.

Current well-being was assessed at the start (t1) and end (t2, approximately 130 min after start) of the controlled air regime (dosing/recirculation rate) on the Basler emotional state scale (Hobi, 1985). Using a bipolar seven-point rating scale, 16 adjectives capture the four subscales vitality (e.g., *tired - fresh*), intrapsychic equilibrium (e.g., *calm - nervous*), social extraversion (e.g., *talkative - secretive*), and vigilance (e.g., *inattentive - attentive*). All subscales showed an acceptable to good internal consistency (Cronbach's Alpha = .707-.890).

Perception of air quality was assessed by different scales. First, pleasantness of air quality, temperature, and air movement was measured in the middle (t1, approximately 70 min after start) and at the end (t2, approximately 130 min after start) of the controlled air regime (dosing/recirculation rate) on a five-point Likert scale (1 = *very unpleasant*, 5 = *very pleasant*). Pleasantness of air quality consists of the four items overall air quality, fresh air, humidity, and odors, whereas temperature and air movement consist of one item each. These items were adopted from the Ideal Cabin Environment (ICE) Questionnaire (Perera, 2010) and the Cost-effective Open-Plan Environments (COPE) project (Veitch et al., 2007). Second, participants rated the acceptability of air quality based on a two-part visual analogue scale from *clearly acceptable* to *just acceptable* and – after a small break of the scale – *just not acceptable* to *clearly not acceptable* right at t2 (Wargocki, 2001). To calculate this visual analogue scale, percentage of maximum possible score (POMP) was used (Cohen et al., 1999). Third, odor intensity was assessed by one item (*How would you assess the odor intensity in this flight?*) on a 5-point scale (1 = *no odor*, 5 = *overwhelming odor*) right after exposure, too.

As ratings of current well-being and air quality can be influenced by a variety of other factors, the following control variables were considered. Health on day of trial was measured by one item (*Taking everything into consideration, how would you describe your health today?*) on a five-point Likert scale (1 = *poor*, 5 = *excellent*) based on McDowell (2010), whereas the physical and mental health status in the past four weeks was assessed by the Short-Form-8-Health Survey (SF-8) by 8 items (e.g., *During the past 4 weeks, how much did physical health problems limit your physical activities (such as walking or climbing stairs)?*) on different four-, five- or six-point scales with higher values showing worse health (Ware et al., 2001; German version Ellert et al., 2005). This scale shows a good internal consistency (Cronbach's Alpha = .815). Moreover, subjects were asked if they currently smoke on a four-point scale (1 = *I've never smoked before*, 4 = *yes, daily*; Gößwald et al., 2012) and how they would rate the quality of their sleep the night before the trial on a four-point Likert scale (1 = very *poor*, 4 = *very good*; Robert-Koch-Institut, 2008). Negative affectivity was measured by the shortened negative subscale of the Positive and Negative Affect Schedule (PANAS) by 5 items (e.g., *afraid*) on a five-point Likert scale (1 = *not at all*, 5 = *extremely*; Watson et al., 1988). This scale shows an acceptable internal consistency (Cronbach's Alpha = .754). Like environmental sensitivity, negative affectivity was separated into tertiles for the sensitivity analysis. The lower two tertiles (range 1–1.4) are considered to present low negative affectivity, whereas the upper tertile (range 1.6–5) presents higher negative affectivity.

2.4. Statistical analyses

To test for comparability of exposure and environmental sensitivity groups, non-parametric tests for independent samples (Mann-Whitney U tests) were used for two groups. To compare three groups, Kruskal-Wallis tests were used. Due to nominal scaling, differences in sex and smoking were calculated by using the Chi^2 test. As method of choice for experimental designs, hypotheses were tested using two-way ANCOVA with repeated measures and Bonferroni adjustment for post hoc comparisons. Furthermore, main and interaction effects were considered to explore how participants with low versus higher environmental sensitivity react to different exposure levels. Covariates were centered to correctly represent the within-subjects effects (Schneider et al., 2015). Crude models were additionally computed.

In addition, sensitivity analyses for sex and negative affectivity were performed. To explore the differences between men and women with increased sensitivity, all analyses were performed separately for both sexes. To test if the results were indeed predicted by environmental sensitivity and not by a more general person-related variable, the controlled models were also computed with negative affectivity.

P-values below .05 were considered significant and *p*-values below .10 as a trend. In addition, effect sizes were reported as partial η^2 for ANOVAs. In line with Ellis (2010), based on Cohen (1992), we considered effect sizes of $\eta^2 \geq 0.01$ as small effects, of $\eta^2 \geq 0.06$ as medium-sized effects, and of $\eta^2 \geq 0.14$ as big effects. Effect sizes reported as *r* above 0.10 were considered as small, above 0.30 as medium and above 0.50 as large (Cohen, 1992). All statistical analyses were performed using SPSS 26.

3. Results

3.1. Sample characteristics

The sample consisted of 503 participants (221 females, 282 males) with a mean age of 42.8 years (*SD* = 14.5, range 18–77 years) and rather high educational level (45.9% with qualification for university entrance/A level, 0.6% left school without graduation). During on-site screening, five participants were screened out before exposure started

because of pre-existing conditions, a surgery a few months ago and a too late appearance. Two female participants left the chamber during exposure (one medium/one high exposure condition) because of nausea and headache; transient symptoms were later confirmed by the onsite physician as almost surely not being related to the air-related exposure. Three subjects had to be excluded due to missing values regarding the chemical odor sensitivity scale. Therefore, 500 subjects were used in all further calculations. 334 individuals were allocated to the group with low environmental sensitivity and 166 individuals to the group with higher environmental sensitivity. These two groups were evenly distributed across the three exposure levels (χ^2 (2) = 3.03, p = .220) and randomization to exposure group was mostly successful (see Table 2). The participants did not differ with regard to age, BMI, or smoking behavior. However, combinations of exposure and environmental sensitivity group showed some differences. In general, more women reported higher environmental sensitivity. This effect was driven by significant differences in low and medium exposures levels and a – albeit not significant - different proportion in the high exposure group. In low and medium exposure levels, generally more women were in the higher than in the low environmental sensitivity group resulting in significant differences. If the proportion of men and women in the two sensitivity groups was similar, no significant differences between the sexes could be found as shown in the high exposure group. People with higher sensitivity to environmental stimuli showed poorer self-assessed health on day of trial, as well as poorer physical and mental health during the past four weeks. Furthermore, participants with higher sensitivity showed higher negative affectivity than participants with low sensitivity. This effect was mainly driven by a significant difference in the low exposure condition. With regard to differences between the exposure groups, a significant difference in sleep quality before trial was determined. Participants in the medium exposure group reported poorer sleep quality before trial than people in the low or high exposure group. All sample characteristics in the three different exposure levels as well as the two environmental sensitivity groups are presented in Table 2. In consideration of the differences, sex, negative affectivity, physical and mental health, health on day of trial, and sleep quality before trial were included as control variables in the model. As the air quality levels were produced differently in the two projects, this was also controlled for by using a binary variable. In addition, results were controlled regarding age. Moreover, crude models were calculated and can be found in the appendix (table A2 and A.3).

To ensure that environmental sensitivity is an independent construct, correlations with the relevant control variables were analyzed. Environmental sensitivity correlated positively with sex (r = 0.277***), negative affectivity (r = 0.215***), and lower physical and mental health (r = 0.246***). That is higher values in environmental sensitivity were associated with poorer physical and mental health. In addition, a negative correlation with health on day of trial (r = −0.142***) was detected. All correlations with environmental sensitivity showed low effect sizes (Cohen, 1992) with less than 8% shared variance (see appendix, table A1).

3.2. Well-being

For intrapsychic equilibrium, analyses of variance showed two interaction trends regarding environmental sensitivity and measurement time (F (2,466) = 2.66, p = .071$^+$, partial η^2 = 0.011) as well as environmental sensitivity and exposure level (F (2,466) = 2.47, p = .086$^+$, partial η^2 = 0.010; see Table 3 and Fig. 1). Intrapsychic equilibrium decreased less over time for subjects with low environmental sensitivity than for those with higher environmental sensitivity. People with low environmental sensitivity indicated a lower intrapsychic equilibrium in the medium exposure level whereas people with higher environmental sensitivity reported the highest intrapsychic equilibrium

Table 2
Characteristics of the study population.

	Low exposure			Medium exposure			High exposure			Overall difference between ES (p-value)	Overall difference between exposure (p-value)
	Low ES (M (SD))	Higher ES (M (SD))	Difference between ES (p-value)	Low ES (M (SD))	Higher ES (M (SD))	Difference between ES (p-value)	Low ES (M (SD))	Higher ES (M (SD))	Difference between ES (p-value)		
Sex			**.002**[**b]			**.002**[**b]			.306[b]	<**.001**[***b]	.899[b]
male	60	21		73	24		76	26			
female	31	33		42	37		52	25			
Age	43.99 (16.18)	41.91 (14.27)	.318[a]	40.35 (14.89)	44.23 (13.09)	.063[a]	43.11 (13.68)	43.88 (13.61)	.635[a]	.437[a]	.419[c]
BMI	24.44 (3.99)	23.95 (4.43)	.216[a]	24.88 (6.01)	24.61 (4.02)	.844[a]	24.94 (5.09)	24.89 (4.53)	.752[a]	.732[a]	.798[c]
Smoking			.848[b]			.200[b]			.097[b]	.384[b]	.292[b]
I've never smoked before	45	30		49	25		63	17			
no, not anymore	18	11		24	21		36	18			
yes, occasionally	20	9		25	9		8	8			
yes, daily	8	4		17	6		21	8			
Environmental sensitivity sum score	2.81 (2.37)	12.70 (5.16)	<**.001**[***a]	2.79 (2.19)	13.21 (4.98)	<**.001**[***a]	2.41 (2.22)	14.55 (5.33)	<**.001**[***a]	<**.001**[***a]	.170[c]
Negative affectivity	1.25 (0.37)	1.50 (0.45)	<**.001**[***a]	1.39 (0.41)	1.52 (0.56)	.334[a]	1.44 (0.51)	1.55 (0.59)	.322[a]	**.002**[**a]	.066[c]
Physical and mental health	14.12 (4.01)	16.59 (4.62)	**.002**[**a]	15.12 (4.46)	16.64 (4.49)	**.035**[*a]	14.98 (4.53)	17.20 (5.05)	**.008**[**a]	<**.001**[***a]	.465[c]
Health on day of trial	4.26 (0.85)	4.00 (0.58)	**.003**[**a]	4.09 (0.70)	4.02 (0.72)	.510[a]	4.13 (0.64)	3.84 (0.62)	**.004**[**a]	<**.001**[***a]	.099[c]
Sleep quality before trial	2.92 (0.73)	2.72 (0.60)	.051[a]	2.57 (0.76)	2.59 (0.80)	.695[a]	2.88 (0.66)	2.90 (0.70)	.810[a]	.452[a]	<**.001**[***c]

*p ≤ .05; **p ≤ .01; ***p ≤ .001; ES = environmental sensitivity, M = Mean, SD = Standard deviation.
[a] Mann-Whitney U test for group comparisons.
[b] Chi^2 test for group comparisons.
[c] Kruskal-Wallis test for group comparisons.

Table 3
Effect of increased sensitivity to environmental stimuli on current well-being.

	Time	Low exposure		Medium exposure		High exposure		MT		ES		Exposure		MT*ES		MT* Exposure		ES* Exposure	
		EMM	SE	EMM	SE	EMM	SE	F	p	F	P	F	p	F	p	F	p	F	p
Vitality (1–7)[a]																			
Low ES	t1	4.72	0.10	4.61	0.08	4.69	0.08	16.45	**<.001*****	16.42	**<.001*****	0.43	.653	0.04	.839	1.58	.206	0.56	.573
	t2	4.61	0.10	4.52	0.09	4.41	0.08												
High ES	t1	4.30	0.13	4.42	0.12	4.34	0.14												
	t2	4.02	0.13	4.27	0.12	4.06	0.14												
Intrapsychic equilibrium (1–7)[b]																			
Low ES	t1	6.00	0.09	5.95	0.08	6.14	0.07	*29.64*	**<.001*****	1.32	.252	0.43	.649	2.66	.071+	0.41	.523	2.47	.086+
	t2	5.80	0.09	5.74	0.08	5.88	0.08												
High ES	t1	5.92	0.12	5.95	0.10	5.90	0.12												
	t2	5.60	0.12	6.00	0.12	5.63	0.13												
Social extraversion (1–7)[c]																			
Low ES	t1	3.70	0.13	3.64	0.12	3.77	0.11	0.58	.445	1.40	.237	0.74	.476	0.22	.639	0.79	.455	1.54	.215
	t2	3.80	0.13	3.58	0.12	3.69	0.11												
High ES	t1	3.68	0.17	3.73	0.16	3.35	0.19												
	t2	3.66	0.17	3.62	0.16	3.31	0.19												
Vigilance (1–7)[d]																			
Low ES	t1	5.00	0.11	4.96	0.10	4.96	0.09	*45.87*	**<.001*****	7.82	**.005****	0.07	.937	0.42	.517	1.41	.244	0.06	.938
	t2	4.69	0.12	4.65	0.10	4.63	0.10												
High ES	t1	4.76	0.15	4.60	0.13	4.74	0.16												
	t2	4.38	0.16	4.45	0.14	4.23	0.17												

Two-way ANCOVA with repeated measures, models controlled for centered variables age, sex, study sample, negative affectivity, physical and mental health, health on day of trial, and sleep quality before trial; in brackets: min-max; ES = Environmental sensitivity, MT = Measurement time, EMM = Estimated marginal mean, SE = Standard error, F = F-value, italic if partial $\eta^2 \geq 0.06$; p = level of significance: +p ≤ .10; *p ≤ .05; **p ≤ .01; ***p ≤ .001; t1 = start of controlled air regime (dosing/recirculation rate), t2 = end of controlled air regime (dosing/recirculation rate).

[a] Higher values = higher vitality.
[b] Higher values = higher intrapsychic equilibrium.
[c] Higher values = higher social extraversion.
[d] Higher values = higher vigilance.

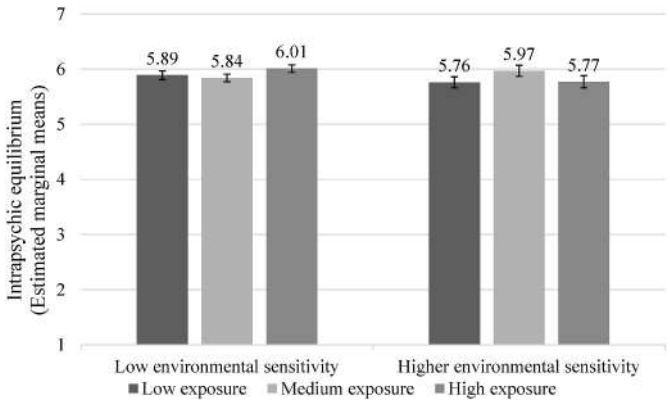

Fig. 1. Interaction effect of intrapsychic equilibrium.

in this condition. However, differences were very small.

For two subscales of current well-being a main effect of environmental sensitivity was found: People with higher sensitivity to environmental stimuli exhibited lower current well-being regarding vitality (F (1,466) = 16.42, $p < .001$***, partial $\eta^2 = 0.034$) and vigilance (F (1,467) = 7.82, $p = .005$**, partial $\eta^2 = 0.016$) during exposure. No main effect of exposure could be found. Moreover, no significant differences in post hoc comparisons were identified. Therefore, the detected effects are not attributable to exposure. Assessment of vitality intrapsychic equilibrium, and vigilance decreased significantly over time in all groups irrespective of exposure or environmental sensitivity. Compared to the controlled model, main effects of environmental sensitivity were additionally found in the crude model for intrapsychic equilibrium and social extraversion (see appendix, table A2).

3.3. Perception of air quality

As shown in Table 4, no interaction effects or main effects of exposure were found regarding perception of air quality. Individuals with higher environmental sensitivity rated the pleasantness of air quality (F (1,476) = 7.55, $p = .006$**, partial $\eta^2 = 0.016$) and air movement (F (1,474) = 5.11, $p = .024$*, partial $\eta^2 = 0.011$) worse than individuals with low environmental sensitivity. They were also prone to perceive odors more intensively (F (1,485) = 2.76, $p = .097^{+}$, partial $\eta^2 = 0.006$). Pleasantness of air quality and air movement decreased significantly over time in all groups irrespective of exposure or environmental sensitivity. Besides these main effects of environmental sensitivity found in the controlled model, the crude models showed main effect trends of environmental sensitivity for pleasantness of temperature and acceptability and one trend for an exposure main effect – subjects with higher environmental sensitivity tended to rate the temperature as less pleasant and acceptable; moreover, subjects in the high exposure condition tended to rate the temperature as less pleasant as subjects in the low exposure condition (see appendix, table A3).

3.4. Sensitivity analysis

3.4.1. Sex

Since the sex distribution differs in low and higher environmental sensitivity groups and previous studies suggest differences between men and women, a sensitivity analysis was performed. This is to test whether males and females with higher environmental sensitivity react differently to the exposures. For current well-being, differences between men and women were only found for intrapsychic equilibrium while the three other subscales showed comparable patterns for both sexes. Men with low environmental sensitivity showed higher intrapsychic equilibrium than men with higher environmental sensitivity (F (1, 267) = 7.07, $p = .008$**, partial $\eta^2 = 0.001$; see appendix, table A4). In contrast, for women an interaction effect of repeated measure and exposure for intrapsychic equilibrium was found (F (2,206) = 4.87, $p = .009$**, partial $\eta^2 = 0.045$; see appendix, table A.5). During first measurement, women in the medium exposure group indicated the lowest intrapsychic equilibrium whereas women during second measurement in the medium exposure group indicated the highest intrapsychic equilibrium.

Perceptions of air quality showed a number of differences between men and women. Men with low environmental sensitivity rated the air quality as more pleasant than men with higher environmental sensitivity (F (1, 271) = 7.02, $p = .009$**, partial $\eta^2 = 0.025$; see appendix, table A.6), while no differences were seen for women. Moreover, for men, but not for women, an interaction effect of repeated measurement and environmental sensitivity was found for pleasantness of air quality (F (1, 271) = 4.42, $p = .036$*, partial $\eta^2 = 0.016$). Men with low environmental sensitivity perceived increasing pleasantness of air quality whereas men with higher environmental sensitivity showed the opposite pattern. Overall, men with low environmental sensitivity perceived the air quality as better than the men with high environmental sensitivity. In contrast, for women but not for men main effects of environmental sensitivity were found for pleasantness of temperature (F (1,210) = 7.66, $p = .006$**, partial $\eta^2 = 0.035$) and air movement (F (1, 211) = 6.81, $p = .010$**, partial $\eta^2 = 0.031$) which were rated better by women in the low environmental sensitivity group than by women in the higher environmental sensitivity group (see appendix, table A.7). In addition, main effects of exposure for pleasantness of temperature (F (2,210) = 3.30, $p = .039$*, partial $\eta^2 = 0.030$) and air movement (F (2,210) = 3.40, $p = .035$*, partial $\eta^2 = 0.031$) driven by the medium exposure group were found for women but not for men. Overall, no systematic differences between men and women could be found.

3.4.2. Negative affectivity

As literature showed a strong association between negative affectivity and environmental sensitivity, negative affectivity instead of environmental sensitivity was used as main factor. In contrast to environmental sensitivity, negative affectivity indicated a main effect for intrapsychic equilibrium: Subjects with low negative affectivity reported a higher intrapsychic equilibrium (F (1,466) = 4.27, $p = .043$*, partial $\eta^2 = 0.009$). They also tended to report higher social extraversion and higher vitality (see appendix, table A.8). In contrast to environmental sensitivity, no main effects of negative affectivity could be found for vigilance. For none of the variables regarding perception of air quality main effects of negative affectivity or interactions were found (see appendix, table A.9).

4. Discussion

This study explored the effects of low-level environmental sensitivity during acute exposure to different air quality levels. Some differences between individuals with low and higher sensitivity to environmental stimuli regarding current well-being and perception of air quality could be observed. Regardless of the level of exposure, individuals with higher environmental sensitivity showed less vitality and vigilance than individuals with lower environmental sensitivity. In addition, and also regardless of the level of exposure, individuals with higher environmental sensitivity perceived the temperature and air movement as less pleasant than individuals with lower environmental sensitivity. Thus, these results support hypothesis 1 and 3, although the differences are rather small. A moderating role of environmental sensitivity for the effects of exposure on current well-being or perception of air quality could not be demonstrated: Individuals with higher environmental sensitivity did not show a stronger reaction to higher exposure levels compared to individuals with lower environmental sensitivity. Hypothesis 2 and 4 must therefore be rejected. In addition and contrary to what is reported in literature, sensitivity analyses showed no systematic differences between men and women but only few scattered effects. For example, men, but not women, with low environmental sensitivity rated the air quality

Table 4
Effect of increased sensitivity to environmental stimuli on perception of air quality.

	Time	Low exposure		Medium exposure		High exposure		MT		ES		Exposure		MT*ES	MT* Exposure		ES* Exposure	
		EMM	SE	EMM	SE	EMM	SE	F	p	F	P	F	p	F	p		F	p
Pleasantness of air quality (1–5)[a]																		
Low ES	t1	3.57	0.08	3.61	0.07	3.66	0.06	7.10	**.008****	7.55	**.006****	0.33	.719	0.09	.770		1.08	.342	0.73	.480
	t2	3.41	0.08	3.56	0.07	3.64	0.07													
High ES	t1	3.40	0.10	3.41	0.90	3.45	0.10													
	t2	3.39	0.10	3.42	0.10	3.27	0.11													
Pleasantness of temperature (1–5)[a]																		
Low ES	t1	3.32	0.12	3.32	0.10	3.39	0.10	1.71	.192	2.00	.159	0.34	.711	0.42	.520		0.17	.842	0.35	.708
	t2	3.38	0.12	3.31	0.11	3.26	0.10													
High ES	t1	3.29	0.15	3.29	0.14	3.09	0.16													
	t2	3.17	0.16	3.22	0.14	3.05	0.17													
Pleasantness of air movement (1–5)[a]																		
Low ES	t1	3.59	0.10	3.63	0.08	3.69	0.08	6.70	**.008****	5.11	**.024***	0.15	.859	0.38	.538		0.33	.721	0.57	.566
	t2	3.49	0.10	3.61	0.09	3.57	0.08													
High ES	t1	3.49	0.12	3.52	0.11	3.43	0.13													
	t2	3.43	0.13	3.37	0.12	3.24	0.14													
Acceptability (0–100)[b]																		
Low ES		80.72	1.69	82.63	1.52	83.71	1.44			1.10	.297	0.15	.862						2.15	.118
High ES		83.08	2.19	80.91	2.07	78.09	2.36													
Odor intensity (1–5)[c]																		
Low ES		1.63	0.09	1.63	0.08	1.56	0.07			2.76	.097^{+}	0.50	.610						0.39	.679
High ES		1.67	0.11	1.82	0.10	1.72	0.12													

Two-way ANCOVA (with repeated measures), models controlled for centered variables age, sex, study sample, negative affectivity, physical and mental health, health on day of trial, and sleep quality before trial; in brackets: min-max; ES = Environmental sensitivity, MT = Measurement time, EMM = Estimated marginal mean, SE = Standard error, F = F-value, italic if partial $\eta^2 \geq 0.06$; p = level of significance: $+p \leq .10$; $*p \leq .05$; $**p \leq .01$; $***p \leq .001$; t1 = middle of controlled air regime (dosing/recirculation rate), t2 = end of controlled air regime (dosing/recirculation rate).

[a] Higher values = higher pleasantness of air quality, temperature, and air movement, respectively.

[b] Higher values = higher acceptability of air quality.

[c] Higher values = overwhelming odor.

as more pleasant. Moreover, they perceived increasing pleasantness of air quality whereas men with higher environmental sensitivity showed the opposite pattern. This suggests that the response of men with higher environmental sensitivity exacerbates during exposure, whereas it improves for individuals with low environmental sensitivity. Sensitivity analyses regarding person-related variables (in comparison with environmental sensitivity) indicated that for current well-being environmental sensitivity showed stronger effects, but negative affectivity showed more main effects at 10% error level; thus, showing no clear superiority of one concept over the other. This result is similar to results from Cheung et al. (2022) about the Big Five in terms of the very small effects of person-related variables. Overall, for prediction of environmental perception environmental sensitivity is more suitable than other person-related variables. Results suggest that even a small increase in environmental sensitivity leads to effects. It can be assumed that environmental sensitivity is a relevant factor regarding the evaluation of the environment, but not regarding the evaluation of the current well-being. Depending on the study aim, it seems recommendable to use one of the two variables. Environmental sensitivity could be included in research as a control variable when the environment is assessed or when assessments are used in an exposure study. Otherwise, the results might be biased. Even though environmental sensitivity showed only small effects, it can have an impact as presented in the present trial.

Although we investigated a just slightly elevated level of environmental sensitivity in this study, the results are in line with MCS related results from previous studies. We did not find differences in the evaluation of odor intensity which is in line with Andersson et al. (2014). In addition, we could not find any differences regarding pleasantness of temperature, or acceptability between individuals with low and higher environmental sensitivity. However, our results suggest that people with higher environmental sensitivity perceive air quality and air movement as less pleasant. Similarly, Alobid et al. (2014) and Ojima et al. (2002) found differences regarding the pleasantness of odor between subjects with MCS and the control groups. This differential results regarding the evaluation of aspects of air quality might in part be due to different assessment methods but also show that people are indeed capable to differentiate between the (intended) underlying constructs of pleasantness, acceptability and satisfaction. To sum up, we cannot confirm that people react differently at different exposure levels or that reactions increase at higher exposures. It seems that no clear reference to real environment exists, but that it is more likely to be a psychological phenomenon. This supports psychological theories about the etiology of increased environmental sensitivity. Different from assumptions from toxicological theories (e.g., Genius, 2010; Terr, 1987), the reactions are less likely to be triggered by exposure, but rather an endogenous self-induced effect (Rossi and Pitidis, 2018) or a learning effect (Van den Bergh et al., 2017). These results are also supported by Bornschein et al. (2008): Twenty individuals with MCS and 17 controls were exposed to a solvent mixture or clean air in six random-order sessions (double-blind). Individuals with MCS did not differ regarding sensitivity, specificity, and efficiency from controls, that is, they were no more able to differentiate between real and placebo conditions than controls. Thus, no direct relation to the real environmental conditions could be found either.

Most studies found that more women are affected by MCS (e.g., Andersson et al., 2008; Berg et al., 2010; Hausteiner et al., 2005) and we also see this in our study population, even for only slightly elevated levels of environmental sensitivity. Furthermore, it could be demonstrated that individuals with increased environmental sensitivity report a poorer general well-being (Georgellis et al., 2003; Johnson and Colman, 2017) as well as a poorer current well-being. Compared to Johnson and Colman (2017), no systematic sex differences in well-being between individuals with low and higher environmental sensitivity were detected.

As a subscale of current well-being, vigilance showed lower values for individuals with higher environmental sensitivity. Other studies also found that the attention of people with MCS or IEI is impaired (e.g., Bornschein et al., 2007; Ziem and McTamney, 1997). Bornschein et al. (2007) assumed that people with IEI are only selectively attentive because they are mainly occupied with their physical sensations. Witthöft et al. (2006) also found this selective attention for people with IEI in their study. In an emotional stroop test, interference indices and recognition performance for IEI-trigger-related words were lower than for symptom-related words (Witthöft et al., 2006).

Since the air quality levels were produced differently in the two projects from which the data was pooled, a bias cannot be ruled out. A general difference (independent of exposure level) between the studies was found for pleasantness of temperature and air movement; the study that let the VOC and CO_2 naturally develop by reducing fresh air supply showed better ratings than the study where both were dosed into the supply air. To control for this, we added the projects as control variable in all analyses. Another difficulty could be temperature and humidity in the middle exposure. Although in general rather comparable, in the middle exposure both were highest on average, and it cannot be ruled out that these also had an effect. Especially since the standard deviations of the different exposure levels must be noted and the relative humidity – as an uncontrolled environmental factor – was very low. However, Grün et al. (2012) determined that low humidity is not related to perceived symptoms. Nevertheless, no effects were found between low and high exposure level regarding air quality. In addition, exposure levels could bias the results due to the classification into three conditions. Especially the allocation of CO_2 needs improvements due to the focus on VOC. As already described in the introduction, odor is important regarding environmental sensitivity. Thus, we decided to focus on VOCs. However, the levels of TVOC might have been too low to cause effects and categorization of conditions was quite arbitrary so that group differences were rather small and "poor" air quality ratings were still good. Due to the relatively low TVOC levels, it is possible that some subjects did not detect the odors and therefore did not react. Many studies on MCS used detectable odors such as mandarin, perfume, lavender oil, acetone, butanol, or methanol (e.g., Hillert et al., 2007; Azuma et al., 2013). Compared to control groups, patients with MCS process odors differently (Hillert et al., 2007; Azuma et al., 2013). Among other things, Azuma et al. (2013) report that this difference is caused by changes in the regional cerebral blood flow (rCBF) in the prefrontal cortex. In their study, there was initially no increase in rCBF when non-odorant was presented, but over time rCBF increased during non-odorant condition. That is, patients with MCS were no longer able to distinguish between non-odorant and odor condition (Azuma et al., 2013) and responded independent of the exposure level. To examine in the present study whether and which odors the subjects perceived, they were asked by an open question to describe the odor if they perceived one. Common responses from 223 subjects were musty, metallic, neutral, fresh, or sweaty odor. However, some subjects smelled aftershave, perfume, floral odor, mandarin, tobacco smoke, disinfectant, or exhaust fumes which were also mentioned in the studies by Hillert et al. (2007) and Azuma et al. (2013). Therefore, it can be assumed that there were detectable odors despite the low exposure level. Another limitation relates to the dichotomization of the chemical odor sensitivity scale to focus on group differences between low and higher environmental sensitivity, as it resulted in a loss of information. Levels of environmental sensitivity were also very low in the sample. However, it can be assumed that people with a very high environmental sensitivity do not participate in air quality assessment studies as they have been informed about the topic in advance. Nevertheless, we did not specifically exclude these people. In general, effect sizes demonstrated rather small, but significant effects. Based on our sample size estimation, power was sufficient, and we are fairly sure that the effects are reliable. However, further research is necessary. Furthermore, an expectation bias could distort the results because the subjects knew that different levels of air conditions existed but not in which condition they participated. A focus of the participants on this information could influence the actual valuation.

5. Conclusion

This study explored the effect of slightly higher levels of sensitivity to environmental stimuli on perception of air quality and well-being in randomized and controlled exposure trials during different exposure conditions. Although self-reported environmental sensitivity was only slightly elevated, participants showed poorer well-being and worse perception of quality during exposure but independent of exposure level. Differential effects were found for women and men, but no systematic pattern emerged. Accordingly, general expectations and previous findings on environmental sensitivity between the sexes could not be confirmed. It is expected that environmental sensitivity will also influence other variables and that the number of affected people will continue to increase. For example, Steinemann (2018) mentioned an increase of self-reported and diagnosed MCS over the last years even though no explanations are given, whereas Platts-Mills (2015) reports a shift to indoor lifestyle as one reason for the aspect of increasing prevalence of many allergies over the last decades. Therefore, environmental sensitivity beyond clinical phenomena should be further investigated. In addition, there should be further studies that confirm the psychological etiology and investigate the associations with other person-related variables.

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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.2022.114045.

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Occurrence of *Naegleria fowleri* and their implication for health - a look under the One Health approaches

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ABSTRACT

One Health approaches are becoming increasingly necessary in the world we live in. Human beings, animals, plants and the environment are intrinsically interconnected and when some intervention occurs, mainly through the action of man himself, everyone suffers the consequences. The objective of this review was to collect data about the occurrence and dispersion of *Naegleria fowleri,* an amphizoic free-living amoeba, and its implications for health approaches through the One Health concept. *N. fowleri* is an opportunistic amoeba, better known as brain-eating amoeba, which causes Primary Amoebic Meningoencephalitis. This amoeba is widely distributed around the world, being isolated from different matrices of natural or anthropogenic environments with temperatures above 30 °C with an upper limit of 45–46 °C. Highly lethal, it has claimed numerous humans patients and only five people have survived the disease so far. Our results indicate that climate change plays a major role in the growth and dispersion of the pathogen in the environment, causing damage to humans and animals. Changes in temperature, antimicrobial resistance, possible transport of other microorganisms by the amoeba, conventional treatments with chlorination, among others, were addressed in our study and should be considered in order to raise questions and possible solutions to this problem that involves health as a whole. The diagnostic methods, prospection of new anti-*Naegleria* drugs and the control of this parasite in the environment are specific and urgent issues. We know that the human-animal-plants-environment spheres are inseparable, so it is necessary to turn a directed look at the One Health approaches related to *N. fowleri*.

1. Introduction

Free-living amoebae (FLA) are protozoa widely distributed and have been isolated from the most diverse environments, such as tap water, swimming pools, cooling towers, air conditioners, dust, contact lenses and storage cases, among others. The genera *Naegleria*, *Acanthamoeba, Balamuthia* and *Sappinia* are part of this group (Khan, 2006). They are considered amphizoic and can become pathogenic if given the opportunistic (Trabelsi et al., 2012). They have two forms of life, the trophozoite through which they move, feed and reproduce and the cyst that is the form of resistance, protecting them from unfavorable conditions for their survival such as changes in temperature, pH and salinity (Khan, 2006; Schuster et al., 2004). One of the genera that have drawn attention for being more vulnerable to climate change and for being highly lethal is *Naegleria* (De Jonckheere, 2012).

This genus comprises several non-pathogenic species such as *Naegleria australiensis* and *Naegleria italica* (Visvesvara et al., 2007). In contrast the highly pathogenic *Naegleria fowleri* in most cases it leads to death. It is known popularly as the "brain-eating amoeba'' which has afflicted humans and animals (Mungroo et al., 2019). It is a thermophilic microorganism, supporting temperatures above 45 °C (Visvesvara et al., 2007). According to De Jonckheere (2011) *N. fowleri* is present on all

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continents except Antarctica. It is mainly found in lakes, ponds, and groundwater but not in sea water. The movement of the sediment at the bottom of these waters through boisterous play, causes the motile flagellated forms and trophozoites to enter the nose of individuals (mostly young males). When these encounter the nasal passages, they travel a path that will take them to the human olfactory epithelium and pass the cribriform plate to the brain through the olfactory cell axon (Baig et al., 2014; Visvesvara, 2013). *N. fowleri* settles in the meninges causing Primary Amoebic Meningoencephalitis (PAM), whose symptoms are similar to those of bacterial or viral meningitis: severe frontal headache, fever, neck stiffness, vomiting, mental confusion, hallucinations and cerebral hemorrhages. The course of this infection is approximately 7 days with a fatal outcome in the overwhelming majority of cases (Siddiqui and Khan, 2014; Heggie, 2010). In the USA alone, according to Centers for Disease Control (CDC), 151 cases of PAM were recorded in the period between 1962 and 2020, with only 5 survivors (CDC, 2021a). More cases have emerged around the world lately, not only through swimming, but also due to the use of neti pot or ablution rituals (Ghanchi et al., 2016). In recent years, cases among animals have also been reported more frequently. So far, there is no effective treatment against this disease (Mahmood, 2015; Siddiqui and Khan, 2014).

In the context, it is understood the need for a One Health approach linking the health of humans, animals, plants and the environment (McEwen and Collignon, 2018). Zinsstag et al. (2021) defined One Health as any added value for saving both human and animal lives, reducing costs and providing sustainable social and environmental services with interdisciplinary cooperation in favor of human and animal health. The four international agencies: the Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (OIE), the UN Environment Programme (UNEP) and the World Health Organization (WHO), have signed a agreement (Quadripartite MoU) to integrate human, animal, plant and ecosystem areas, reinforcing national and regional health systems and services (WHO, 2022). Depending on view and approaches, the One Health concept may be related with "One Medicine, One Medicine-One Health, One World-One Health, EcoHealth, and Planetary Health" (Pettan-Brewer et al., 2021). Several factors can influence the environment, causing instability. According to Ellwanger et al. (2020) global warming has consequences for the spread of infections, due to anthropogenic actions (emission of greenhouse gases from industries and extensive use of fossil fuels). The indiscriminate use of antimicrobials in human and animal health, pesticides and wildlife trade can lead to the transmission of pathogens that are often lethal (Hernando-Amado et al., 2019; Essack, 2018; Cunningham et al., 2017). Within this context there are microorganisms that have an ecological role in nature, but that, due to changes in the environment, can become pathogenic, such as free-living amoebae (FLA) (Samba-Louaka et al., 2019). According to Angelici et al. (2021), there is a need for a multidisciplinary and transdisciplinary integrated health approaches to understand and mitigate infectious diseases caused by neglected protist pathogens, which is in line with the One Health vision. In this review, we take a One Health approaches on occurrence of *N. fowleri* and their implications on human, animal, plant and environmental health.

2. Occurrence and prevalence of *N. fowleri* in the environment

N. fowleri is a thermophilic FLA whose occurrence has been predominantly documented in soil and freshwater samples, with emphasis on warm freshwater. Its isolation was carried out from various water bodies, such as surface water (Panda et al., 2015), groundwater (Rusiñol et al., 2020), well water (Baquero et al., 2014), geothermal water (Moussa et al., 2013), roof-harvested rainwater (Waso et al., 2018), tap water (Yousuf et al., 2013) and plumbing (Isaac and Sherchan, 2020; Cope et al., 2019). Isolation of *N. fowleri* was also performed from soil samples from various sites, including surface water sediment (Gunarathna et al., 2021), garden and field soil (Maclean et al., 2004), sewage sludge (Lawande et al., 1979a,b), as well as desert dust (Lawande, 1983). Furthermore, *N. fowleri* was also isolated from biofilm (Zbikowska et al., 2014).

In the present study, we found that the overall prevalence of *N. fowleri* in natural and anthropogenic environmental matrices is 16.70% (95% CI = 14.07–21.82) (Fig. 1, and Figure S1 in supplementary information). This prevalence was determined based on results from 47 studies (Table 1) systematically retrieved from databases (Web of Science, Scopus, PubMed, Science Direct, EMBASE, ProQuest, and CAPES journals) between August 5 and 9, 2022, using the combination of the terms "*Naegleria fowleri*", "identification" and "isolation" as a search strategy.

Despite the general prevalence value of *N. fowleri* being relatively higher in artificial sampling sources (20.43%) than in natural ones (18.44%), the prevalence values did not differ significantly in these two groups of sample sources ($p = 0.7599$). The values of the global prevalence of *Naegleria* spp. and *N. fowleri* as a function of the type of sampling source are summarized in Table 2 and in Figure S2 (Supplementary information). An overall prevalence of 23.27% and 26.42% in water sources for *N. fowleri* and *Naegleria* spp., respectively, has been previously reported in the literature (Saberi et al., 2020). An overall prevalence of 30.95% for *Naegleria* spp. in swimming pools and recreational waters was also recently reported in the literature (Chaúque et al., 2022).

The highest prevalence values of *N. fowleri* were obtained in North America (24.18%) and Central America (23.88%), and lower values were obtained in and Asia (4.36%) and Latin America (2.00%) (Fig. 1). Among countries, the highest prevalence values were obtained in Belgium (34.63%), USA (24.11%), Egypt (24.27%) and Mexico (23.88%), while the lowest in Sri Lanka (1.92%), India (1.26%) and Turkey (1.03%) (Table 2, Figure S3).

Our findings on the geographic distribution of *N. fowleri* prevalence, while helping to explain the worldwide distribution, do not consistently reflect the epidemiology of MAP cases worldwide (Gharpure et al., 2021). On the other hand, the prevalence of *N. fowleri* as a function of sampling sources is consistent with the literature, which associates MAP cases mainly with exposure to contaminated water sources (Gharpure et al., 2021).

Although water is considered the most frequent vector of infection, it is possible that people also become infected by inhaling dust containing *N. fowleri* cysts, as reported in Nigeria (Lawande et al., 1979a,b). The so-called "dry infections" are of concern mainly in very arid regions, where they are already responsible for cases of PAM. Dust contaminated with *N. fowleri* can also come into contact with the eyes and then amoebas can pass through the nasolacrimal ducts to the brain (Maciver et al., 2020).

Interestingly, our findings show a high prevalence of *N. fowleri* also in certain sampling sources from some countries that report low PAM cases. This may be related to the low diversity and nature (e.g. high water temperature) of the sample sources studied, lower number of studies performed and lower frequency of risk activities (e.g. recreation in undisinfected natural water sources) in the referred countries. According to Yoder et al. (2012) the frequency of exposure to *N. fowleri* can be inferred not only from reports of the occurrence of amoebas in environmental matrices, but also from the prevalence of antibodies in humans.

3. Ecological role of *N. fowleri*

Aquatic ecosystems have long been altered due to human action. Water flow deviation for irrigation of plantations, extensive livestock and artificial eutrophication, are examples of how this action affects the environment, affecting not only animals and plants, but also the human being who will have contact with opportunistic microorganisms, which cause diseases such as *N. fowleri* (Callisto et al., 2001). FLA influence the aquatic food chain in relation to species abundance, biomass, and

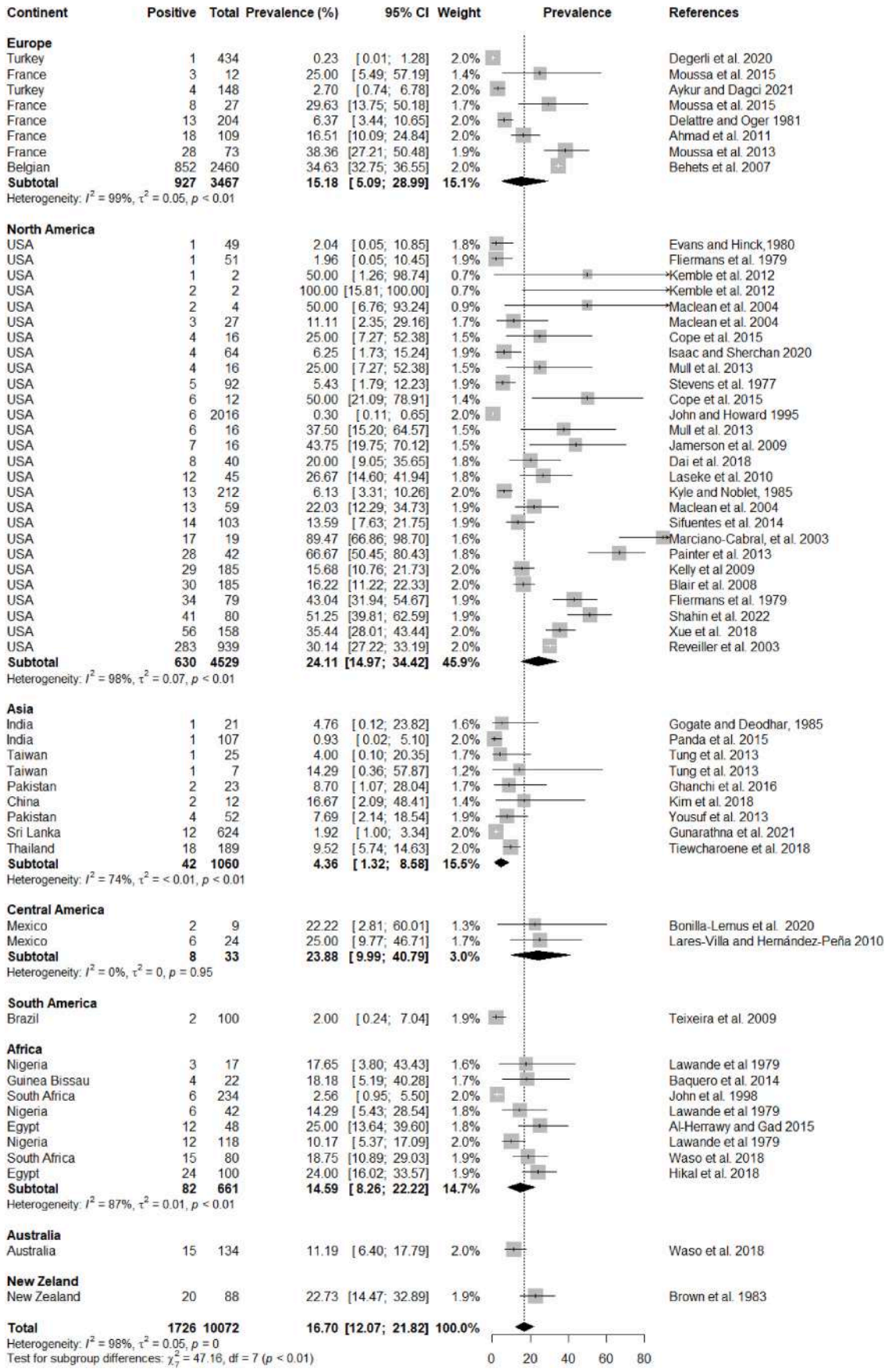

Fig. 1. Forest plot of the world and continental prevalence of *Naegleria fowleri* in the environment.

Table 1
Number of positive samples of *Naegleria fowleri* in different sampling sources.

References	Country	Sample source	Analyzed samples	Positive samples
Ahmad et al. (2011)	France	River water	109	18
Al-Herrawy and Gad (2015)	Egypt	River water	48	12
Aykur and Dagci (2021)	Turkey	Various waters sources	148	4
Baquero et al., 2014	Guinea-Bissau	Well water	22	4
Behets et al. (2007)	Belgian	Cooling water	2460	852
Blair et al. (2008)	USA	Well water	185	30
Bonilla-Lemus et al. (2020)	Mexico	Irrigation channels	9	2
Brown et al. (1983)	New Zealand	Thermal pool	88	20
Cope et al. (2015)	USA	Household samples	12	6
Cope et al. (2015)	USA	Various waters sources	16	4
Dai et al. (2019)	USA	Well water	40	8
Değerli et al., 2020	Turkey	Thermal pool	434	1
Delattre and Oger (1981)	France	Heated water	204	13
Evans and Hinck, 1980	North-east Arkansas	Lake water and sediment	49	1
Fliermans et al. (1979)	USA	Heated water	79	34
Fliermans et al. (1979)	USA	Surface water	51	1
Ghanchi et al. (2016)	Pakistan	Water treatment plants	23	2
Gogate and Deodhar, 1985	India	Swimming pool	21	1
Gunarathna et al. (2021)	Sri Lanka	Surface water	624	12
Hikal et al. (2018)	Egypt	Swimming pool	100	24
Isaac and Sherchan (2020)	USA	Drinking water distribution system	64	4
Jamerson et al. (2009)	USA	Lake water	16	7
John and Howard (1995)	USA	Surface water	2016	6
John et al. (1998)	South Africa	Surface water	234	6
Kelly et al. (2009)	USA	Well water	185	29
Kemble et al. (2012)	USA	Lake sediment	2	2
Kemble et al. (2012)	USA	Lake water	2	1
Kim et al. (2018)	China	Surface water	12	2
Kyle and Noblet (1985)	USA	Lake water	212	13
Lares-Villa and Hernández-Peña (2010)	Mexico	Surface water	24	6
Laseke et al., 2009	USA	Warm ground water	45	12
Lawande et al. (1979)	Nigeria	Sewage sludges	42	6
Lawande et al. (1979)	Nigeria	Soil	118	12
Lawande et al. (1979)	Nigeria	Various waters sources	17	3
Maclean et al. (2004)	USA	Soil	27	3
Maclean et al. (2004)	USA	Surface water	59	13
Maclean et al., 2004	USA	River water	4	2
Marciano-Cabral et al. (2003)	USA	Bathroom and kitchen pipes and sink traps	19	17
Moussa et al. (2013)	France	Hot spring water	73	28
Moussa et al. (2015)	France	Hot spring sediment	12	3
Moussa et al. (2015)	France	Hot spring water	27	8
Mull et al. (2013)	USA	Lake sediment	16	6
Mull et al. (2013)	USA	Lake water	16	4
Painter et al. (2013)	USA	Lake water	42	28
Panda et al. (2015)	India	Surface water	107	1
Reveiller et al., 2003	USA	River water	939	283
Shahin et al. (2022)	USA	Lake sediment	80	41
Sifuentes et al. (2014)	USA	Surface water	103	14
Stevens et al. (1977)	USA	Heated water	92	5
Teixeira et al. (2009)	Brazil	Floor dust	100	2
Tiewcharoene et al. (2018)	Thailand	River water	189	18
Tung et al. (2013)	Taiwan	Hot springwater	25	1
Tung et al., 2013	Taiwan	Surface water	7	1
Waso et al. (2018)	Australia	Rainharvested water	134	15
Waso et al. (2018)	South Africa	Rainharvested water	80	15
Xue et al. (2018)	USA	Lake water	158	56
Yousuf et al. (2013)	Pakistan	Tap water	52	4

biodiversity, playing a key role in the balance of the aquatic environment (Xu et al., 2009). A higher bacterial richness and abundance (*Alphaproteobacteria* and *Saprospirae*) are present in aquatic environments and may affect the growth of *N. fowleri* (Stahl and Olson, 2020). FLA feed on various microorganisms and can contribute to the recycling of nutrients, thus impacting the dynamics of biofilms regulating bacterial populations (Martinez-Urtaza et al., 2016; Thomas et al., 2010). Amoebae feed preferentially on bacteria, fungi, and algae. There are reports of predation of *N. fowleri* by other FLA such as *Balamuthia mandrillaris* and by rotifers that have a predilection for protozoan cysts (Stahl and Olson, 2020). One example is a correlation of *N. fowleri* with *Enterococci* as reported by Isaac and Sherchan (2020), which demonstrated the association of the amoeba with the food source (bacteria) in water distribution systems. FLA can also harbor viruses (Stahl and Olson, 2020).

Another food source for protists, especially amoebae, are the cyanobacteria. Anthropogenic eutrophication in freshwater systems is the main responsible for promoting the proliferation of cyanobacterial blooms which cause environmental and economic consequences. Global warming can be the cause by bloom proliferation and the spread of these cyanobacteria in freshwater systems (El-Shehawy et al., 2012). The availability of prey is a determining factor in relation to the vertical distribution of *N. fowleri* in warm lakes (Maciver et al., 2020). Thus, the eutrophication of water can result in the proliferation of cyanobacteria, favoring the proliferation of FLA, including *N. fowleri*, and the chances of occurrence of PAM. Another example are the Red tides that were very well described health risks through the One Health concept causing neurological symptoms in humans and animals associated with climate change and excessive use of fertilizers. (Heil and Muni-Morgan, 2021; Havens, 2015).

4. *N. fowleri* and physicochemical parameters in water

Many factors can directly influence the occurrence and dispersion of *N. fowleri* in the aquatic environment. Some still need further studies, others are already well mapped, so we highlight some of the most important points below.

Table 2
Global prevalence of *Naegleria fowleri* (%) by country and sampling source.

Sobgrups	Prevalence (95% CI)
Country	
Turkey	1.03 (0.00; 4.78)
India	1.26 (0.00; 6.63)
Sri Lanka	1.92 (1.00; 3.34)
Brazil	2.00 (0.24; 7.04)
Taiwan	4.64 (0.00; 16.78)
Pakistan	7.80 (2.37; 15.42)
South Africa	8.75 (0.00; 30.44)
Thailand	9.52 (5.74; 14.63)
Nigeria	11.22 (6.71; 16.58)
China	16.67 (2.09; 48.41)
Guinea-Bissau	18.18 (5.19; 40.28)
France	20.84 (9.27; 35.23)
New Zealand	22.73 (14.47; 32.89)
Mexico	23.88 (9.99; 40.79)
USA	24.11 (14.97; 34.42)
Egypt	24.27 (17.61; 31.60)
Belgian	34.63 (34.75; 36.55)
Sample source	
Cooling water	34.63 (32.63; 36.55)
Drinking water distribution system	6.25 (1.73; 15.24)
Floor dust	2.00 (0.24; 7.04)
Heated waters	15.23 (0.66; 42.11)
Hot spring water	22.63 (4.88; 47.42)
Hot spring sediment	25.00 (5.49; 57.19)
Household samples	72.81 (29.69; 99.86)
Irrigation channels	22.22 (2.81; 60.01)
Lake water and sediment	2.04 (0.05; 10.85)
Lake sediment	50.27 (38.78; 61.74)
Lake water	33.01 (12.63; 56.69)
Rainharvested water	14.34 (7.71; 22.52)
River water	19.72 (10.34; 30.87)
Sewage sludge	14.29 (5.43; 28.54)
Soil	9.99 (5.40; 15.64)
Surface water	5.83 (1.18; 12.85)
Swimming pool	14.61 (1.33; 36.50)
Tap water	7.69 (2.14; 18.54)
Thermal pool	7.18 (0.00; 42.48)
Various waters sources	11.29 (0.29; 31.15)
Warm ground water	26.67 (14.60; 41.94)
Water treatment plants	8.70 (1.07; 28.04)
Well water	16.04 (12.62; 19.77)

4.1. Temperature and pH

N. fowleri is a thermophilic amoeba associated with elevated temperatures (30–46 °C) (Maciver et al., 2021; De Jonckheere, 2011). Trophozoites and cysts can survive at temperatures of 50–65 °C. Cysts are more resistant, and can survive at 4 °C for 6 months, hibernating in lakes sediments and re-emerging when conditions become favorable (Maciver et al., 2020; Stahl and Olson, 2020). In the 20th century there were significant changes in the average temperature in the USA, with elevations of 0.7 °C–1.1 °C. Amoebae that live in the ground are introduced into lakes and ponds as rainfall increases. As the average temperature rises, cyanobacteria and eubacteria grow, becoming a food source for *N. fowleri*, and consequently increasing the probability of *N. fowleri* proliferation in freshwater bodies (Cooper at el., 2019). Prolonged periods of heat and drought caused by raising temperatures in freshwater bodies promote amoeba densities to increase, likely causing an increase in PAM, particularly among people using recreational waters (Siddiqui and Khan, 2014). It is important to highlight that the climate changes currently perceived, often characterized by hotter and longer summers, increase the practice of recreational activities in natural surface water resources (rivers, lakes) located around human settlements. These water resources are increasingly enriched in organic matter, fertilizers and other contaminants (eg iron, phosphorus, nitrogen) which, in combination with the increase in water temperature, favor the proliferation of aquatic microorganisms, including *N. fowleri*. All these aspects increase the risk of human and other animal exposure to *N. fowleri* as well as the likelihood of developing PAM.

There is no consensus about the relationship between pH and the occurrence of *N. fowleri* in the environment. Studies by Lam et al. (2019) reveal that in fresh water with a pH below 2, *N. fowleri* immediately becomes unviable, but remains viable at pH 3 for 72 h, persisting for at least 96 h at pH 4–11. At a pH of 12, *N. fowleri* are viable for 24h and rapidly lose viability with increasing pH. In vitro, the pH range tolerated by potentially pathogenic FLA went between 4.6 and 9.5 (Carter, 1970).

4.2. Light and turbidity

In the context of sunlight, there is no consensus that this act on the capture of prey by *N. fowleri*, as it has already been found in sediments that often do not receive sunlight. There was also no correlation between turbidity and the presence or abundance of *N. fowleri* in fresh or brackish waters, whether recreational, potable or from wells. As turbidity can occur in suspended sediments or nutrients, availability of prey for grazing by living amoebae free in biofilms may increase (Stahl and Olson, 2020). There are reports of PAM cases that have been associated with high water turbidity. For example, in 2010 in Minnesota, a young woman had PAM after swimming in a warm lake with cloudy water and algae blooms. Similar cases have also been reported in Indiana and Kansas (Cooper et al., 2019). In 2013, Moussa and collaborators verified the existence of a correlation between *N. fowleri* and turbidity through a geothermal survey of water and sediments.

4.3. Salinity

Freshwater amoebae adapt to higher salinity over time. Although *N. fowleri* has been found in brackish water, it is not known to survive in marine environments. It has already been verified that this microorganism tolerates up to 2% of NaCl. Human tissue tone is equivalent to 0.9% NaCl, so this would explain how *N. fowleri* is able to tolerate these concentrations by surviving the passage through the cribriform plate to the brain. Brackish waters in coastal environments can be a source of human exposure if the viability of *N. fowleri* in higher salinities is considered, even for a short time (Maciver et al., 2020).

5. Water treatment: chlorination x presence of iron

According to the World Health Organization (WHO), for effective disinfection, the residual concentration of free chlorine must be equal to or greater than 0.5 mg/L, with at least 30 min of contact time (at 20 °C) at pH less than 8.0 (Mahmood, 2015). Studies by Miller and collaborators (2017) demonstrated the elimination of *N. fowleri* from both biofilm and bulk water in an operational drinking water distribution system due to the presence of a constant free chlorine residual above 1.3 mg/L.

One of the problems related to the control of opportunistic pathogens is the increase in temperature, as there is a reduction in the levels of chlorine used in water distribution storage networks and cooling tower systems (Canals et al., 2015; Cross and Latorre, 2014). In biofilms, *N. fowleri* is more resistant to disinfectants such as chlorine and chloramines (Bright et al., 2016). Another method used in microbial water decontamination that has aroused interest, and its effectiveness has been widely demonstrated against all groups of microorganisms, including trophozoites and protozoan cysts, is the solar water disinfection (SODIS) (Chaúque and Rott, 2021a; McGuigan et al., 2012). Photolysis of sodium hypochlorite and sodium chloride by UV radiation has also been shown to be highly effective in inactivating trophozoites and cysts of *Acanthamoeba castelanii* and *Cryptosporidium parvum* (Chaúque and Rott, 2021; Zhou et al., 2014). The development of large-scale solar water disinfection systems will allow this technique to be applied to reduce human exposure to *N. fowleri* through the treatment of drinking water as well as recreational water (Chaúque and Rott, 2021a).

Some cases of PAM were associated with tap water and, therefore the Environmental Protection Agency (EPA) in the USA regulated tap water

chlorination levels for certain contaminants, but *N. fowleri* was not one of those and in 2002 two cases occurred due to the use of water from a geothermal well and, in 2013 a boy contracted PAM after playing on a water slide supplied with *N. fowleri* contaminated municipal water (Cooper et al., 2019). In France, there is a launch limit for industrial cooling water. In the case of Guadeloupe, it is allowed to discharge water if it contains less than 100 *N. fowleri* per liter. Once this limit is exceeded, the use of disinfectants in the water is permitted. If the number does not exceed the limit, the amoebae are released in the water bodies, being able to reach long distances and encyst in the face of unfavorable conditions. This can result in very high cyst concentrations in sediments. During the summer, the surface of the water is heated due to the greater incidence of sunlight, allowing the amoebae to emerge from the cysts and proliferate (De Jonckheere, 2011). Data collected between 2004 and 2005 reveal spills of cooling water with approximately 1.724 *N. fowleri* per liter occurred from power plants in Belgium. Global warming, in addition to cooling water, is considered the second impact of human activity, which increases the probability of *N. fowleri* proliferation in water (De Jonckheere, 2011). Water and sanitation systems are very sensitive to extreme climate change events, particularly in urban contexts (Cissé et al., 2016; Sherpa et al., 2014).

The presence of Iron in water can increases *N. fowleri* pathogenicity and destroys chlorine and soluble. Iron is a vital element for the development of the vast majority of living beings, participating in processes that occur in cells, such as respiration and DNA synthesis, in addition to binding to proteins (Arroyo et al., 2015). Common worms such as *Necator americanus* or *Ancylostoma duodenale* are known to feed on the blood of their hosts and extract the Iron necessary for their survival (Farid et al., 1969). *N. fowleri*, was found concentrated in the iron-rich layers in lakes. Iron was used not only for their locomotion and growth, but would also be involved in their pathogenicity (Maciver et al., 2021). During the invasion of *N. fowleri* through the nasal cavities, the parasite travels through tissues of the olfactory bulb and neuroepithelium that have iron-binding proteins, thus being able to obtain the chemical element from the tissues of the host (Maciver et al., 2021). Erosion, voluminous rainfall and iron-rich soils can be a perfect combination for the growth of *N. fowleri*, due to the rise in temperature in surface waters, which has been occurring since the 1980s in Europe (Maciver et al., 2021).

6. PAM: risk habits

N. fowleri is the species of the *Naegleri*a genus that infects humans and causes the PAM which is a relatively rare, highly lethal and under detected brain infection (Angelici et al., 2021; Siddiqui and Khan, 2014). People (particularly healthy children and youth) who swim in warm waters (lakes) or non-chlorinated recreational waters are most at risk, particularly during periods of high temperature, usually in summer (Ruszkiewicz et al., 2019; Mahmood, 2015). The increasing use of untreated water or wells in very arid regions and of water storage tanks of water has also been a factor of concern (Cope et al., 2016; Siddiqui and Khan, 2014). Slime, dirt, and high ambient temperatures are likely to favor the increased multiplication of *N. fowleri* in these storage tanks (Ganchi et al., 2014). Another worrying situation refers to ablution (Wudu), which is a religious practice that consists of washing different parts of the body (hands, mouth, nose, ears, face, arms and feet) with emphasis on rinsing the nostrils. This practice is prevalent among practitioners of the Islamic religion (Siddiqui and Khan, 2014). As such nasal irrigation could take place with amoeba contaminated tap water (Mahmood, 2015), this activity could be a communal source for potential outbreaks (Ganchi et al., 2014). In 2012, in the USA, the first death of a Muslim male patient who performed ablution was reported (CDC, 2013). The use of neti pot, mainly among Ayurveda practitioners has also been reported as a vehicle of pathogen transmission in many studies (Mahmood, 2015; Ghanchi et al., 2014). In animal's cases, the cattle can lick their nostrils and transfer amoebae in the nasal cavity (Daft et al., 2005).

Although the most common route of entry of *N. fowleri* into the host is through trophozoite-laden water (wet infection), it is also possible that there is contact with dry cysts present in dust (dry infection). In Zaria, Nigeria, *N. fowleri* is reported to be isolated from the nostrils of 2 out of 50 children during the hartmattan, which is a dry, dusty wind from the Sahara. In Borno State, 400 miles away, 3 out of 50 children carried viable *N. fowleri* in their nostrils (Maciver et al., 2020). Other causes should be investigated in relation to the host, such as genetic causes, biochemical alterations, underlying diseases that can lead to damage or abnormalities in the nasal mucosa (containing abnormal levels of immune factors), and rigorous, or routine nasal irrigation (Siddiqui and Khan, 2014).

7. PAM cases

Due to population growth, over population, climate change and deforestation, there have been changes in the interactions between humans, animals and plants, thus enabling infectious diseases to become emerging and sometimes zoonotic (Cavalcante et al., 2020). For a long time *N. fowleri* was known to cause PAM only in humans. But for some years now, mainly thanks to molecular biology, there have been reports of cases of PAM in animals caused by *N. fowleri* (Henker et al., 2021). Diagnosis of *N. fowleri* infection also in animals has a very large impact on public health because of human exposure to the environment and the high fatality rate of PAM (Yaw et al., 2019). The concept of One Health can be applied in these cases, since humans and animals are involved. Regarding plants, there is a report of isolation of *Naegleria australiensis* in irrigation water for vegetable crops production (Reyes-Batlle et al., 2019). PAM can be confused with bacterial meningitis due to symptomatology, so the diagnosis is difficult. Only about 27% of cases are diagnosed before death and most were diagnosed *post mortem* through immunohistochemical or molecular assays. The mortality rate is high, above 97% (Ruszkiewicz et al., 2019; Capewell et al., 2015). The incubation period for the disease varies from 2 to 15 days. Symptoms include severe headache, fever, nausea, vomiting, neck stiffness, hallucinations, mental confusion, seizures and coma. Death usually occurs within a week to 10 days (Ruszkiewicz et al., 2019).

7.1. In humans

The first register of a PAM case dates from 1937. Until 2018, a total of 381 cases of PAM caused by *N. fowleri* were registered around the world, with only five survivors: four in the USA, 1978 (1), 2013 (2), 2016 (1), and one in Mexico (2003) (CDC, 2021b). In the USA alone (1962–2020) 145 cases were identified through CDC's Free-Living Amoeba Surveillance System and 11 USA cases were identified through case reports before 1962. During this period, only in 7 years no case reports was registered (Gharpure et al., 2021). Typical cases of PAM in the USA are related to young male victims exposed to *N. fowleri* during recreational times. Boys generally play more in the water, sometimes stirring up lake sediments where *N. fowleri* trophozoites and cysts may be. In India or Pakistan, in addition to this type of contact, there is the ritual of ablution and lack of chlorination, making the risk of contracting the disease more comprehensive, reaching the general population (Maciver et al., 2020).

7.2. In animals

PAM also affects animals, mainly cattle that graze in flooded areas and become contaminated as they dip their nostrils into the water when drinking. Generally, the source of PAM infection in cattle is related to stagnant warm water in canals, areas of pasture or drinking troughs providing an environment for *N. fowleri* to proliferate. It is likely that the animals, when drinking water, transferred amoebae to the anterior part of the nasal cavity by licking their nostrils (Daft et al., 2005). Three cases

were identified in Brazil: one Holstein-Gir steer, in the state of Paraíba (Pimentel et al., 2012); two other cases in cattle occurred in southern Brazil, in the city of Glorinha (Henker et al., 2019, 2021). Other cases of PAM have also been reported elsewhere in the world; Benterki et al. (2016) reported a case in a cow and a sheep in Algeria, Yaw et al. (2019) in a black rhinoceros and Lozano-Alarcón et al. (1997) in a tapir.

8. Treatment of PAM: most common drugs used

Due to the high mortality rate of this disease, there is no consensus on a completely effective drug to eradicate it. Only 5 people survived PAM, so researchers around the world have been striving to produce a drug that will save victims of the disease, but most of the information is based on case reports or *in vitro* studies (Grace et al., 2015). The drugs used in the treatment of *N. fowleri* infections are fluconazole, miconazole, azithromycin, rifampin, clotrimazole, itraconazole, ketoconazole, and chlorpromazine, with varying degrees of efficacy, which was informed in case reports (Grace et al., 2015; Kim et al., 2008). Lately, the most used drugs are amphotericin B and miltefosine. These antimicrobial drugs have been used empirically and indiscriminately in veterinary and human medicine resulting in antimicrobial resistance. Recent public policies and government laws have been trying to regulated this controversial reality (PAN-BR, 2018).

Amphotericin B is a macrolide polyene produced through a fermentative process by the soil actinomycete, *Streptomyces nodosus*. It has been clinically used since 1959, after FDA approval (Hamill, 2013). Amphotericin B has been studied *in vitro*, used in several case reports and is recommended by CDC. It has been used intravenously or intrathecally, including in all the treatments of patients who survived PAM (Capewell et al., 2015; Vargas-Zepeda et al., 2005). Therefore, amphotericin B is considered a primary drug of choice in the treatment of PAM and can be used alone or in combination with other drugs (Cooper et al., 2019). A high dose is required, and its use may be associated with renal toxicity and anemia, among other adverse effects (Bashyal et al., 2017). The recommendation of CDC for the conventional intravenous treatment with amphotericin B is 1.5 mg/kg/day in 2 divided doses for 3 days followed by 1 mg/kg/day once daily for an additional 11 days (total of 14 days of therapy). The CDC-recommended dose for the conventional intrathecally amphotericin B treatment is 1.5 mg/day for 2 days followed by 1 mg/day for an additional 8 days (total of 10 days of therapy) (Cooper et al., 2019).

Miltefosine whose chemical name is hexadecyl-2-(trimethylazaniumyl) ethyl phosphate, the antiparasitic used in the treatment of leishmaniasis, has been used in PAM cases having been successful in treating two survivors (Dorlo et al., 2012). The phospholipid and alkyl phosphocholine components that allow this drug to penetrate the blood-brain barrier and concentrate in brain tissue (Alli et al., 2021; Cooper et al., 2019). The route for drug administration is oral: weight <45 kg - 50 mg twice daily or weight >45 kg - 50 mg three times daily, with the dose maximum of 2.5 mg/kg/day for a total of 28 days (Cooper et al., 2019). These drugs were not able to reduce the mortality rates caused by *N. fowleri*, so the importance of testing new drugs in the fight against this disease is extremely important.

9. Antibiotic resistance

One of the main global health challenges of the present century is antimicrobial resistance (Hernando-Amado et al., 2019). Natural ecosystems have been undergoing changes due to anthropic action, thus affecting their dissemination. Global warming is probably acting on microorganisms, humans, animals, and interacting vectors (Hernando-Amado et al., 2019). An example of this would be the intercontinental distribution of bacterial pathogens caused by the alteration of ocean currents caused by El Niño (Martinez-Urtaza et al., 2016).

According to Soares (2020), antibiotic residues from hospital effluents and pharmaceutical industries, from their use by the agribusiness industry, contribute to bacterial resistance and the development of resistance genes to these compounds.

The spread and maintenance of antibiotic resistance genes depends on their interaction between bacterial populations or between human and animal hosts (Baquero, 2017). Most antibiotics used for therapeutic purposes are released into water and can act as chronic pollutants. Its use in agriculture is worrying, because two-thirds of the general use of antibiotics is destined for animal production (Hernando-Amado et al., 2019; Done et al., 2015). Tertiary processes, such as chlorination or ultraviolet treatment, can induce horizontal gene transfer, triggering the spread of antibiotic resistance genes (Hultman et al., 2018). In this context and through One Health we can infer that bacteria carried by FLA may be resistant to antibiotics or perhaps exchange resistance genes with the protozoan, which may increase the virulence of both microorganisms. More studies are needed to elucidate this issue, especially in *N. fowleri.*

10. Use of plants and algae for effective drug formulation against PAM

There is a growing interest in the discovery of new drugs that can be used to fight infectious diseases. Plant-based products have played a key role in the treatment of humans and animals throughout history. There is a need to develop new anti-*Naegleria* compounds with low toxicity and high efficacy compared to drugs currently used to combat PAM and the use of plant extracts, already tested against other protozoan becomes a viable alternative, (Arberas-Jiménez et al., 2022; Panatieri et al., 2017; Castro et al., 2013).

Larrea tridentata, known as creosote bush or chaparral, presents in its composition antiparasitic secondary metabolites successfully used in other parasites such as *Trypanosoma brucei rhodesiense, T. cruzi, Leishmania donovani and Plasmodium falciparum* (Schmidt et al., 2012). *L. tridentata* was used in experiments with *N. fowleri* and proved to be more potent than the drug Miltefosine commonly used against PAM (Bashyal et al., 2017).

Algae have been used as a source of drugs to combat PAM such as *Laurencia johnstonii,* being red algae from which cyclolaurane-type sesquiterpenes are extracted, effective against *Naegleria* species. One of its compounds, debromolauinterol, reduced ATP by 99.98% in relation to the control, inducing programmed cell death in *N. fowleri* (Arberas-Jiménez et al., 2022).

11. Detection of *N. fowleri*

Several techniques have been used to detect microorganisms. Environmental studies of *N. fowleri* are mainly based on detecting surface water and sediment from lakes, ponds, and rivers (Al-Herrawy and Gad, 2015). The culture methods serve to recover and amplify *N. fowleri* from water samples. This is performed on plates containing non-nutrient agar, with a lawn of *E. coli* as the growth substrate and incubation at 42–44 °C (Visvesvara et al., 2007). After growth on plates, the material can be scraped into distilled water for the observation of the emission of flagella within 1–2 h and the differentiation of *Naegleria* from other FLA (Behets et al., 2003).

In humans, *N. fowleri* can be diagnosed by a lumbar puncture, where the collecting cerebrospinal fluid is analyzed for polymorphonuclear leukocytes and *N. fowleri* trophozoites through the staining with Wright-Giemsa and Gram stain (Siddiqui et al., 2016; Visvesvara, 2013). In addition, molecular biology and biochemistry can provide detailed knowledge about protist pathogens. Postmortem samples of brain fragments are macerated, the DNA extracted and the primers ITS1 and ITS2 used to amplify the 5.8S region of the rRNA genes and internal transcribed spacer regions of *Naegleria*. The molecular identification was positive for *N. fowleri* in Southern Brazil (Henker et al., 2021). In the same municipality - Glorinha, (29° 55′ 12.7″ S 50° 44′ 48.4″ W) RS, Southern Brazil, another case of PAM in cattle had already been

registered by the same author, but the technique used for diagnosis was immunohistochemistry (Henker et al., 2019). Although desirable, the flagellation test and visualization of morphology alone are not able to accurately differentiate *N. fowleri* from other species of the genus *Naegleria*. Therefore, molecular assays such as conventional PCR or real-time PCR (qPCR) have replaced the immunological assays previously used for this purpose (Ahmad et al., 2011; Madarová et al., 2010; Qvarnstrom et al., 2006; Behets et al., 2003; Marciano-Cabral et al., 2003; Pélandakis et al., 2000). The qPCR can detect *N. fowleri* in water samples minimizing the possible errors that can occur when determining the concentration of the microorganism (De Jonckheere, 2011), in addition to presenting high specificity and sensitivity (Wang et al., 2017) such as demonstrated in studies of Isaac and Sherchan (2020) where *N. fowleri* was only detected in 4 of 64 samples (6%).

The use of metagenomics for the detection of FLA has been increasingly explored, as for example in a study by Rusiñol et al. (2020), this investigation identified the presence of *Naegleria* spp. in samples collected from water of reservoirs, groundwater, river water and reuse water and *N. fowleri*, was detected in groundwater. These tools are indispensable for the diagnosis and treatment of clinical cases of PAM, in addition to being useful in the detection of outbreaks and preventing deaths of humans and animals (Angelici et al., 2021). Zysset-Burri et al. (2014) used the tools of transcriptomic and proteomic data from virulent *N. fowleri* trophozoites to identify potential targets involved in amoebic pathogenicity and virulence. In addition, a mass spectrometry approach has been used to identify protein biomarkers for pathogenic *N. fowleri* using matrix-assisted laser-desorption-ionization-time-of-flight (MALDITOF) (Moura et al., 2015). Genomic information is an important tool to understand and develop actions for the prevention and treatment of diseases caused by protozoa such as *N. fowleri* (Faktorova et al., 2020) and provide information about the pathogenicity and virulence of an organism (Cope and Ali, 2016).

12. *Naegleria* spp. and endosymbionts

FLA feeds mainly by phagocytosing particulate organic material and microorganisms and digestion takes place within phagolysosomes. Some of phagocytosed microorganisms can resist digestion being internalized, therefore called “amoeba-resistant microorganisms” (ARMs). Within amoebic cells, ARM, in addition to surviving, can multiply, recombine its genetic material and regulate the host’s metabolism (Oliveira et al., 2019). Thus amoebae serve as reservoirs or “Trojan horses” carrying microorganisms and performing the selection of virulence traits in addition to adaptation of the microbes to macrophages (Greub and Raoult, 2004). Studies by Dos Santos et al. (2022) revealed the presence of an endosymbiont in *Acanthamoeba* in a CL user patient who had severe keratitis in both eyes.

Naegleria may also serves as natural reservoirs for pathogenic bacteria in addition to dispersing diseases in the environment, as is already the case in other FLA such as *Acanthamoeba* (Maschio et al., 2015; Marciano-Cabral and Cabral, 2007). Laseke et al. (2010) reveal the presence of *N. fowleri* and bacterial communities in warm groundwater aquifers. Studies on the presence of endosymbionts such as *Legionella* in *Naegleria fowleri* are found in the literature (Dobrowsky et al., 2016; Zbikowska et al., 2014; Newsome et al., 1985) and in chemically treated water and sewage systems *Naegleria* cysts that contained *Legionella* were resistant to chlorination and biocides (Declerck et al., 2005). Virus-like particles were found inside *N. gruberi* (Stahl and Olson, 2020) and *Simkania nevegensis* (*in situ* interaction), *Acidovorax temperans*, and *Waddlia* sp. (both *in vitro* interaction) were found in *Naegleria* (Thomas et al., 2008; Balczun and Scheid, 2017).

13. Global warming impact and climate changes on the dispersion and proliferation of *N. fowleri*

Global warming generally refers to the increase in the average global temperature over the last few decades, and is induced by the greenhouse effect resulting primarily from CO_2 emissions from the wordwide burning of fossil fuels and deforestation (El-sayed and Kamel, 2020). In addition, the increase in temperatures also affects the dynamics of predators and competitors (Scheid, 2019). According to IPCC et al. (2018) human activity has warmed the world by about 1 °C since the pre-industrial era, and the impacts of this warming have already been felt in many parts of the world. Although these changes do not occur at the same speed everywhere, some have already suffered from temperatures increases above 1.5 °C. Reaching 2 °C instead of 1.5 °C of global warming, in addition to posing greater risks to human and animal health, would lead to an increase in heavy rainfall events, raising the risk of flooding. In the last 50 years, the rise of just 0.5 °C in global temperatures has contributed to changes in the distribution of plants and animals and more frequent wildfires. Similar changes can be expected with further increases. Changes in climate are associated with changes in the geographic range, seasonality and intensity of transmission of many infectious diseases (Semenza and Menne, 2009). Meteorological and climatic events are related to the increase in morbidity and mortality according to IPCC et al. (2014).

Changes in climate and environment can affect the dispersion and abundance of organisms to the dynamics of a population influencing the occurrence of infectious diseases in people, domestic animals, and wildlife, some of them provoked by *N. fowleri* and their pathogenic endocytobionts (Scheid, 2019). It Global warming is believed to transform the world into a breeding ground for parasites mainly the zoonotics with are vulnerable to climate change becoming more transmissible. The protistan parasites have an undisputed global health impact (Angelici et al., 2021). According to Siddiqui and Khan (2014), the rise in temperatures that has occurred in recent years due to global warming, lack of wastewater management and basic sanitation, in addition to antimicrobial resistance, may be the cause of an increase in deaths from infectious diseases, in which PAM is also inserted. High temperatures in freshwater environments can promote the expansion of *N. fowleri* (Fig. 2) causing it to occupy more and more specific niches (Angelici et al., 2021), and influencing the increase in recreational activities and consequently in cases of PAM (Siddiqui and Khan, 2014). Both drought and precipitation can cause erosion and eutrophication of fresh waters and can favor the proliferation of *N. fowleri*. In arid regions, the use of rainwater on roofs, groundwater or the use of artesian wells that may be exposed to *N. fowleri* may be used (Maciver et al., 2020). Due to the increase in precipitation, amoebae that live in the soil are introduced into lakes and ponds. Climate change may expand the amoeba’s natural habitat (Cooper et al., 2019). Other extreme climatic events (cyclones and floods) also increase the human exposure to waterborne pathogens both for the direct effect on the environment and by modifying human behavior (Angelici and Karanis, 2019).

14. Conclusions

N. fowleri is the pathogen responsible for PAM, a rare disease but highly lethal for affected humans and animals around the world. The incidence of PAM has been increased probably due to climate change effects. This parasite is widely distributed, and has been isolated from several environments, mainly those involving water, being the habitat where there is a greater probability of infection. Many studies have been carried out in the hope of finding a drug that is capable of eliminating this microorganism and saving the patient affected by PAM. Although more modern and effective techniques are used in the identification of *N. fowleri*, a different approach to this issue is necessary. The One Health approaches in this context involve *N. fowleri*, humans and animals affected by the disease. The study of prospect of new drugs, whose dosage does not contribute to antimicrobial resistance, is extremely important through the One Health approaches as well. The understanding of how human action interferes in the ecosystem through climate change and global warming leading to a dispersion of the

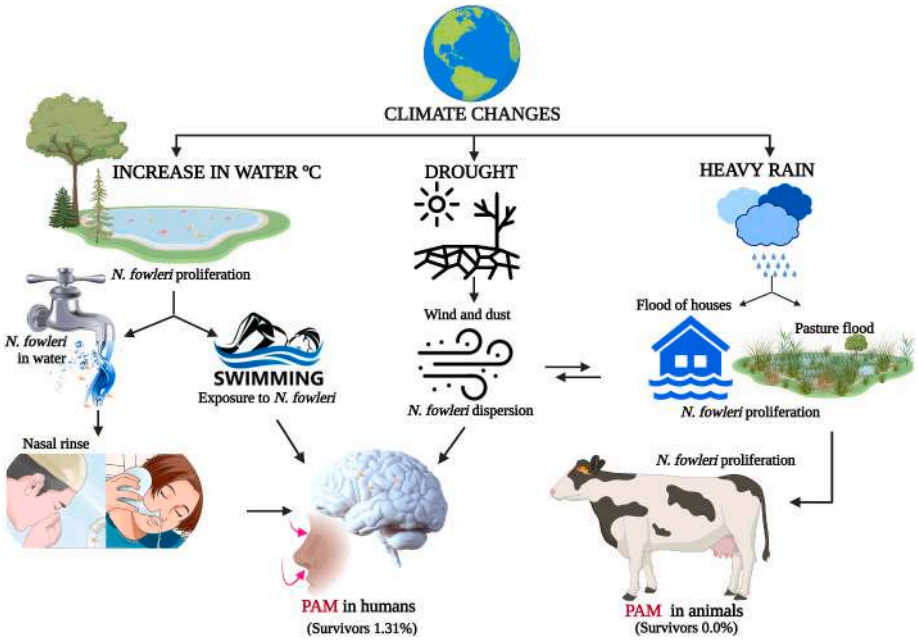

Fig. 2. Diagram of the action of climate change on environments, favoring the proliferation and dispersion of *N. fowleri* and its implications for human and animal health within the One Health approaches.

pathogen is relevant information to outline strategies for effective solutions for health as a whole.

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Declaration of competing interest

No declared.

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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.2022.114053.

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Passive smoking and urinary oxidative biomarkers: A pilot study of healthy travelers from Los Angeles to Beijing

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ABSTRACT

There is a great heterogeneity in smoking prevalence and tobacco control policy across different countries. However, it is unknown whether this heterogeneity could cause increased passive smoking and adverse health effects among international travelers. In this pilot study, we collected 190 urine samples from 26 Los Angeles residents before (LA-before), during (Beijing), and after (LA-after) a 10-week visit to Beijing to measure biomarkers of passive smoking (cotinine), exposure to polycyclic aromatic hydrocarbons (OH-PAHs), and oxidative stress (malondialdehyde, 8-isoprostane, and uric acid). The geometric mean concentrations of urinary cotinine were 0.14, 1.52, and 0.22 μg/g creatinine in LA-before, Beijing, and LA-after, respectively. Likewise, OH-PAH levels were significantly higher in Beijing as compared to LA-before or LA-after, in association with the urinary cotinine levels. One-fold increase in urinary cotinine levels was associated with 10.1% (95% CI: 5.53–14.8%), 8.75% (95% CI: 2.33–15.6%), and 25.4% (95%CI: 13.1–39.1%) increases in urinary levels of malondialdehyde, 8-isoprotane, and uric acid, respectively. OH-PAHs mediated 9.1–23.3% of the pro-oxidative effects associated with passive smoking. Taken together, our findings indicate that traveling to a city with higher smoking prevalence may increase passive smoking exposure, in association with pro-oxidative effects partially mediated by PAHs.

1. Introduction

Active smoking caused 6.4 million deaths worldwide in 2015 with cardiovascular diseases as the primary cause (GBD 2015 Tobacco Collaborators, 2017). Passive smoking, which refers to involuntary exposures to tobacco smoke that were either exhaled by smokers (secondhand smoke, SHS) or adhered to the surfaces of clothing, hair, furnishings, and dust (thirdhand smoke, THS) (Sleiman et al., 2010), is thought to induce cardiovascular effects nearly as large as active smoking (Barnoya and Glantz, 2005), and likely to impact adversely a greater portion of the population beyond smokers. It has been estimated that in 2004, more than 1/3 of global population was exposed to SHS at home or workplaces, which caused 603,000 deaths; 379,000 of which were due to ischemic heart disease (Öberg et al., 2011). Remarkably, substantial heterogeneity in SHS exposures across countries has been documented, with higher exposures in developing countries (Centers for Disease Control and Prevention (CDC), 2007; Öberg et al., 2011; Warren et al., 2011), likely due to higher smoking prevalence and the lack of regulatory policies (King et al., 2013; Thomas et al., 2008). It is less understood, however, whether this heterogeneity could lead to increased exposures to SHS, and subsequent adverse health effects among international travelers who usually spend short time at their destinations.

Previous studies have shown that even brief (minutes to hours) passive smoking could markedly perturb the cardiovascular system with increased oxidative stress as one of the underlying mechanisms

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(Barnoya and Glantz, 2005), which is largely attributed to its content of a large number of toxic redox active components. To date, at least 90 toxic components have been identified in SHS with biological half-lives ranging from milliseconds (free radicals) to years (cadmium) (Brewer et al., 2017; Talhout et al., 2011), some of which could react with gaseous pollutants, leading to the formation of even more toxic products in THS (Sleiman et al., 2010). Of note, polycyclic aromatic hydrocarbons (PAHs), a group of toxic byproducts of incomplete combustions, are of high abundance in tobacco smoke and thought to exert significant effects on the health of passive smokers. However, concrete evidence is lacking to what extent the adverse health effects of passive smoking could be attributable to PAHs exposure.

The United States (U.S.) and China are among the world's most populous countries, with marked differences in the exposure to tobacco smoke. The number of residences traveling between both countries increased rapidly in recent years, reaching 1.31 and 2.59 million in the U.S and China in 2015, respectively (Yearbook of Tourism Statistics, 2018). In our previous studies, we have identified a marked increase in personal exposures to PAHs among healthy young adults who traveled from Los Angeles to Beijing (Lin et al., 2016, 2019, 2021), a city with higher smoking prevalence, population density, and air pollution levels (Los Angeles County Department of Public Health, 2010; Zhang et al., 2016). Furthermore, we observed significant increases in the circulating levels of a panel of oxidative biomarkers, after the participants spent 6–8 weeks in Beijing, which reversed almost completely 4–7 weeks after the participants returned to Los Angeles, and were positively associated with urinary levels of PAHs metabolites (Lin et al., 2019; Lu et al., 2021). Importantly, typical oxidative stress markers have been associated with all major cardiovascular diseases (Daiber et al., 2021). However, our panel of lipid oxidation products in the blood failed to associate with urinary cotinine levels, suggesting that the observed effects were likely due to other sources of PAHs such as air pollution (Lin et al., 2019; Lu et al., 2021) or that other biomarkers would be required to reflect pro-oxidative effects potentially induced by SHS. Therefore, the health effect of increased passive smoking exposures during the travel remained largely unknown.

In the current study, we studied this group of travelers and focused on urinary oxidative biomarkers such as 8-isoprostane, malondialdehyde (MDA) and uric acid, which were measured in 190 urine samples, repeatedly collected from 26 healthy nonsmoking participants. This study aims to determine (i) whether changes in passive smoking exposures during the travel were associated with urinary biomarkers of oxidative stress; and (ii) to what extent the oxidative effect of passive smoking exposure was mediated by PAHs exposure.

2. Methods

Study Participants. The study is based on a summer research program between Peking University (PKU) and University of California, Los Angeles (UCLA), in which students from UCLA visit PKU and stay there for 10 weeks in each summer. All participants in this study (n = 26) were healthy non-smoking adults recruited in 2014 or 2015 with details described previously (Lin et al., 2019). Demographic information and written informed consent were obtained from all participants with study purpose and risk explained. The study was performed in accordance with guidelines and approval of the Institutional Review Board of both UCLA (Protocol# IRB17-001812) and PKU (Protocol# IRB00001052-14037).

Sample Collection. For each participant, up to nine urine samples were collected before the travel (LA-before), in Beijing (2–10 weeks after arrival in Beijing), and 4–10 weeks after returning to Los Angeles (LA-after). Because PAHs and nicotine are metabolized and excreted rapidly with a half-life less than one day (Benowitz, 1996; St.Helen et al., 2012), each urine was collected at least one week after the previous collection. Each urine collection was coupled with a questionnaire survey that included time spent near smokers in the past three days, and various activities related to PAHs exposure in the past three days, such as (i) cooking behaviors (time for cooking and barbecuing), (ii) dietary intakes of food rich in PAHs (consumption of barbecue or baked food), and (iii) traffic-related behaviors (driving hours, public transportation usage, and time spent near heavy traffic). All the urine samples were collected using 90-mL polypropylene specimen containers in the morning after fasting for at least 8 h. The samples were aliquoted to 15-mL polypropylene centrifuge tubes and were transported through flight (~12 h) with dry ice. All the urine samples were stored at −20 Celsius and kept away from light until laboratory analysis.

Laboratory Analysis. We used a gas chromatograph and mass spectrometer (GC-MS; Agilent 7890A-5975C) with an isotope dilution method to measure the concentration of cotinine and 11 hydroxylated PAHs in the urine including 1- and 2-hydroxylated naphthalenes (OH-NAPs), 2-hydroxylated dibenzofuran (2-OH-DBF), 2- and 3-hydroxylated fluorenes (OH-FLUs), 1-, 2-, 3-, 4- and 9-hydroxylated phenanthrenes (OH-PHEs), and 1-hydroxylated pyrene (1-OH-PYR). To determine urinary cotinine, 0.5 mL urine was spiked with deuterium-labeled (+/−)-cotinine and alkalinized with 0.5 mL NaOH (4M)/KCl (1M) solution. The aliquant was then extracted with 2 mL dichloromethane four times and concentrated under nitrogen flow for instrumental analysis (Lin et al., 2019). To determine urinary OH-PAHs, after spiked with deuterium- or ^{13}C- labeled hydroxylated PAHs (i.e., $^{13}C_6$-2-OH-NAP, $^{13}C_6$-3-OH-FLU, $^{13}C_6$-3-OH-PHE and d_9-1-OH-PYR) as surrogate standards, 2 mL of urine was incubated with β-glucurnonidase-sulfatase (*Helix pomatia*, Sigma-Aldrich, St. Louis, MO), followed by liquid-liquid extraction, diazomethane derivation, purification by silica column chromatography and instrumental analysis (Lin et al., 2016, 2021). Two OH-NAPs and two OH-PHEs (i.e., 3-OH-PHE and 9-OH-PHE) could not be distinctly separated in chromatograms; therefore, their summed concentrations were reported as ΣOH-NAPs and 3+9-OH-PHEs, respectively. The concentration of 2-OH-DBF was corrected by $^{13}C_6$-3-OH-FLU, while other analytes were corrected by corresponding surrogate standards. After correction, the recovery of all analytes in spiked samples was 99.6 ± 7.6% (range 85.2–110.7%), and the relative standard deviation was 7.6 ± 4.3% (range 2.6–15.7%). The limit of quantification (LOQ) of different OH-PAHs ranged from was 7.5 pg/mL to 28.5 pg/mL with detailed information listed in Table S1. All the OH-PAHs were detected in at least 97.4% of the urine samples (Table S1). Blank samples were prepared for each eight urine samples, and negligible contamination was observed for all analytes. Concentrations of metabolites from the same PAHs were summed as an exposure indicator for the precursor compound.

Urinary creatinine was measured by a spectrophotometer under a wavelength of 510 nm based on the Jaffe reaction (Toora and Rajagopal, 2002). Urinary MDA concentrations were measured by a high-performance liquid chromatograph (HPLC; Waters2695) coupled with a UV detector under a wavelength of 532 nm, after the reaction with 2-thiobarbituric acid (TBA) (Lin et al., 2016). Urinary concentrations of 8-isoprostane and uric acid were determined based on the same methods that we have previously used for blood testing (Lin et al., 2019; Lu et al., 2021). Briefly, urinary 8-isoprostane was measured with a commercial ELISA kit (R&D, Detroit, MI) according to the manufacturer's instructions. Each urine sample was analyzed in duplicate. Urinary uric acid was measured with a metabolomic approach, and the results were confirmed with the authentic standard. Briefly, urine samples were thawed on ice, followed by centrifugation by 10,000 rpm at 4 °C for 10 min. 50 μL supernatant was transferred to a new tube, added with 50 μL methanol and vortexed for 1 min. After centrifugation by 10,000 rpm at 4 °C for 10 min, 80 μL supernatant was transferred into the sample vial and diluted with 40 μL water. Samples were analyzed by high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS, 1290II-6550; Agilent) with a 10-cm Zorbax Eclipse C18 column (2.1 mm i.d., 1.8 μm particle size; Agilent) and electrospray ionization in negative mode. Urinary levels of cotinine, OH-PAHs, MDA, and 8-isoprostane were normalized by

creatinine levels, to reduce interferences of diluted/concentrated spot urine samples.

Statistical Analysis. Analytes not detected in urine samples were substituted with 1/2 limit of quantification (LOQ) for statistical analysis. Logarithmic transformation was performed for biomarkers with a right-skewed distribution. Depending on the distribution of variables, Student's t-test or Mann-Whitney U were used to investigate the difference between two variables, and Pearson or Spearman correlation tests were used to test associations between two variables when the control of intra-individual variability was not necessary. Otherwise, we built different mixed effects models with random intercept at the participant level as follows. First, we modeled biomarker concentrations as a function of phases as categorical variables to study the changes of biomarker during the travel. Second, we modeled urinary OH-PAH levels as functions of cotinine, year, questionnaire variables, and the interactions of city with cotinine and year, to study the effects of passive smoking on PAHs exposure while controlling other factors. Third, we tested the associations between exposures and health biomarkers in mixed effects models with random intercept at both participant and phase levels.

If significant associations were observed between urinary levels of cotinine, OH-PAHs, and markers of oxidative stress (i.e., MDA, 8-iso-prostane, and uric acid), additional mediation analyses were performed to examine whether OH-PAHs mediate the pro-oxidative effects of cotinine. Both mediator and outcome models were linear mixed-effects models with random intercept at the participant level and fixed effects of phases as categorical variables. We used quasi-Bayesian approximation with a simulation of 5000 iterations to estimate the total effects, average direct effects (ADE, the average direct effect of cotinine on the outcome), and average causal mediation effects (ACME, the average casual mediation effects of cotinine on the outcome attributable to OH-PAHs) (Tingley et al., 2014). Alpha was set at 0.05, and all tests were two-tailed. Data were analyzed with the software R with *lme4*, *lmeTest*, and *mediation* packages (www.r-project.org).

3. Results

Demographic characteristics. The mean (standard deviation) age and body mass index of the 26 study participants (14 females and 12 males) were 23.8 (±5.6) years and 21.6 (±2.4) kg/m^2, respectively (Table S2). All participants were healthy young non-smokers and most of them were Asian. No participants were recorded with any heart disease, metabolic disorder, kidney disease, blood coagulation disorders, rheumatological disease or chronic inflammation. Of the 26 participants, 22 provided urine samples in all three phases while four provided urine samples only in two phases with urine samples absent in either LA-before (n = 3) or LA-after (n = 1).

Passive smoking exposures. The urinary cotinine was detected in all samples collected in Beijing but only in 80.9% of those in Los Angeles. The geometric mean concentration of urinary cotinine was 1.52 (IQR: 0.88–2.25) μg/g creatinine in Beijing, significantly higher ($p < 0.001$) than that in LA-before (geometric mean: 0.14; IQR: 0.06–0.24 μg/g creatinine) or LA-after (geometric mean: 0.22; IQR: 0.09–0.40 μg/g creatinine; Table 1). This trend was consistent in both 2014 (Fig. 1A) and 2015 (Fig. 1B). Correspondingly, 49.0% of urine samples in Beijing were collected after participants had spent time near smokers in the past three days, markedly higher than the frequencies in LA-before (3.0%) or LA-after (3.3%). All this data indicates elevated passive smoking exposures in Beijing.

To our surprise, the cotinine concentration in urine collected from participants who reported to spend time near smokers in Beijing (n = 47) was comparable with those who did not (n = 49, $p = 0.54$; Fig. S1). Consistently, we observed no associations between urinary cotinine concentrations and self-reported time near smokers after controlling for phase, year, and other activities ($p = 0.83$; Table S3).

Associations between passive smoking and PAHs exposures. Similar to the changes in urinary cotinine levels, traveling from Los Angeles to Beijing led to significant increases in the urinary concentrations of all OH-PAHs except for ΣOH-NAPs ($p < 0.001$; Table 1), which reversed, at least partially, after participants returned to Los Angeles. Of note, the concentrations of most OH-PAHs and cotinine in LA-after were significantly higher as compared with LA-before ($p < 0.05$); however, the differences between LA-before and LA-after were much smaller than the differences between the two cities. These results strongly suggest that participants were subjected to elevated exposures to PAHs in Beijing.

The urinary levels of cotinine were positively associated with the concentrations of ΣOH-PHEs and 1-OH-PYR in the urine ($p < 0.001$), but not with the other OH-PAHs (Table 2). On the other hand, time spent on barbecuing activities and public transportation was positively associated with OH-PAHs ($p < 0.05$), suggesting that behavioral changes related to diet and outdoor air pollution could have also influenced the exposure to PAHs. Since both cotinine concentrations and time spent in public transportation were markedly increased from Los Angeles to Beijing (Table S4), it is likely that both passive smoking and behavioral changes contributed to higher exposure to PAHs in Beijing. Remarkably, the concentrations of 2-OH-DBF, ΣOH-FLUs, and ΣOH-PHEs remained significantly higher in Beijing than in LA-before even after adjusting for urinary cotinine concentrations and behavioral changes (Table 2), which likely reflect the effects of the more severe air pollution in Beijing as previously reported (Lin et al., 2019).

Associations between passive smoking and oxidative biomarkers. We observed significant increases in urinary MDA concentrations in Beijing ($p < 0.001$; Fig. 2A). However, unlike urinary OH-PAHs and cotinine, the increase in MDA concentration in Beijing did not return to baseline levels after the participants returned to Los Angeles (LA-after; Fig. 2A). To our surprise, the concentration of 8-iso-prostane did not increase in Beijing but instead, it increased after returning to Los Angeles (LA-after) as compared with levels in LA-before or in Beijing ($p < 0.001$; Fig. 2B). We did not observe significant changes in urinary uric acid concentrations in the study period (Fig. 2C). Of note, the changes of 8-isoprostane and uric acid levels in the urine were markedly different from those in the blood. As reported previously, we found that blood levels of 8-isoprostane and uric acid were higher in

Table 1
Descriptive statistics of urinary exposure biomarkers in Beijing and Los Angeles before (LA-before) and after the trip (LA-after).

	geometric mean (IQR)[a]			*p*-value for the difference[b]		
	in LA-before (n = 33)	in Beijing (n = 96)	in LA-after (n = 61)	Beijing vs. LA-before	Beijing vs. LA-after	LA-before vs. LA-after
Creatinine	1455 (1184–2256)	1512 (1130–2301)	1446 (997–2272)	0.46	0.38	0.97
Cotinine	0.14 (0.06–0.24)	1.52 (0.88–2.25)	0.22 (0.09–0.40)	<0.001	<0.001	0.038
ΣOH-NAPs	2.70 (1.74–3.41)	3.62 (2.42–4.98)	3.48 (1.88–5.58)	0.12	0.97	0.13
2-OH-DBF	0.12 (0.08–0.18)	1.11 (0.83–1.60)	0.14 (0.11–0.22)	<0.001	<0.001	0.30
ΣOH-FLUs	0.19 (0.12–0.35)	1.21 (0.88–1.71)	0.28 (0.19–0.34)	<0.001	<0.001	0.007
ΣOH-PHEs	0.38 (0.27–0.61)	1.34 (1.01–1.83)	0.51 (0.38–0.70)	<0.001	<0.001	0.007
1-OH-PYR	0.08 (0.04–0.11)	0.20 (0.14–0.29)	0.12 (0.07–0.17)	<0.001	<0.001	0.002

[a] Unit: μg/L for creatinine; μg/g creatinine for the others.
[b] Tested by mixed-effect model with random intercepts of study participant.

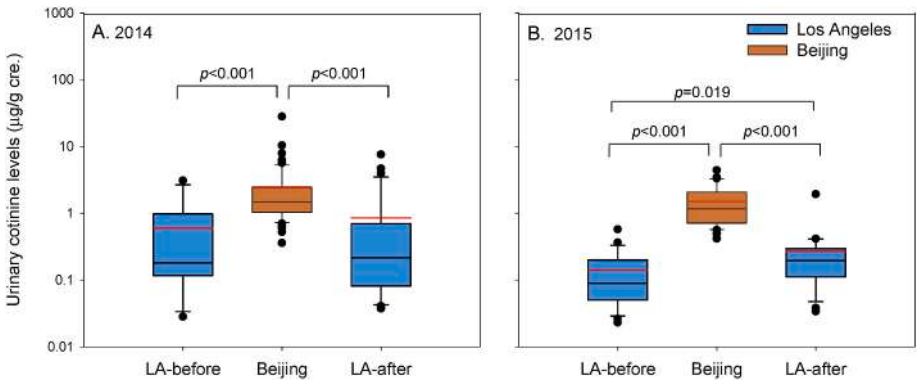

Fig. 1. Urinary cotinine concentrations in LA-before, Beijing, and LA-after in 2014 (panel A) and 2015 (panel B). Blue and orange plots indicate data in Los Angeles and Beijing, respectively; black line - median; red line - mean; box - 25th and75th percentiles; whiskers - 10th and 90th percentiles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Determinants of logarithmic urinary OH-PAHs concentrations in multivariable mixed-effect model with random intercepts of study participant.[a]

Variables	ΣOH-NAP		2-OH-DBF		ΣOH-FLUs		ΣOH-PHE		1-OH-PYR	
	β(SE)	*p*-value	β(SE)	*p*-value	β(SE)	*p*-value	β(SE)	*p*-value	β(SE)	*p*-value
Log_{10} cotinine	0.12 (0.07)	0.061	−0.01 (0.06)	0.91	0.07 (0.05)	0.21	**0.18 (0.05)**	**<0.001**	**0.24 (0.06)**	**<0.001**
Log_{10} cotinine × City (Beijing = 1)	0.03 (0.11)	0.78	0.14 (0.11)	0.21	0.01 (0.09)	0.88	−0.05 (0.09)	0.61	−0.19 (0.11)	0.078
Beijing vs. LA-before	−0.01 (0.10)	0.93	**1.01 (0.10)**	**<0.001**	**0.70 (0.08)**	**<0.001**	**0.45 (0.08)**	**<0.001**	0.09 (0.10)	0.37
LA-after vs. LA-before	0.05 (0.06)	0.38	0.09 (0.06)	0.14	**0.15 (0.05)**	**0.003**	**0.12 (0.05)**	**0.016**	**0.13 (0.06)**	**0.031**
Year (2015 vs. 2014)	−0.11 (0.10)	0.30	0.08 (0.08)	0.29	0.06 (0.07)	0.40	**0.17 (0.06)**	**0.006**	−0.01 (0.09)	0.90
Year × City (Beijing in 2015 = 1)	−0.10 (0.09)	0.25	**−0.30 (0.09)**	**<0.001**	**−0.21 (0.08)**	**0.005**	−0.14 (0.07)	0.051	−0.04 (0.09)	0.61
Time in cooking (h)	0.02 (0.02)	0.30	−0.01 (0.02)	0.73	**−0.03 (0.02)**	**0.034**	−0.00 (0.01)	0.76	−0.02 (0.02)	0.21
Time in barbecuing (h)	**0.16 (0.06)**	**0.006**	0.10 (0.06)	0.080	**0.11 (0.05)**	**0.024**	**0.18 (0.05)**	**<0.001**	**0.27 (0.06)**	**<0.001**
Barbecue intake (kg)	**−0.18 (0.07)**	**0.013**	−0.00 (0.07)	0.98	−0.01 (0.06)	0.88	**−0.13 (0.06)**	**0.026**	**−0.16 (0.07)**	**0.023**
Baked food intake (kg)	−0.03 (0.02)	0.22	−0.01 (0.02)	0.68	−0.01 (0.02)	0.41	−0.03 (0.02)	0.073	−0.01 (0.02)	0.57
Driving time (h)	−0.00 (0.01)	0.95	**−0.03 (0.01)**	**0.003**	−0.00 (0.01)	0.82	−0.01 (0.01)	0.33	−0.00 (0.01)	0.75
Time near heavy traffic (h)	0.01 (0.02)	0.67	−0.00 (0.02)	0.81	−0.02 (0.01)	0.25	−0.00 (0.01)	0.69	−0.01 (0.02)	0.44
Time in public transportation (h)	0.01 (0.01)	0.68	0.02 (0.01)	0.12	**0.04 (0.01)**	**<0.001**	0.01 (0.01)	0.21	**0.03 (0.01)**	**0.006**
Time near smokers (h)	−0.00 (0.02)	0.98	−0.01 (0.02)	0.58	−0.01 (0.01)	0.60	−0.01 (0.01)	0.46	0.00 (0.02)	0.82

[a] β, SE, and p-value were estimated in full models including all variables.

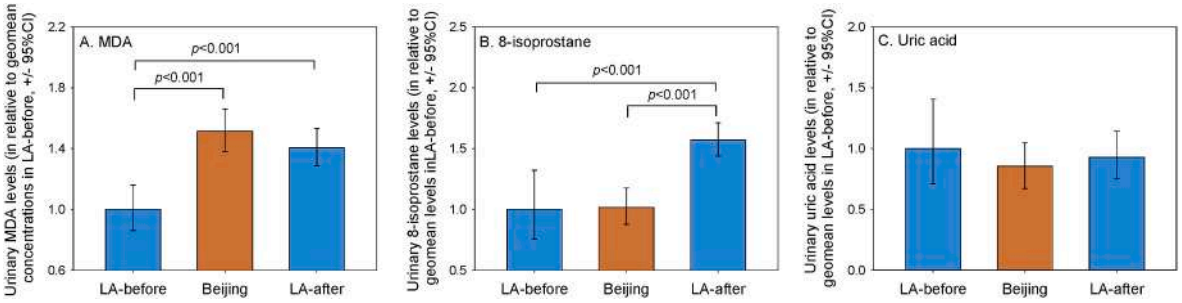

Fig. 2. Urinary concentrations of MDA (panel A), 8-isoprostane (panel B), and uric acid (panel C) in LA-before, Beijing, and LA-after. Blue and orange plots indicate data in Los Angeles and Beijing, respectively. Between-phase differences were tested in mixed-effect models with random intercepts of study participant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Beijing as compared with LA-before or LA-after, while no statistical difference was observed in either biomarker between LA-before and LA-after (Fig. S2) (Lin et al., 2019; Lu et al., 2021).

We found that one-fold increase in urinary cotinine concentrations was associated with 10.1% (95% CI: 5.53–14.8%), 8.75% (95% CI: 2.33–15.6%), and 25.4% (95%CI: 13.1–39.1%) increases in urinary concentrations of MDA, 8-isoprotane, and uric acid, respectively (Fig. 3A–C). Likewise, urinary OH-PAHs concentrations were positively associated with urinary MDA, 8-isoprostane, and uric acid concentrations as well (Fig. S3). Adjusting for OH-PAHs concentrations extenuated but did not annihilate the association of urinary cotinine with MDA, 8-isoprostane, or uric acid concentrations (Fig. 3A–C) and vice versa (Fig. S3). Given the short half-time of cotinine and PAHs in human body (i.e. several hours) (Benowitz, 1996; St.Helen et al., 2012), these associations suggested oxidative effects of short-term exposures to passive smoking and PAHs. We further performed mediation analysis to

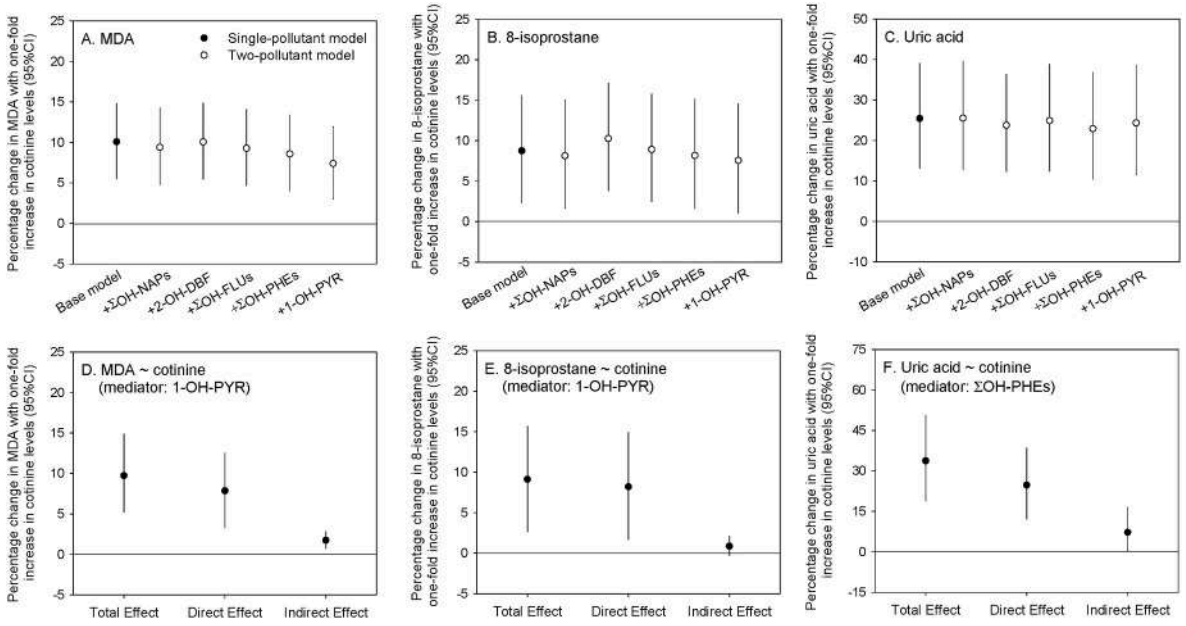

Fig. 3. Changes of urinary MDA (panel A), 8-isoprostane (panel B), and uric acid (panel C) levels in association with one-fold increases in urinary cotinine levels, and the mediating effects of urinary OH-PAH levels (panel D-F). Associations were tested in mixed-effect models with random intercepts of study participant and phase. The mediation analysis was based on mixed-effects models controlled for fixed effects of phases as categorical variables and random intercepts of study participant.

evaluate to what extent the oxidative effects of passive smoking were attributable to PAHs. The results indicated that exposures to PAHs mediated 18.3% (95%CI: 7.28–39.0%), 9.05% (95%CI: 3.53%–41.0%), and 23.3% (95%CI: 1.95–48.0%) of passive smoking's effects on MDA, 8-isoprostane, and uric acid levels, respectively (Fig. 3D–F).

Given the significant decreases in exposures, but not urinary levels of oxidative biomarkers from Beijing to LA-after, we explored whether the elevation of oxidative biomarkers in LA-after as compared to LA-before was associated with the accumulation of exposures in Beijing, indicated by the average concentrations of urinary cotinine and OH-PAHs in Beijing. We found that the difference in average 8-isoprostane concentrations between LA-after and LA-before was significantly associated with the average cotinine concentration in Beijing ($p < 0.05$), but not with OH-PAHs concentrations (Fig. S4). These results suggested that the lasting pro-oxidative effects after participants returned to Los Angeles may be related to exposures to passive smoking in Beijing.

4. Discussion

In this study, we followed 26 healthy non-smoking travelers between Los Angeles and Beijing and have found that traveling to the city with higher smoking prevalence resulted in increased exposure to passive smoking, in association with increases in urinary oxidative biomarkers. These results provide the first evidence in support of potentially adverse health effects of passive smoking among a ubiquitous but underappreciated population – international travelers. Our results also indicate that PAHs are important toxic components mediating the pro-oxidative effects of passive smoking.

Increased oxidative stress has been frequently observed in individuals with cardiovascular diseases (Liguori et al., 2018). Blood and urinary biomarkers of oxidative stress have been shown to be partly predictive of future cardiovascular events and mortality in initially healthy subjects, after adjustment for established cardiovascular risk factors (Daiber et al., 2021). In general, oxidative biomarkers have demonstrated good sensitivity in the detection of early effects induced by environmental factors or exposures to toxic agents before the development of cardiovascular pathology (Daiber et al., 2021; Niemann et al., 2017). Compared with blood, urine samples are easy to collect and have low content of organics and metals, which minimizes the artifactual generation of oxidative products *in-vitro* (Il'yasova et al., 2012). Indeed, we were able to collect multiple urine samples in contrast to only one blood sample from each participant at each phase. We observed increased urinary levels of MDA and cotinine after participants traveled from Los Angeles to Beijing, and increased urinary levels of 8-isoprostane after participants returned to Los Angeles (LA-after), as well as robust associations between passive smoking exposure and urinary levels of MDA, 8-isoprostane, and uric acid, suggesting that increased passive smoking exposure likely contribute to increased oxidative stress among international travelers.

Urinary oxidative biomarkers have been suggested as convenient surrogates of those in the blood as indicators of systemic oxidative stress (Il'yasova et al., 2012); however, we found that changes of oxidative biomarkers in the urine among travelers from Los Angeles to Beijing were different to those in the blood. Thus, while traveling from Los Angeles to Beijing increased the plasma levels of 8-isoprostane by 20.8% and uric acid by 10.5% (Fig. S2) (Lin et al., 2019; Lu et al., 2021), it did not influence the urinary levels of 8-isoprostane or uric acid but only after the individuals returned to Los Angeles in the case of the former. Of note, the increase in blood levels of uric acid in Beijing was accompanied by decreased xanthine levels (Fig. S2) (Lu et al., 2021) and therefore it likely resulted from increased oxidation of xanthine by xanthine oxidase. Uric acid itself is a major free radical scavenger in the blood (Feig et al., 2008) that may counteract non-enzymatic lipid peroxidation through scavenging singlet oxygen, peroxyl and hydroxyl radicals (Sautin and Johnson, 2008), probably impeding an increase in the urinary levels of 8-isoprostane in Beijing (Fig. 2B). When plasma levels of uric acid returned to baseline in LA-after (Fig. S2), there was an increase in the urinary 8-isoprostane levels (Fig. 2B). It is possible that the buffering effects of uric acid were insufficient to prevent an increase in urinary MDA, a more terminal product of lipid peroxidation from both enzymatic and non-enzymatic pathways, in Beijing as well as LA-after (Fig. 2A).

We noted that the increase in 8-isoprostane levels in LA-after was consistent across different years and different time points (Fig. S5). Similar results were also observed by a previous study where nine volunteers traveling from Germany to China for 8–42 days exhibited

elevated urinary 8-isoprostane levels four weeks after returning to Germany (Wu et al., 2017). Importantly, the increase in 8-isoprostane levels from LA-before to LA-after in our study was positively associated with the average cotinine level in Beijing (Fig. S4), suggesting that pro-oxidative effects induced by passive smoking may persist several weeks after the exposure cessation. Increases in urinary 8-isoprostane levels have been reported in subjects with coronary artery disease (Schwedhelm et al., 2004) and in association with increased cardiovascular mortality due to coronary artery disease and strokes (Roest et al., 2008). Furthermore, a meta-analysis including 242 studies unveiled increases in blood and/or urinary 8-isoprostane levels for hypertension, chronic heart failure, coronary artery disease and ischemic stroke compared to control groups (van 't Erve et al., 2017). Altogether, this data raises the possibility that participants in our cohort could have had an increased cardiovascular risk due to passive smoking exposures.

Previous animal studies have also shown that tobacco smoke exposure induces oxidative damages not only in the systemic circulation (Van Der Vaart et al., 2004), but also in multiple tissues including kidney (Cigremis et al., 2004; Van Der Vaart et al., 2004), which is possibly attributed to the accumulation of redox-reactive chemicals. While our results suggested PAHs mediating 9.1–23.3% of the pro-oxidative effects of passive smoking, previous studies have shown short biological half-lives of PAHs (i.e., <24 h) (St.Helen et al., 2012). Indeed, the increase in 8-isoprostane levels from LA-before to LA-after was not associated with average OH-PAHs levels in Beijing (Fig. S4). Therefore, the lasting pro-oxidative effects of passive smoking may relate to other redox active chemicals with longer biological half-lives. For example, cadmium, a tobacco smoke component with pro-oxidative effects, has been shown to accumulate in the kidney with a clearance half-life of 25 years (Bernhoft, 2013). Additionally, the slow translocation of cadmium from circulation to organs (half-life = 75–128 days) (Bernhoft, 2013) may also explain why urinary 8-isoprostane was not increased until participants returned to Los Angeles.

Although this study only focused on the pro-oxidative effects of passive smoking, passive smoking may perturb the cardiovascular system through other mechanisms, given its significant contributions to exposures to PAHs, a group of chemicals that have been shown to promote inflammation and subsequent atherosclerosis in animal models (Curfs et al., 2005); and associate with inflammatory biomarkers and the 10-year atherosclerotic cardiovascular risk in epidemiological studies (Delfino et al., 2010; Hu et al., 2018). Among the study participants, exposures to PAHs have also been found to associate with circulating C-reactive protein levels as previously reported (Lin et al., 2019). It is important to note that passive smoking has been thought to be a less important contributor to PAHs exposure than air pollution and diet, especially among population without known smokers in their families or working places (Yu et al., 2015; Zhang et al., 2014). However, we observed significant elevation in urinary cotinine concentrations in Beijing even among participants who reported no proximity to active smokers (Fig. S1), in strong associations with ΣOH-PHEs and 1-OH-PYR levels ($p < 0.001$; Table 2). These results suggest that passive smoking might have been an ignored contributor to PAHs exposures among the non-smokers in Beijing. Indeed, nicotine has been detected in the air of 44 public places tested in a previous study (e.g., hospitals, secondary schools, and restaurants) with a median concentration of 3.0 μg/m^3 (Stillman et al., 2007). This level is comparable to that of smoking-permitted workplaces in the U.S. and might cause notable passive smoking exposures among visitors to those places (Hammond, 1999).

In this study, participants' urinary cotinine levels were higher in Beijing than in Los Angeles, likely due to a higher prevalence of smoking (30.2%) (Zhang et al., 2016) as compared with Los Angeles (14.3%) (Los Angeles County Department of Public Health, 2010). To explore whether smoking prevalence could predict the change of passive smoking exposures due to international travels, we compared the urinary cotinine concentrations among travelers in Los Angeles and Beijing with other non-smokers from 20 countries (Aquilina et al., 2010; Den Hond et al., 2015; Joo et al., 2017; Mori et al., 2011; Tranfo et al., 2017), and explored their relationship with national smoking prevalence (Table S5) (Ng et al., 2014). Remarkably, we found a positive association between urinary cotinine levels and smoking prevalence ($\rho = 0.49$, $p < 0.05$; Fig. 4), supporting the use of smoking prevalence in departure and destination countries to roughly estimate changes of passive smoking exposures during travels.

Effective June 2015, the Beijing government banned smoking in all the indoor areas of public places, including workplaces and public transportation as well as outdoor areas of schools and hospitals. Our data obtained in Beijing, in the summers of 2014 and 2015 reflect passive smoking exposures before and after the ban policy, respectively. Although we observed a trend towards decreased exposures in Beijing from 2014 to 2015 as evidenced by decreased urinary cotinine concentrations (geometric mean in a unit of μg/g creatinine: 1.68 in 2014 and 1.21 in 2015, respectively), the difference didn't reach statistical significance, and the urinary cotinine concentrations and self-reported time near smokers remained much higher in Beijing than in Los Angeles in 2015. These results suggest that the regulatory policy didn't reduce the passive smoking exposure in this study significantly. However, it is important to note that our study was conducted only 1–3 months after the policy implementation, and future studies are warranted to evaluate the long-term compliance and health benefits of these regulatory policies.

Although the results of exposure biomarkers (i.e., cotinine and OH-PAHs) has clearly indicated that traveling from Los Angeles to Beijing may be accompanied by considerable risk in passive smoking exposure, it is important to note that these findings on oxidative biomarkers are only associative due to our limited capacity to control potential confounders in this natural experiment. Even if we have attempted to control the effects of unmeasured exposure on the associations between passive smoking and oxidative biomarker using a random intercept of phase, this approach cannot control the within-city variations of potential confounders and the between-phase difference in oxidative biomarker remained subject to confounding effects. Particularly, although we were able to partially address the confounding effects of combustion-originated air pollution with urinary 2-OH-DBF and ΣOH-FLUs, we cannot control the pro- and/or anti-oxidative effects of dietary factors and psychological stress (Ghezzi et al., 2018; Romieu et al., 2008), which were previously found to change during the travel as well (Lin et al., 2019).

Strengths and Limitations. In this natural experiment, we performed repeated measurements among the same travelers, allowing each participant to serve as his/her own control. The built-in "crossover" feature (i.e., from LA-before to Beijing vs. from Beijing to LA-after) allowed us to control the effects due to travel itself and the exposures during the flights. Thus, the observed effects were most likely attributable to the exposures in each city. Despite these strengths, our study has several limitations. First, the population size in this study is relatively small (n = 26). However, in our previous study on the same cohort (Lin et al., 2019), we successfully identified significant increases (false discovery rate <5%) in a panel of oxidative biomarkers in the blood after traveling from Los Angeles to Beijing, suggesting that the sample size of our study would provide enough statistical power to detect significant pro-oxidative effects given the drastic difference in exposures between the two cities. Nevertheless, our results have an exploratory nature and future studies with larger sample size and multiple locations are warranted to confirm the findings of this pilot study. Second, changes in urinary levels of cotinine depend not only on the degree of exposure to passive smoking, as determined in here, but also on the activity of inducible cytochromes P450 responsible for nicotine metabolism (Bramer and Kallungal, 2003), which was not assessed in our study. Third, our study only depicts the scenario of traveling from a less-polluted to a more-polluted city. The health effects of passive smoking exposures during a reversed travel remain unclear. Finally, we

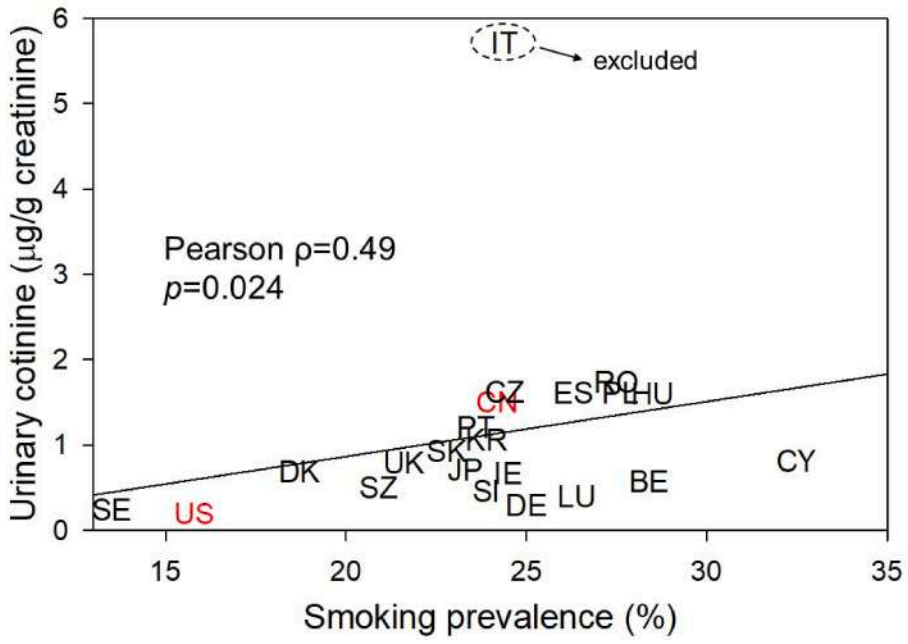

Fig. 4. Correlation between population's geometric mean concentration of cotinine in urines and the corresponding national smoking prevalence in 2012. Detailed information on the population was shown in Table S5. National Smoking prevalence data was obtained from Ng et al. Red symbols indicate data from our study. Abbreviations: US – United States; SE – Sweden; LU – Luxembourg; SI – Slovenia; SZ - Switzerland; BE - Belgium; IE – Ireland; DK – Denmark; JP – Japan; UK – United Kingdom; CY – Cyprus; SK – Slovak Republic; KR – Korea; PT – Portugal; CN – China; HU – Hungary; PL – Poland; ES – Spain; CZ – Czech Republic; RO – Romania; IT – Italy. Data in Italy was considered as an outlier and was removed from the correlation analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

did not measure ambient levels of nicotine in the different microenvironments, hence the location where exposures occurred was unclear. While future studies are warranted to address these limitations, our results are relevant in the design of policies aimed at reducing passive smoking exposures.

5. Conclusions

In this pilot study, we have shown that healthy young adults traveling from Los Angeles to Beijing were inadvertently exposed to elevated levels of tobacco smoke, which led to increased exposure to PAHs in the destination city. Passive smoking exposures were significantly associated with systemic pro-oxidative effects of cardiovascular relevance as indicated by urinary oxidative biomarkers, with 9.1–23.3% of effects mediated by PAHs. Future studies are needed to better understand the reversibility of pro-oxidative effects induced by passive smoking, the impact of regional differences in smoking control on exposures to passive smoking among travelers, and to determine whether these effects influence the risk of developing cardiovascular diseases.

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Declaration of competing interest

None.

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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.2022.114048.

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Serum concentration trends and apparent half-lives of per- and polyfluoroalkyl substances (PFAS) in Australian firefighters

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ABSTRACT

Background: Per- and polyfluoroalkyl substances (PFASs) are persistent manmade compounds used in aqueous film forming foam (AFFF). The extensive use of AFFF has led to widespread environmental PFAS contamination and exposures of firefighters.
Objectives: To determine PFAS blood serum concentration trends and apparent serum half-lives in firefighters after the replacement of AFFF.
Methods: Current and former employees of an Australian corporation providing firefighting services, where AFFF formulations had been used since the 1980s up until 2010, were recruited in 2018–2019 to participate in this study. Special focus was put on re-recruiting participants who had provided blood samples five years prior (2013–2014). Participants were asked to provide a blood sample and fill in a questionnaire. Serum samples were analysed for 40 different PFASs using HP LC-MS/MS.
Results: A total of 799 participants provided blood samples in 2018–2019. Of these, 130 previously provided blood serum in 2013–2014. In 2018–2019, mean (arithmetic) serum concentrations of perfluorooctane sulfonate (PFOS, 27 ng/mL), perfluoroheptane sulfonate (PFHpS, 1.7 ng/mL) and perfluorohexane sulfonate (PFHxS, 14 ng/mL) were higher than the levels in the general Australian population. Serum concentrations were associated with the use of PFOS/PFHxS based AFFF. Participants who commenced service after the replacement of this foam had serum concentrations similar to those in the general population. Mean (arithmetic) individual apparent half-lives were estimated to be 5.0 years (perfluorooctanoic acid (PFOA)), 7.8 years (PFHxS), 7.4 years (PFHpS) and 6.5 years (PFOS).
Conclusion: This study shows how workplace interventions such as replacement of AFFF can benefit employees at risk of occupational exposure.

1. Introduction

Per- and polyfluoroalkyl substances (PFASs) are a group of manmade fluorinated organic compounds widely used in industrial and consumer products such as stain repellents, food packaging materials, waxes, adhesives, and aqueous film forming foams (AFFFs) used to extinguish fuel fires. Due to the widespread use of PFASs, these substances have been detected worldwide in the environment, wildlife, and humans (Ahrens and Bundschuh, 2014; Jian et al., 2017; Paul et al., 2009).

The major PFAS exposure routes for humans are ingestion of contaminated food and drinking water and inhalation of dust and air (Gao et al., 2015; Haug et al., 2011; Nilsson et al., 2013; Trudel et al., 2008; Worley et al., 2017; Zhou et al., 2014). The most studied PFASs are the perfluoroalkyl acids (PFAAs) perfluorooctanoic acid (PFOA), perfluorohexane sulfonate (PFHxS) and perfluorooctane sulfonate (PFOS), which are persistent and bioaccumulative (Jian et al., 2018). Previous biomonitoring of individuals after the end of elevated PFAS exposure showed estimated half-lives of 1.8–3.9 years for PFOA,

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2.9–15.5 years for PFHxS, and 2.9–5.4 years for PFOS (Brede et al., 2010; Li et al., 2018; Olsen et al., 2007; Worley et al., 2017; Xu et al., 2020). Recently, the half-life of lesser studied perfluoroheptane sulfonate (PFHpS) was reported to be 1.5–2.9 years (Li et al., 2019; Xu et al., 2020). The estimated half-lives show variations between study populations, as well as inter-individual variation within study populations, which have not yet been adequately explored. This variation suggests that some individuals may be at greater risk of accumulating high concentrations of PFASs. Epidemiological studies have suggested that PFAA exposure may be associated with several adverse health effects, and populations impacted by elevated PFAS exposure may experience elevated psychosocial stress (Bach et al., 2015; Ballesteros et al., 2017; Calloway et al., 2020; Kirk et al., 2018; Lazarevic et al., 2021; Steenland et al., 2010; Zhao et al., 2020).

Firefighters may be occupationally exposed to PFASs through inhalation of PFASs present in smoke, air and dust during firefighting, and at firefighting stations. However, the main PFAS exposure is thought to be through inhalation of aerosolized foam, contamination of personal protective equipment (PPE), as well as direct or indirect skin contact and hand to mouth transfer during the use of fluoro-surfactant based firefighting foams, such as AFFFs (Peaslee et al., 2020; Seow, 2013; Shaw et al., 2013; Tao et al., 2008). Elevated serum PFAS concentrations, in comparison to a reference population, have been reported in firefighters from different parts of the world (Dobraca et al., 2015; Graber et al., 2021; Khalil et al., 2020; Leary et al., 2020; Rotander et al., 2015; Seow, 2013; Shaw et al., 2013). However, these studies are predominately cross-sectional analyses of PFAS exposure in firefighters. Although Laitinen et al. (2014) assessed PFAS serum concentrations in eight firefighters over a three month period, the short temporal period and the limited number of participants limits any conclusions. Thus, temporal exposure trends of firefighters to PFAS are largely unknown.

AFFF containing PFASs have been used globally since the 1960s to extinguish hydrocarbon-fuel and chemical solvent fires during aviation incidents, such as aircraft crashes and storage tank fires, and during training exercises (Rotander et al., 2015; Taniyasu et al., 2015). Due to concerns over PFASs exposure and the subsequent introduction of restrictions to reduce exposure, a movement to replace AFFF with non-fluorinated firefighting foams was initiated. In Australia, where AFFF has been used since the 1970s, several agencies have replaced fluoro-surfactant based firefighting foams in the past two decades (Personal Communication with Airservices, 2018; NSW, 2021). To determine whether the replacement of firefighting foams successfully reduces PFAS exposure, it is necessary to monitor PFAS serum concentrations in firefighters over time.

In 2013–2014, Rotander et al. (2015) assessed serum PFAS levels in 149 employees of an Australian organisation offering aviation rescue and firefighting services at airports. The organisation used AFFF for firefighting training and emergencies until approximately 2010, when AFFF foams were replaced by fluoro-surfactant free firefighting foam. At the time of serum collection in 2013–2014, mean serum PFOS and PFHxS concentrations among participating firefighters were six to ten times higher than the mean concentrations of the general Australian population (Rotander et al., 2015). PFAS serum concentrations ranged from 3.4 to 391 ng/mL for PFOS, 0.7–277 ng/mL for PFHxS and 0.3–18 ng/mL for PFOA (Rotander et al., 2015).

In the present study, current and former firefighting staff from the same organisation previously investigated by Rotander et al. (2015) in 2013–2014 (referred to as the "2013–2014 Study") were approached to participate in a second exposure study. Participants in the earlier study were specifically targeted for recruitment. The overall aim of the study was to determine if the implemented work health and safety measures, such as replacement of AFFF, had been effective in reducing PFAS exposure given that staff continued to work at PFAS contaminated sites. The objectives were to: 1) assess the PFAS serum concentration in current and former firefighting staff; 2) compare the concentrations of PFASs in the participants in this study to the 2013–2014 study, and estimate individual apparent half-lives; and, 3) assess associations between PFAS concentrations and half-lives with potential explanatory factors explored in the questionnaire.

2. Methods

2.1. Study origin and overview

Airservices Australia (Airservices) is an organisation providing Aviation Rescue and Firefighting (ARFF) services across 27 major airports in Australia. Various types of firefighting foams have been used for firefighting and training purposes across the sites serviced by Airservices (Fig. 1). Lightwater AFFF (3 M), a PFAA based AFFF formulation produced by electrochemical fluorination (ECF), was used since early 1980 up until 2001 when the phasing out of this foam commenced. The cessation was a gradual process across the sites and was finalised before 2003. However, full replacement of this foam was delayed until 2005 at one site. Lightwater AFFF foam was replaced by Ansulite AFFF (ANSUL® 6% AFFF), a telomere-based foam. Ansulite AFFF was replaced by a fluorine-free foam formulation (Solberg RF6) in 2010 (for contractual reasons, the transition to this foam was delayed until 2019 at two sites). Solberg has been used at all sites since (Personal Communication with Airservices 2018; Backe et al., 2013; SOLBERG, 2011; Weiner et al., 2013). In 2018, Airservices commissioned the current study to assess whether the replacement of AFFF had been effective in reducing PFAS exposure among employees.

The study design and methods of the current study is described elsewhere; Nilsson et al., 2022 (study development, design, and logistics), and Nilsson et al. (2021) (laboratory methods and Quality Assurance/Quality Control (QA/QC)). Ethics approval for this study was granted by The University of Queensland Medical Research Ethics Committee (approval number 2018001790). Briefly, all current and former Airservices ARFF employees were invited to participate, including previous participants of the 2013–2014 Study. Participation was voluntary and all participants provided written or oral informed consent. Information on demographics, health history, lifestyle habits and work history were captured by a questionnaire (available in Nilsson et al., 2022). Individual PFAS results as well as study results were reported back to the participants at the end of the study.

2.2. Sample collection and laboratory method

Blood samples were collected between December 2018 and October 2019. Following biochemical analysis (including analysis for total protein (albumin + globulin) which was included in statistical models as a potential confounder (Han et al., 2003)), the serum was transported to a research laboratory for PFAS analysis, as previously described in Nilsson et al. (2021). Quantitative analysis was performed using high-performance liquid chromatography (HPLC, Nexera, Shimadzu Corp., Kyoto Japan), coupled to a tandem mass spectrometer (SCIEX Triple Quad 6500+, Concord, Ontario, Canada) equipped with an electrospray ionisation source and using scheduled multiple reaction monitoring mode (sMRM). The sMRM parameters are listed in Table S1, **Supplementary Material.** Reported concentrations are for linear isomers, with the exception of PFOS where the total concentration of the sum of both linear and branched isomers is measured.

2.3. Quality Assurence/Quality Control

Several QA/QC measurements were included in the PFAS analysis; blanks (ultrapure water and acetonitrile) to monitor for potential contamination, intra- and inter-batch duplicates, and pooled serum replicates to monitor the precision, and standard reference materials (NIST 1957) to monitor the accuracy in and between all batches. Stored serum samples from the 2013–2014 study (n = 120) were reanalysed in the same batches as the serum samples collected in the current study,

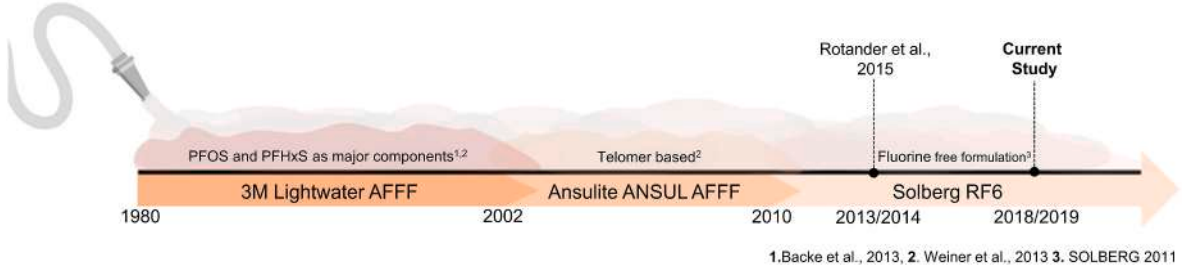

Fig. 1. Approximate timeline of foam usage at the firefighting training facilities investigated in this study.

and a total of 10 randomly selected samples were included in an inter-laboratory comparison with two other labs. Mean coefficients of variation (CV) of all quality control duplicates and replicates were below 11% for all detected PFASs, and the standard reference serum was within acceptable limits of reference values (Z-score $-2<x>2$). The mean CV for reanalysed stored serum samples ranged from 4% to 8% for PFOA, PFHxS and PFOS, and the mean CV of the interlaboratory comparison was 16%. The research laboratory participated in two inter-laboratory comparison studies (AMAP and G-EQUAS), with successful performance (details available in Nilsson et al. (2021)). Mean recovery of mass labelled internal standards, and spiked native standards were 97% and 95% respectively. Method detection limits (MDLs) were determined using EPA guidelines (40 CFR 136 Appendix B), set as 3.14 times the standard deviation of seven spiked replicates (calf serum spiked at 0.5 ng/mL and human pooled serum spiked at 1 ng/mL). The MDLs for all analysed PFASs ranged from 0.04 to 0.1 ng/mL and are listed in Table S2, Supplementary Material.

2.4. Data analysis and half-life calculations

For comparison with the PFAS serum levels in the general population in Australia, data from Toms et al. (2019) was used. Toms et al. (2019) present the most recently reported PFAS data for the general Australian population, which were obtained from pooled serum samples from southeast Queensland in 2016–2017 (Toms et al., 2019). The 95th percentile (p95%) for PFOA, PFNA, PFDA, PFHxS and PFOS was estimated from the pooled serum samples, using the p95%:AM ratios derived from US National Health and Nutrition Examination Survey (NHANES) datasets (described further in Toms et al. (2019)). This data is referred to as 'the general Australian population' from here on. Comparisons were always made with the matching age group (16–30, 31–45, 46–60, >60). Individuals were considered as having elevated concentrations of PFASs if they fell above the p95% concentration of the general Australian population. PFHpS was not reported by Toms et al. (2019). Therefore, the 95th percentile of PFHpS was estimated by using the mean:p95% ratios for PFOA, PFHxS and PFOS data, and applying this to unpublished PFHpS data from the same round of pooled biomonitoring samples used in Toms et al. (2019).

PFAS concentrations below MDL were not included in graphs, but were treated as MDL/$\sqrt{2}$ for the calculation of distribution parameters, in statistical analysis, following standard scientific convention (Harel et al., 2014). Where means are presented, we refer to the arithmetic mean, unless stated otherwise. All statistical analysis were conducted using IBM SPSS (version 25 or later, Chicago, IL) or GraphPad Prism (9.0.0) software. A p-value of <0.05 was considered statistically significant. PFAS concentrations were transformed to the natural logarithm to approximate the normal distribution. General Linear Model, Analysis of Covariance (GLM ANCOVA) was used to assess any associations between PFAS concentrations and exposure and demographic factors, such as age, gender, the number of years worked with the specific foams, years since stopped working (at Airservices) and blood donation. The selection of factors for inclusion in the model was based on their potential of predicting PFAS serum concentrations based on existing literature and the availability of sufficient information in the questionnaire. When determining the association between PFAS concentration and foam history, only employment history at Airservices was assessed as no further information was provided regarding other workplaces. Participants who stated that they had worked in an alternative job (not at Airservices) where there was potential contact with AFFF were excluded from the main analyses. All-inclusive data is shown in the **Supplementary Material** and did not change main findings. Bivariate correlations and variance inflation factors (VIF) were used to check multicollinearity prior to analysis. No independent variables were highly correlated (Pearson's $r < 0.8$, VIF <3) (Kock and Lynn, 2012). Models were adjusted for serum total protein (albumin + globulin) as serum proteins have been found to provide binding sites for PFAAs (Han et al., 2003). Thus, the levels of serum proteins in an individual's blood could potentially affect the degree to which PFAAs are circulating freely, rather than bound. This may have an impact on the bioaccumulation/rate of elimination of PFASs.

Unfortunately, due to the very complex work history of the firefighters, it was not possible to assess role specific factors in the above-mentioned models. The majority of participants had held several roles during their employment, oftentimes at the same time. However, a separate multiple linear regression analysis was conducted assessing the association between PFAS serum concentrations and the number of years working with 3 M Lightwater AFFF in emergency vehicle technicians and firefighters as these two roles had a sufficient number of participants representing each role. These models were adjusted for age.

For the 130 participants with longitudinally collected data, the reduction of PFAS concentration was calculated as a percent change from measurements of the reanalysed samples collected in the 2013–2014 study. Where stored serum samples were not re-analysed ($n = 10$), the measured concentrations from the 2013–2014 study were used. PFHpS was not quantified in the 2013–2014 study, therefore only the 120 participants with re-analysed samples were included for the temporal assessments of PFHpS. Apparent serum half-life was calculated on an individual level to be able to assess inter-individual variation. First order elimination was assumed and the (apparent) serum half-life ($T_{1/2}$) was calculated as:

$$T_{1/2} = \frac{\ln(2)}{\frac{\ln\left(\frac{Serum\ concentration\ in\ 2013-2014}{Serum\ concentration\ in\ 2018-2019}\right)}{Time\ between\ serum\ collections}}$$

Individual apparent half-lives were estimated in all participants with longitudinal data, where there was a decrease in concentration between the two serum collections (participants with a change lower than the analytical uncertainty ($\approx< \pm 10\%$) were not included for the estimation of half-lives). Apparent half-lives were also calculated separately for those with and without elevated serum PFAS concentration in their initial sample, when comparing the initial concentration to the 95th percentile of the general Australian population as described above (Toms et al., 2019).

Multiple linear regression analysis was used to assess any relationships between individual half-life with potential explanatory variables such as age, initial PFAS concentration, blood donation and BMI and the

models were adjusted for ratio change in serum total protein over the time assessed.

To account for the possible influence of background exposure, half-lives were additionally estimated using the same calculations as described above, after subtracting for general background levels. The background levels consisted of the mean concentrations obtained from age specific pools from the general Australian population in 2013–2014, and 2016–2017 respectively ((Eriksson et al., 2017; Toms et al., 2019) + unpublished data). As these background levels are not strictly comparable to the data in the current study (pooled vs. individual values, different years etc.), this half-life calculation is not considered as a main result, and interpretation should be made cautiously.

3. Results

3.1. Demographics of the study population

General characteristics of the study population are presented in Table 1 (Additional lifestyle characteristics are presented in the Supplementary Material (Table S3)). In total, 799 current or former ARFF staff provided blood. Of these, 97.5% were male (779/799), with ages ranging from 21 to 82 years (mean age 52). Of the 799 participants, made up of 544 current and 244 former employees, 783 (783/799) filled in the questionnaire. The participants represented several different positions; officers, firefighters, instructors, and emergency vehicle technicians (EVT). A total of 494 (62%) participants commenced employment prior to 2005, used as a cut-off value to represent the time while Lightwater AFFF was in use. Although Lightwater AFFF was fully replaced at the majority of stations by 2003, the replacement of Lightwater AFFF was delayed at one station. Of the 494 participants who commenced service prior to 2005, approximately 8% confirmed that they worked or attended training at that particular station prior to the replacement of Lightwater AFFF. A total of 140 (18%) participants commenced employment between 2005 and 2010 when Ansulite was in use, and 135 (17%) participants commenced employment after 2010, when all fluorine containing firefighting foam had been replaced by fluorine free foam at the majority of stations. Two stations did not replace Ansulite until 2019. Of the 135 participants who commenced employment after 2010, approximately 21%, confirmed that they had, at some point attended training, or worked at a station where Ansulite was still in use.

3.2. PFAS detection frequency and serum concentrations

Of the 40 PFASs analysed, nine PFASs (PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFBS, PFHxS, PFHpS and PFOS) were detected in more than 15% of the study participants. The detection frequencies and concentrations of these PFASs are presented in Table 2. PFASs with lower detection frequency (<15%) included PFBA, PFPeA, PFHxA, PFDoDA, PFTreDA, PFPeS, PFNS, FOSA, FOSAA and N–Et FOSAA (Table S4, Supplementary Material). PFOA, PFHxS and PFOS were detected in all participants. Median concentration was 1.5 ng/mL (range 0.08–15 ng/mL) for PFOA, 6.5 ng/mL (range 0.08–168 ng/mL) for PFHxS and 14 ng/mL (range 0.14–191 ng/mL) for PFOS. PFNA (range 0.09–2.1 ng/mL), PFDA (range 0.09–3.0 ng/mL) and PFHpS (range <0.08–16 ng/mL) were detected in more than 90% of participants. Strong correlations ($r > 0.93$) were found between PFOS and PFHxS, PFOS and PFHpS as well as PFHpS and PFHxS. Weaker correlations were found between these three compounds and PFOA ($r < 0.33$) (Fig. S1, Supplementary Material).

3.3. Comparison with PFAA concentrations in the general population

Mean serum concentrations of PFHxS, PFHpS and PFOS of the full study population were approximately five to seven times greater (PFHxS: 14 vs 2.1 ng/mL; PFHpS: 1.7 vs. 0.24 ng/mL; PFOS: 27 vs 5.7 ng/mL) compared to the mean concentrations of the general Australian

Table 1
Study population basic demographics[a] and employment information, Airservices Exposure Study, 2018–2019.

		n (%)[b]	
Gender	Male	779 (97.5)	
	Female	20 (2.5)	
Age	Mean	52	
	Min	22	
	Max	82	
BMI[c]	<18.5	0 (0)	
	18-<25	149 (18.6)	
	25-<30	424 (53.1)	
	≥30	205 (25.7)	
	No information	21 (2.6)	
Year Commenced Service According to Foam Usage[d]	Lightwater AFFF (<2005)	494 (61.8)	
	Ansulite AFFF (2005–2010)	140 (17.5)	
	Solberg RF6 (>2010)	135 (16.9)	
	No information	30 (3.8)	
			Did not wear any PPE[e]
Have Held Role As	Officer	332 (41.6)	32%
	Senior Officer	143 (17.9)	36%
	Fire Fighter	744 (93.1)	27%
	Instructor	102 (12.8)	8%
	Emergency Vehicle Technician (EVT)	43 (5.4)	74%
	Other	21 (2.6)	
Other potential AFFF exposure sources	Have held other job (not at Airservices), with potential AFFF contact	173 (21.7)	
	Have lived within 5 km of a defence base	184 (23.0)	
	Have lived within 5km of an airport	286 (35.8)	

Percentage may not add to 100% because of rounding.

[a] ; Lifestyle demographics is presented in Table S3, Supplementary Material.

[b] Of the 799 participants, 783 filled in the questionnaire, although some were incomplete. For the information presented in the current table, 19–30 participants did not provide sufficient information.

[c] BMI was calculated using self-reported height and weight.

[d] ; The cessation of Lightwater use was a gradual process at Airservices, that started in 2001 and finalised before 2003, in all stations but one, where 3 M Lightwater was used up until 2005. Therefore, 2005 is used as a cut-off year in this study, to ensure everyone with potential Lightwater AFFF exposure is captured.

[e] ; Calculated from all participants who provided information about PPE use during their employment. For reference, see the questionnaire question 24; Additional question 2., available in Nilsson et al. (2022). The % of participant that did not use PPE was calculated from the number of participants who answered the question. The mean across all roles within the particular position was calculated. In total, n = 287 (Officer), n = 127 (Senior Officer), n = 679

(Firefighter), n = 81 (Instructor), n = 39 (EVT) provided information about their PPE in at least one of their positions.

population. Over 52% of the study cohort had PFHxS, PFHpS and/or PFOS concentrations above the estimated 95% percentile. Elevated concentrations were especially apparent in the older age-groups (>46 years) (Fig. S2, Supplementary Material). Serum levels of PFHpA, PFOA, PFNA, PFDA and PFUnDA in the firefighting staff were consistent with the most recent data of PFAS levels in the general Australian population (Toms et al., 2019) & unpublished data).

PFAA concentrations were assessed in relation to when each participant commenced employment at Airservices, according to the different foam usage periods; commencement prior to 2005 (use of Lightwater AFFF), between 2005 and 2010 (use of Ansulite) and after 2010 (use of Solberg). Although the cessation of Lightwater was completed by 2003 at the majority of stations, this was a gradual process and the timing differed between stations. To ensure the inclusion of everyone with potential for direct exposure to Lightwater AFFF during employment at Airservices, 2005 was used as a cut-off value in this assessment (based on personal communication, 2018). Study participants who commenced service prior to 2005 showed the highest serum concentrations of PFOS, PFHxS and PFHpS, while participants who started their employment with Airservices between 2005 and 2010 (use of Ansulite) or after 2010 (use of Solberg), showed concentrations similar to the general Australian population. As specified above, two stations delayed the full replacement of Ansulite AFFF. Participants twho confirmed that they had worked, or trained at either of these stations, did not have any observed difference in PFAS serum concentrations compared to those who did not work at these stations during this time (data not shown). Serum concentrations of PFOA were consistent with the general population regardless of the year of commencement (Fig. 2).

3.4. Factors associated with PFAS serum concentrations

Table 3 presents the outcomes of ANCOVA models assessing exposure and demographic factors as potential predictors of PFAA concentration. Positive associations were found between age and PFAA concentration. However, these were only significant for PFOA and PFHpS in participants who commenced employment prior to 2005, and for PFHpS and PFOS in participants who commenced employment after 2005. Although the number of female participants was low (n = 20), lower levels of PFAAs were associated with females (statistically significant for PFHxS, PFHpS and PFOS in participants employed after

Table 2
Detection frequencies (%) and concentrations (ng/mL) of PFASs[a] in the serum of Airservices staff and former members (n = 799). Only PFASs detected in more than 15% of all participants are presented (for age-group specific concentrations see Table S4, Supplementary Material).

		Detection Frequency (%)	Mean[b]	SD	Median	P95	Range
Perfluorocarboxylic acids	**PFHpA**	29%	0.08	0.10	<0.07	0.26	<0.07–2.0
	PFOA	100%	1.7	1.0	1.5	3.4	0.08–15
	PFNA	98%	0.35	0.17	0.32	0.65	<0.09–2.0
	PFDA	92%	0.19	0.18	0.16	0.36	<0.10–3.0
	PFUnDA	30%	0.08	0.05	<0.08	0.18	<0.08–0.54
Perfluorosulfonic acids	**PFBS**	16%	0.05	0.02	<0.05	0.10	<0.05–0.32
	PFHxS	100%	14	17	6.5	46	0.08–168
	PFHpS	91%	1.7	2.0	0.85	5.8	<0.08–16
	PFOS	100%	27	29	14	84	1.14–191

[a] ; Abbreviations are detailed in Table S1, Supplementary Material. Values < MDL were treated as MDL/Sqrt(2) for calculation of distribution parameters.
[b] ; Arithmetic mean.

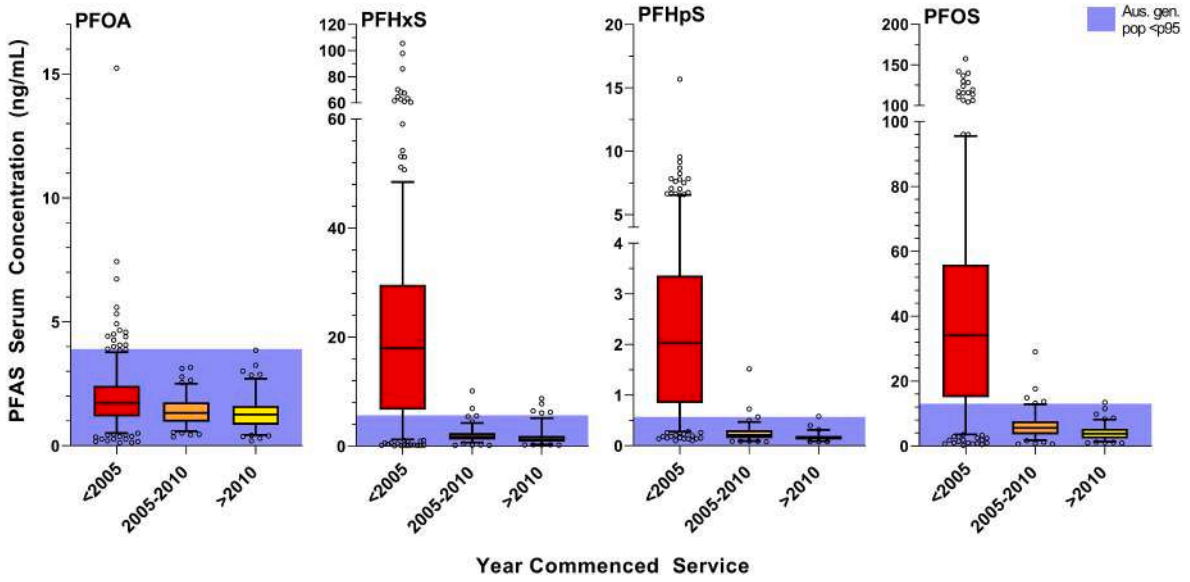

Fig. 2. PFAA concentrations by year of employment commencement at Airservices; commencement prior to 2005 (use of 3 M Lightwater, n = 377), between 2005 and 2010 (use of Ansulite, n = 110) and after 2010 (use of Solberg RF6, n = 110), Airservices exposure study, 2018–2019. Highlighted area presents the concentrations in 95% of in the general Australian population, estimated from pooled serum samples from Southeast Queensland 2016–2017 (Toms et al., 2019) & unpublished data). The graphs exclude participants who stated that they have worked elsewhere where they may have been in contact with AFFF, all-inclusive graphs are presented in the Supplementary Material (Fig. S3).

Table 3
Results from GLM ANCOVA analysis assessing the relationship between measured PFAS concentration and potential predictors; age, gender, years working, years since stopped working and blood donation, in participants with fully completed questionaries[a], Airservices Exposure Study 2018–2019.

		Ln-PFOA				Ln-PFHxS			
		Commenced service				Commenced service			
		<2005		>2005		<2005		>2005	
		B-coefficient (95% CI)	p-value	B-coefficient (95% CI)	p-value	B-coefficient (95% CI)	p-value	B-coefficient (95% CI)	
Age		0.028 (0.017, 0.038)	**0.001**	0.010 (−0.001, 0.021)	0.080	0.039 (0.019, 0.06)	**0.024**	0.015 (0, 0.031)	
Gender[b]		−0.236 (−0.691, 0.220)	0.310	−0.148 (−0.436, 0.141)	0.313	−0.867 (−1.763, 0.03)	0.058	−0.543 (−0.944, −0.141)	
Years of working with 3 M Lightwater		−0.010 (−0.019, −0.002)	**0.021**			0.05 (0.033, 0.067)	**0.000**		
Years of working with Ansulite AFFF/ Solberg				0.010 (−0.011, 0.031)	0.364			0.030 (0, 0.059)	
Years since stopped working		−0.015 (−0.024, −0.007)	**0.000**	0.07 (−0.018, 0.159)	0.119	−0.036 (−0.053, −0.02)	**0.000**	0.074 (−0.049, 0.198)	
No Blood donor		0 (Referent)		0 (Referent)		0 (Referent)		0 (Referent)	
Blood donations/year	<1/yr	0.108 (−0.098, 0.314)	0.302	−0.012 (−0.239, 0.215)	0.917	−0.006 (−0.41, 0.399)	0.978	−0.036 (−0.353, 0.28)	
	1/yr	0.021 (−0.231, 0.273)	0.871	0.061 (−0.199, 0.32)	0.646	−0.142 (−0.638, 0.354)	0.575	0.002 (−0.359, 0.363)	
	2-4/yr	−0.172 (−0.377, 0.034)	0.101	−0.044 (−0.243, 0.156)	0.667	−0.480 (−0.885, −0.076)	**0.020**	−0.346 (−0.624, −0.068)	
	4+/yr	−0.765 (−1.037, −0.492)	**0.000**	−0.794 (−1.048, −0.541)	**0.000**	−1.715 (−2.252, −1.178)	**0.000**	−0.833 (−1.186, −0.48)	
Total Protein[c]		0.014 (0.002, 0.026)	**0.025**	−0.002 (−0.018, 0.014)	0.778	0.026 (0.002, 0.049)	**0.033**	0.009 (−0.013, 0.032)	
Model Adj. R2		0.134		0 .184		0 .411		0.198	

a; n = 436 participants who commenced service prior to 2005, and n = 198 participants who commenced service after 2005, had fully completed questionaries and were included in the models.
b; Gender; Male (0) vs. Female (1).
c; Sensitivity analysis (not shown), excluding total protein from the model did not change the main findings.

2005). Lower PFAS concentrations were associated with increased frequency of blood donations (assessed as number of blood donations per year) across all models.

For participants who started working prior to 2005, the number of years working with Lightwater AFFF, was positively associated with PFHxS, PFHpS and PFOS serum concentrations. Additionally, years since stopped working had a reverse association, i.e., PFAS concentrations decreased after the participants stopped working. No associations with years since stopped working were observed for participants who commenced employment after 2005 and did not work directly with Lightwater AFFF. However, among these participants, the number of years working was associated with PFHpS and PFHxS concentration, but not with PFOS.

Differences in PFAS exposure between two of the main work roles – firefighters and emergency vehicle technicians (EVTs)- were identified. Only participants who had not worked in both roles were included in this assessment. Participants who worked as EVTs (n = 17) prior to 2005, had a higher mean serum concentration of PFHxS (28 vs. 20 ng/mL), PFHpS (3.2 vs. 2.3 ng/mL) and PFOS (48 vs. 38 ng/mL), compared to those who worked as firefighters (n = 344). For both EVTs and firefighters, the PFOS concentration was associated with years of employment (Table S6 and Fig. S4, Supplementary Material). Of the EVTs and firefighters who provided information regarding the use of PPE in the questionnaire (n = 39 EVTs and n = 679 firefighters), 74% and 27% respectively did not wear any PPE for most days when they were in contact with AFFF (Table 1).

3.5. Comparison with aviation rescue and firefighting staff in 2013–2014

In the study population recruited in 2018–2019, mean serum concentrations of PFOA, PFHxS and PFOS were 55–79% lower than the mean concentrations measured in firefighters in 2013–2014 (n = 149) (1.7 vs 4.6 ng/mL (PFOA), 14 vs 33 ng/mL (PFHxS) and 27 vs 74 ng/mL (PFOS)) (Rotander et al., 2015). Compared to the subset of reanalysed samples (n = 120), PFHpS concentrations were 57% lower (1.7 vs 3.9 ng/mL) in the current study population. PFNA, PFDA and PFUnDA were detected in 100%, 99% and 88% of the study population recruited in the 2013–2014 study (Rotander et al., 2015). In the current study population, the detection frequencies were lower; 98% (PFNA), 92% (PFDA) and 30% (PFUnDA), and the mean concentrations were approximately 50% lower for PFNA, 30% lower for PFDA and 10% lower for PFUnDA.

3.6. Individual decrease and apparent half-life estimations

The paired serum samples from participants who took part in both the 2013–2014 and current (2018–2019) study (n = 130 for PFOA, PFHxS and PFOS and n = 120 for PFHpS) were used to assess individual change, and apparent half-lives over this period. All participants showed decreasing levels of PFOA over this time. One participant showed increasing levels (<1%/year) of both PFHpS and PFOS, and two other participants showed increasing levels (2–4%/year) of PFHxS. The mean annual change in serum concentrations from 2013 to 2014 to 2018–2019 was −14% for PFOA, −10% for PFHxS, −11% for PFHpS and −12%/year for PFOS. Individual annual change, in relation to initial PFAS serum concentration is shown in Fig. 3.

Individual apparent half-lives were estimated for all participants with a measurable decrease in PFOA (n = 129), PFHxS (n = 120), PFHpS (n = 117) and PFOS (n = 126) serum concentration (Table 4). For PFOA, mean apparent half-life was 5.0 years (95% CI 4.7, 5.35). Mean apparent half-life for PFHxS was 7.8 years (95% CI 7.3, 8.3), PFHpS apparent half-life was 7.4 (95% CI 6.8–8.0), and PFOS apparent half-life was 6.5 years (95% CI 6.1, 6.9). A large inter-individual variation was observed among participants, with an approximate 3–6 times difference between the 5th and 95th percentiles. Correlation analyses show a relatively strong correlation between PFHxS, PFHpS and PFOS apparent half-lives (Pearsons r: >0.66), while the correlations between these three compounds and PFOA were weaker (Pearsons r: <0.52) (Table S7, Supplementary Material).

Estimation of half-lives, using serum PFAS levels after subtracting general background levels are listed to the right in Table 4. For PFOA, where the levels in the firefighters were not elevated compared to the general population, the half-lives estimated after subtraction of the background exposure were approximately 60% lower (2.0 vs 5.0 years). For PFHxS, PFHpS and PFOS, where the levels in the firefighters were much higher in the firefighters compared to the general population, the half-life estimates after subtraction of background levels were approximately 25% lower for PFHxS (6.0 vs 8.0 years) and PFHpS (5.6 vs 7.4 years), and 10% lower for PFOS (5.7 vs 6.5 years). For all compounds apart from PFOS, the half-life estimates after subtraction of background levels were significantly shorter compared to the (apparent) half-lives without subtraction of background exposure.

Multiple linear regression was used to assess the inter-individual variations of apparent half-lives (Table S9, Supplementary material).

Ln-PFHxS	Ln-PFHxS				Ln-PFOS			
Commenced service	Commenced service				Commenced service			
	<2005		>2005		<2005		>2005	
p-value	B-coefficient (95% CI)	p-value	B-coefficient (95% CI)	p-value	B-coefficient (95% CI)	p-value	B-coefficient (95% CI)	p-value
0.055	0.043 (0.023, 0.063)	**0.000**	0.04 (0.027, 0.054)	**0.000**	0.037 (0.019, 0.054)	0.305	0.029 (0.017, 0.042)	**0.000**
0.**008**	−0.935 (−1.802, −0.068)	**0.035**	−0.747 (−1.103, −0.391)	**0.000**	−0.876 (−1.649, −0.102)	**0.027**	−0.501 (−0.831, −0.172)	**0.003**
	0.047 (0.03, 0.064)	**0.000**			0.037 (0.022, 0.052)	**0.000**		
0.047			0.045 (0.019, 0.071)	**0.001**			0.020 (−0.004, 0.045)	0.293
0.237	−0.044 (−0.06, −0.028)	0.000	0.101 (−0.009, 0.211)	0.070	−0.038 (−0.052, −0.024)	**0.000**		
	0 (Referent)		0 (Referent)		0 (Referent)		0 (Referent)	
0.822	0.016 (−0.375, 0.407)	0.936	−0.027 (−0.308, 0.253)	0.848	0.055 (−0.294, 0.404)	0.757	0.082 (−0.178, 0.341)	0.535
0.992	−0.278 (−0.758, 0.202)	0.255	−0.027 (−0.347, 0.294)	0.870	−0.188 (−0.616, 0.241)	0.389	−0.150 (−0.447, 0.146)	0.319
0.015	−0.561 (−0.952, −0.17)	**0.005**	−0.405 (−0.651, −0.159)	**0.001**	−0.431 (−0.78, −0.082)	**0.016**	−0.319 (−0.546, −0.091)	**0.006**
0.000	−1.447 (−1.966, −0.927)	**0.000**	−0.677 (−0.989, −0.364)	**0.000**	−1.344 (−1.807, −0.881)	**0.000**	−0.702 (−0.992, −0.413)	**0.000**
0.405	0.02 (−0.003, 0.043)	0.087	0.011 (−0.009, 0.031)	0.262	0.021 (0.001, 0.042)	**0.038**	0.006 (−0.012, 0.025)	0.497
	0.416		0 .416		0.381		0.303	

Initial PFOA serum concentration (Ln-transformed) was negatively associated with the length of PFOA apparent half-lives (shorter apparent half-life was associated with higher initial PFOA concentration). This association was not observed for the apparent half-lives of any other PFAA. Blood donation was found to be a significant predictor of PFHxS, PFHpS and PFOS apparent half-life, where blood donors had a shorterhalf-life. BMI or change in BMI was not associated with the apparent half-lives of any compound. Table S8, Supplementary Material additionally present the apparent half-lives where females and participants who are donating blood were excluded and demonstrates a

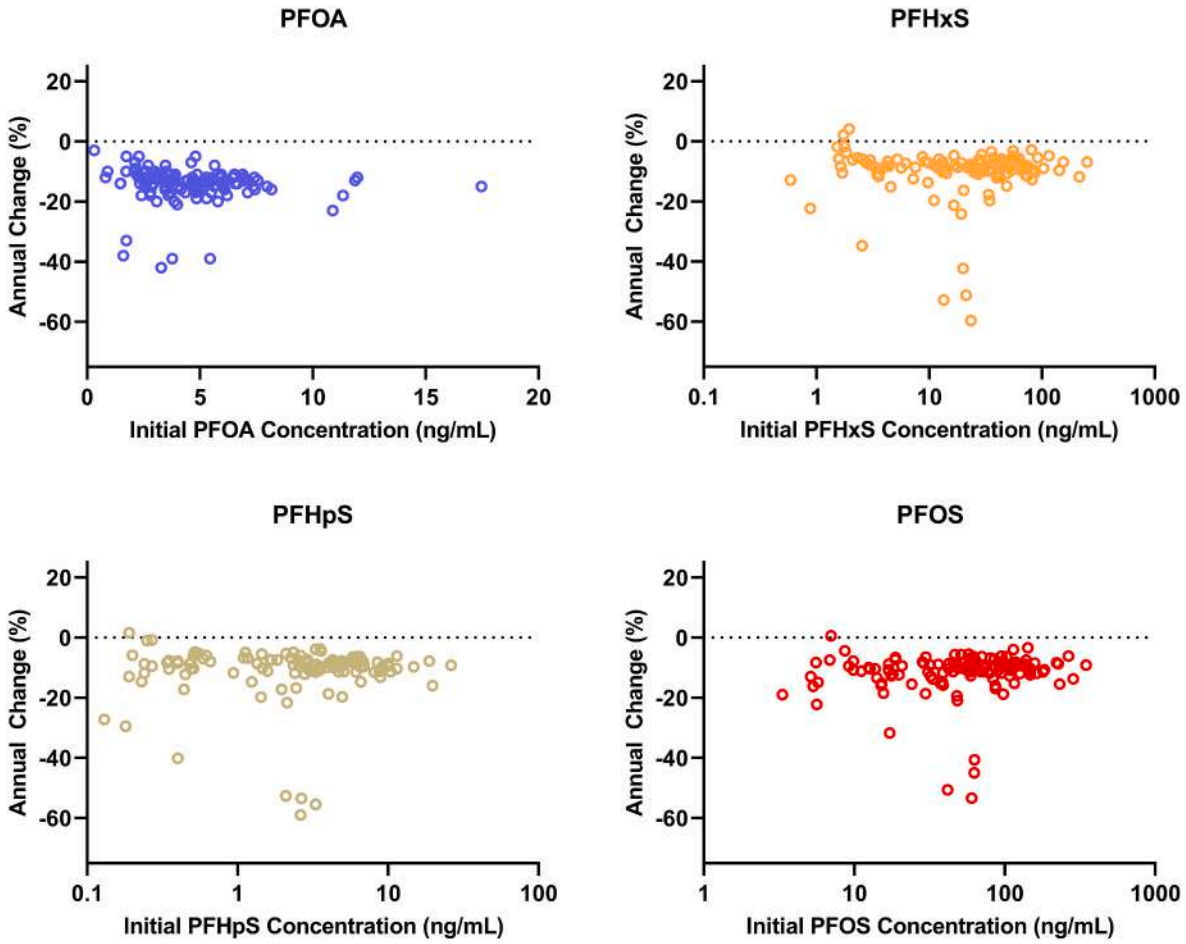

Fig. 3. Percent annual change of PFOA, PFHxS, PFHpS and PFOS serum concentration in relation to initial PFAS serum concentration, in current and former Australian ARFF staff (n = 130 with PFOA, PFHxS and PFOS measurements, n = 120 with PFHpS measurements), Airservices Exposure Study 2018–2019. Annual change between two sample collections, in 2013–2014 and 2018–2019 respectively.

Table 4
Estimated individual half-lives (years) of PFOA, PFHxS, PFHpS and PFOS in Australian firefighters, Airservices Exposure Study, 2018–2019.

		Original serum levels (apparent half-life)				Serum levels after subtraction of background exposure[d]				p-value
T½	Initial Concentration	N[e]	Mean (95%CI)	5th Percentile	95th Percentile	N[e]	Mean (95%CI)	5th Percentile	95th Percentile	
PFOA	All [a]	129	5.0 (4.7, 5.4)	3.0	8.3	118	2.0 (1.7, 2.2)	1.0	4.0	<0.0001
	High[b]	57	4.6 (4.3, 4.9)	3.2	5.8	57	2.0 (1.7, 2.2)	1.0	4.0	<0.0001
	Low[c]	72	5.4 (4.8, 5.9)	2.4	9.3	61	2.0 (1.6, 2.4)	1.1	3.3	<0.0001
PFHxS	All [a]	120	7.8 (7.3, 8.3)	2.7	12.4	111	6.0 (5.5, 6.5)	1.1	11.4	<0.0001
	High[b]	97	7.7 (7.1, 8.3)	2.8	12.4	99	6.7 (6.2, 7.2)	1.6	11.4	0.5468
	Low[c]	23	8.2 (6.7, 9.6)	2.9	12.1	20	2.6 (1.5, 3.6)	1.1	5.9	<0.0001
PFHpS	All [a]	117	7.4 (6.8, 8.0)	2.1	13.1	119	5.6 (5.1, 6.0)	1.8	9.6	<0.0001
	High[b]	101	7.6 (7.0, 8.2)	3.1	13.4	101	5.8 (5.3, 6.3)	1.9	9.5	0.0001
	Low[c]	16	5.9 (4.6, 7.3)	1.8	9.4	17	4.0 (2.7, 5.3)	2.0	9.0	<0.0001
PFOS	All [a]	126	6.5 (6.1, 6.9)	3.0	10.6	123	5.7 (5.2, 6.2)	1.2	10.1	0.8430
	High[b]	115	6.5 (6.1, 6.9)	3.1	10.6	117	5.8 (5.3, 6.3)	1.4	10.1	0.9808
	Low[c]	11	6.7 (4.5, 8.8)	3.0	12.2	6	4.5 (1.4, 7.7)	1.3	10.2	0.9965

[a] ; Participants with longitudinal data, that have had a decrease in concentration between the two serum collections.
[b] ; Participants with initial serum PFAS concentrations greater than the 95th percentile of the General Australian Population in 2016–2017 (Toms et al., 2019).
[c] ; Participants without initial serum PFAS concentrations greater than the 95th percentile of the General Australian Population in 2016–2017 (Toms et al., 2019).
[d] ; Corresponding average background levels were subtracted from measured serum PFAS levels. The background levels used were the age specific mean concentrations obtained from the general Australian population in 2013–2014, and 2016–2017 (Toms et al., 2019 + unpublished data). Age specific mean values were not available for PFHpS in 2013–2014, thus the average for all adults (>16 years) was used for PFHpS when subtracting background levels from the initial sample (Eriksson et al., 2017). If concentrations after subtraction were negative, levels were replaced by MDL/Sqrt(2).
[e] ; Only participants with measurable change in PFAS serum concentrations were included in half-life calculations. These include all participants who had a decrease greater than the analytical uncertainty (±10%). Note that the number of participants changes between the two different calculations.

generally longer half-life when the influence of blood loss is minimised.

4. Discussion

This study provides cross-sectional and longitudinal data of the concentrations of PFASs in current and former ARFF employees in Australia, that have historically used AFFF. This contributes to the understanding of occupational exposure to certain PFASs amongst ARFF employees. Over the last five years, levels of all assessed PFAAs have decreased. However, participants who have used Lightwater AFFF in the past, still have elevated levels of PFHxS, PFHpS and PFOS, compared to the general Australian population, over 15 years after the replacement of this foam.

4.1. PFAA exposure trends in association with work history

PFOS, PFHpS and PFHxS were elevated in those with past occupational exposure to 3 M Lightwater AFFF, when compared to the general Australian population. In this current study, and in the previous 2013–2014 study, there was a strong correlation between these three compounds which suggests that they originate from the same exposure source. The weaker correlation found with PFOA suggests that the exposure source for PFOA is more varied. PFOS, PFHpS and PFHxS are major components in Lightwater AFFF (3M Company, 1999; Backe et al., 2013) which was used by ARFF staff for firefighting and training purposes up until the transition to Ansulite AFFF, which begun in 2001.

As mentioned, the majority of participants worked in different roles during their employment. Therefore, assessments of role-specific exposure were limited to a comparison between those identifying as only working as EVTs or firefighters prior to 2005. It was found that the EVTs had higher PFAS concentrations. EVTs work on maintenance and repair of the fire engines and often came into direct contact with the AFFF concentrate. Further, compared to firefighters, a greater percentage of EVTs stated they wore limited PPE, indicating an increased potential for direct exposure of hands and arms to AFFF concentrate, which may explain the higher PFAS serum levels.

While Ansulite AFFF contains fluorotelomer sulfonic acids (FTSAs) which can potentially degrade to PFOA and shorter chained perfluoro carboxylic acids (PFCAs) ($C \leq 7$), such as PFHpA, PFHxA, PFPeA and PFBA, the PFCAs assessed in this study were consistent with the levels found in the general Australian population (Toms et al., 2019 + unpublished data). This suggests that there was no apparent elevated occupational PFOA exposure. Past exposure to short chained PFASs could not be determined due to their relatively short half-lives, which range from days to months (Xu et al., 2020; Zhang et al., 2013).

Of all 40 PFASs analysed, only 9 PFASs were detected in more than 15% of the participants, where PFOA, PFNA, PFDA, PFHxS, PFHpS and PFOS, were detected in 90% of the participants. Serum levels of PFOA, PFNA and PFDA were consistent with the levels found in the general Australian population, suggesting no apparent elevated occupational exposure to these compounds. Although the lower detection frequency of other PFASs suggest no recent wide-spread occupational exposure, some of these compounds have short elimination half-lives (e.g. PFBS and PFPeS (Xu et al., 2020)) and the elimination half-lives of other compounds (e.g. PFDoDA) are not well studied. The short/unknown elimination half-life of these compounds hinders any conclusions regarding potential occupational exposure to these compounds in the past.

The cross-sectional and longitudinal assessments in this study suggest that elevated occupational exposure to PFOS, PFHpS and PFHxS mainly occurred before 2005 when Lightwater AFFF was in use. Since the replacement of this foam, occupational exposure to these PFASs has decreased. Participants who commenced employment while Lightwater AFFF was still in use had higher PFHxS, PFHpS and PFOS concentrations compared to the rest of the study cohort as well as the general Australian population. Increasing levels of these three PFAAs were further associated with increased number of years working with Lightwater AFFF. On the other hand, PFOA was found to be negatively associated with the number of years working with Lightwater AFFF. We cannot explain this association. Possibly, this association is influenced by unknown confounding factor that was not accounted for in the model, or it may be a chance finding (type II error).

Levels of PFHxS and PFHpS were still positively associated with the length of employment after 2005; the number of years working with Ansulite and/or Solberg. It is likely that those who had been employed for longer also started working shortly after the replacement of 3 M

Lightwater AFFF. These participants may have been exposed to surfaces or dust that were still contaminated at the firefighting stations. The longer apparent half-lives of PFHxS and PFHpS, compared to PFOS, may explain why the association between length of employment after 2005 and higher PFAS concentration was apparent for PFHxS and PFHpS, but not observed for PFOS. The PFAS levels in the participants who commenced service after 2005 and the general Australian population in 2016–2017 (Fig. 2) are consistent, suggesting that the firefighters have not been occupationally exposed to elevated levels of PFASs. However, as discussed further below, the comparison of the current dataset to pooled serum samples from the general Australian population in 2016–2017 may not be strictly comparable. In addition to direct AFFF exposure, which ceased in 2005, a recent study has suggested multiple alternative exposure pathways in firefighters, including the consumption of foods grown at firefighting stations (such as eggs, fruits and vegetables) (Tefera et al., 2022). Assessing such exposure sources was out of the scope of the current study. However, unlike state-based brigades, ARFF stations do not routinely have foods grown onsite for consumption by firefighters (Personal Communication with Airservices, 2022). Nevertheless, compared to the 2013–2014 study cohort, the mean levels of all assessed PFAAs were lower in the current study population, demonstrating a reduction in occupational PFAS exposure over five years. Decreasing trends were also observed on an individual level. Although a few individuals showed an increase in concentration, this increase (<4%/y) is lower than the analytical uncertainty and can be considered as 'no significant change'. Furthermore, participants where non-decreasing PFAS serum measurements were observed had initial PFAS levels consistent with the levels expected in the general Australian population. Thus, their (lack of) change in PFAS serum concentration was likely influenced by background exposure, not occupational exposure.

4.2. PFAA apparent half-lives

The estimates of apparent half-lives of PFHxS and PFOS in this study are in the upper range of previously published estimates in populations after abrupt cessation of exposure (Bartell et al., 2010; Li et al., 2018, 2019; Olsen et al., 2007; Worley et al., 2017; Xu et al., 2020), and the mean apparent half-lives of PFOA (5 years) and PFHpS (7.4 years) were longer than apparent half-lives reported in previous observational studies (PFOA; 1.8–3.9 years (Li et al., 2018; Li et al., 2019; Olsen et al., 2007; Worley et al., 2017; Xu et al., 2020), PFHpS; 1.5–4.7 years (Li et al., 2019; Xu et al., 2020) (reported PFASs half-lives of other studies that are referred to in this manuscript are presented in Table S9, Supplementary Material).

The half-lives estimated in the current study are apparent half-lives, which are influenced by ongoing background exposure. This is evident by the shorter half-lives when subtracting the general background levels (Table 4). The shorter half-lives estimated when subtracting the general Australian population are closer to the previously reported half-lives (Bartell et al., 2010; Li et al., 2018, 2019; Olsen et al., 2007; Worley et al., 2017; Xu et al., 2020). A shorter half-life was particularly evident for PFOA, where the levels in the firefighters were in the range of the PFOA levels expected in the general population. For PFHxS, PFHpS and PFOS (where the contrast in levels between the firefighters and the expected general population levels were comparatively larger), the influence of background exposure was lower. No significant difference in PFOS half-lives was seen between the apparent half-life, and the estimated half-life when subtracting background levels.

When comparing the levels from pooled serum samples obtained in 2010-2011 and 2016–2017 from the general Australian population (adults>16), an approximate 5-year period which is comparable to the current study (Eriksson et al., 2017; Toms et al., 2019), the mean levels of PFOS and PFOA decreased approximately 55%, while the mean levels of PFHpS and PFHxS decreased 27% and 37% respectively. The lower decrease of PFHpS and PFHxS, compared to PFOS, may explain why background exposure levels had a greater influence of the apparent half-lives of PFHpS and PFHxS, compared to PFOS. However, as mentioned, background exposure was assumed from pools from the general Australian population which were not matched to the year of sampling. As levels in the general Australian population have been observed to decrease over time, it is likely that the background exposure is over-estimated in the firefighters' samples from 2018-2019 which were compared to the general population levels obtained from 2016-2017. Further, as discussed above, it is possible that the firefighters, in addition to the assumed background exposure of the general Australian population, may have experienced occupational exposure sources not assessed in the current study, such as contaminated dust at the firefighting stations. Over 20% of participants stated that they had lived in close proximity (5 km) to an airport/defence base. As such, there is a possibility that some firefighters assessed may have experienced a higher PFAS exposure due to their residential location. However, as the majority of firefighters moved regularly, it is unlikely that any potential residential exposure sources were long lasting. Nevertheless, if they experienced elevated exposure, this exposure may not have been substantial enough to increase the PFAS serum concentration but may have contributed to longer apparent half-lives.

The longer apparent half-lives observed in the current study population may further be explained by several other factors. Participant demographics (such as sex and age), the exposure source and the timing of the study (time since exposure, and time between samples) can influence the apparent half-life. A negative association between initial serum concentration and apparent half-life of PFOA was observed, supporting that the apparent half-life is influenced by background PFOA exposure. This was not observed for any other PFAAs (Table S9). The current study commenced several years after the removal of the major exposure sources of PFHxS, PFHpS and PFOS, with initial blood samples obtained approximately 10 years after the commencement of the transition to Ansulite from Lightwater AFFF (2001/2002). The replacement of Lightwater AFFF was a gradual process over 18 months, not an abrupt cessation. Previously acquired stocks of Lightwater AFFF were used up (largely in training) while being concurrently replaced with Ansulite AFFF, a process that differed slightly among stations. Residual contamination of the work environment and equipment may also have existed. The majority of other observational studies conducted the initial serum measurements sooner after an abrupt end of elevated exposure (e.g. 2 weeks (Xu et al., 2020), 6 months (Li et al., 2018)). Additionally, the estimation of apparent half-lives in this study are based on serum samples taken five years apart. A study by Li et al. (2022), assessed the possibility of a change in elimination rate over time since end of exposure, and found that the elimination rates based on the samples collected during the first year of observation after end of exposure were generally faster than the elimination rate based on samples collected later in the study (2–5 years after end of exposure). Further, as discussed in Xu et al. (2020), the length between assessments of individuals serum levels and length of half-lives in previous studies have shown shorter half-lives in studies with a shorter time-window, compared to studies with a longer time-window, especially for PFOA. These findings indicate the possibility of time-dependent elimination rates.

In accordance with the majority of previous observational studies, the half-lives of PFOS were estimated using the 'total PFOS' concentrations (i.e., sum of both linear and branched isomers), in the current study. However, different isomers have been suggested to have different urinary elimination rates, and observational studies where PFOS isomers have been distinguished, have reported isomer specific apparent half-lives (Li et al., 2022; Xu et al., 2020). Direct exposure to Lightwater AFFF-produced by electrochemical fluorination (ECF), a method that yields a mix of 70% linear and 30% branched PFAA isomers, may have led to bioaccumulation of a PFAS isomer profile which is different from that in populations where contaminated drinking water was the main exposure source, consequently explaining some of the differences in apparent half-lives between different populations (3M Company, 1999;

Li et al., 2018; Li et al., 2019; Worley et al., 2017).

The large inter-individual variation of apparent half-lives observed in the current study has also been reported in previous studies (Li et al., 2018; Olsen et al., 2007). Blood donation was associated with both lower levels of serum PFAS concentrations and shorter PFAS half-lives. This study confirmed the finding of lower PFAS concentrations in blood donors compared to those who were not blood donors that was previously reported in the 2013–2014 study (Rotander et al., 2015). Recently, blood donation has also been trialled as a potential treatment for elevated blood levels of PFASs and reductions of serum PFAS concentrations after a one-year clinical trial was reported, further confirming the results found in this study (Gasiorowski et al., 2022; Silver et al., 2021). As discussed by Rotander et al. (2015), this can be explained by the bioaccumulation mechanism of PFAAs. PFAAs have been found to bind to serum protein, such as albumin (Han et al., 2003). As blood is removed through blood donation, bound PFAAs would also be removed, resulting in decreased serum concentrations. Loss of blood through menstruation is also hypothesised to be an elimination route in females, and consistent with previous studies reporting shorter apparent half-lives in females (Li et al., 2018; Olsen et al., 2007; Thompson et al., 2010; Wong et al., 2014).

Gender specific half-lives could not be assessed due to the low number of females ($n = 4$) in the longitudinal portion of the current study. However, lower levels of PFASs in females were found in both this study and in the 2013–2014 study (Rotander et al., 2015). In addition to gender, age and BMI have both been suggested as factors associated with the half-life for several PFASs. More rapid elimination has been reported to be associated with younger ages and lower BMI (Li et al., 2019), however, shorter apparent half-lives were not associated with age and BMI the current study.

The models assessing apparent half-lives in the current study had low r^2 values ($r^2 < 0.5$), which suggests that there are factors that were not assessed, that may explain the variation of half-lives. One example is variances in urinary elimination. Urinary elimination is suggested to be the main elimination route for PFAAs, where organic anion transporters (OATs) and organic anion-transporting polypeptides (OATPs) are responsible for the secretion and resorption in the kidney. These transporters are controlled by hormones; thus, expression levels may vary between individuals (Andersen et al., 2008; Harada et al., 2005; Seithel et al., 2008). Additionally, reduced kidney function may influence the elimination of PFASs. The current study assessed apparent half-lives, which, in addition to physiological changes, are influenced by background exposure. The background exposure may have varied among the study participants, which can explain some of the variation.

4.3. Study limitations and further research

A key strength of this study is the combination of a cross-sectional and longitudinal design. This allowed the assessments of associations between PFAS concentrations and work history and other factors using both cross sectional and longitudinal approaches.

However, there are also some limitations that need to be considered. For example, compared to the cross-sectional assessments, the longitudinal portion of the study had a smaller sample size, which lowered the statistical power of the longitudinal assessments. For apparent half-life estimations, the decrease between only two sample points were assessed. Thus, non-linear decreases in serum concentrations could not be studied and differences in apparent half-lives as a result of a dynamic exposure system could not be assessed. Future follow-up studies, producing more sampling points, would allow investigation of this, as well as increase the statistical power for these estimates.

Additionally, this study relied on a self-reported questionnaire for factors such as work history, blood donation, or BMI. The assessments of the relationship between PFAS concentrations and these factors are dependent on the validity of the self-reports. The majority of participants had very complex work histories, such as working across several different stations and holding different positions, at overlapping times. This made the grouping and analysis complex and constrained the number of participants who could be included at each specific analysis, again limiting the statistical power.

The lack of a directly comparable reference population in the current study is a limitation, making it hard to assess the influence of background PFAS exposure to the firefighters. The most recent data on PFAS concentrations in the general Australian population from 2016-2017 (Toms et al., 2019) was used as the comparator for this study, when assessing the magnitude of elevation of PFAS concentration in firefighters, as well as when subtracting the estimated background exposure levels when estimating half-lives. However, historical data from Australian biomonitoring have shown decreasing levels of several PFASs over time. It is possible that the levels of PFAS within the general population at time of the current study (2018–2019) were lower. If this was the case, the magnitude of elevation of PFAS concentrations reported in this study may have been underestimated, and when estimating the half-lives after the subtraction of background exposure from the PFAS serum concentrations, the subtraction may have been overestimated. On the other hand, the general population estimates are based on both sexes, while the current study population was dominated by men (97.5%), who generally have been found to have higher concentrations of several PFASs compared to women.

The overall rationale of this study was to assess whether the implementation of health and safety measures, such as the replacement of AFFF foam, successfully reduced if not removed the occupational exposure to PFAS. The study provided evidence that occupational exposure to PFAS has been reduced. However, further investigation into the health effects arising from historical exposure is needed.

5. Conclusion

Historical exposure to 3 M LightWater used for aviation firefighting training and operational responses in Australia prior to 2005 was associated with significantly higher serum levels of PFHxS, PFHpS and PFOS in current and former ARFF staff employed during the same time period. The elevated serum concentrations of these PFAAs were observed more than 15 years after the phaseout of 3 M LightWater AFFF. However, the replacement of Lightwater AFFF has reduced and potentially eliminated the elevated exposure. Decreases in concentrations were documented for the subset of study participants who had longitudinal data available. Further, study participants who commenced service after 3 M Lightwater AFFF was fully replaced (after 2005), have similar serum concentrations of PFHxS, PFHpS and PFOS compared to the general Australian population. This study has demonstrated how workplace interventions such as the removal of Lightwater AFFF can benefit employees at risk of occupational exposure.

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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.2022.114040.

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Surveyed from Afar: Household water security, emotional well-being, and the reliability of water supply in the Ethiopian lowlands

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ABSTRACT

Almost half of the world's population is expected to experience water stress in their daily lives by 2050. The impacts of water scarcity on physical and psychosocial health are especially felt in arid regions of Ethiopia where semi-nomadic pastoralist populations are heavily reliant on groundwater for domestic and livestock needs. However, functional water supply infrastructure and reliable service delivery remain a challenge. A cross-sectional water security household survey of 469 heads of households and borehole runtime sensor data in Afar Region, Ethiopia, has three main findings. First, higher levels of household water insecurity experiences (HWISE) and water-related emotional distress (WRED) are positively correlated (0.57, $p<0.01$) and are significantly associated with "limited" water service levels, the non-use of boreholes, and more vulnerable household demographics (female-headed households and lower household incomes). Lower HWISE scores are associated with increased borehole pump usage and reliability, with a cut-off point of 6 h of pump usage per day measured with electronic sensors. Adding additional water points to the dry lowlands of Afar have led to overcrowding and rangeland degradation in the past, highlighting a need to balance increased production for human consumption with livestock use. When it comes to climate resilience and adaptation, ensuring the reliability of what has already been constructed is a top priority for the regional government. Our findings suggest that increasing the reliability and daily usage of existing water supply systems over the short-sighted expansion of sources is worth the investment in services it will take to reach even the most far-flung communities.

1. Introduction

The number of people worldwide experiencing water stress in their daily lives could double by 2050 to half of the global population (Munia et al., 2020). The impacts of water scarcity are especially felt in arid and semi-arid regions like northeastern Ethiopia, where rural populations rely primarily on groundwater for domestic needs, and livestock are a large source of household income. In 2017, only 11% of all Ethiopians had access to safely managed drinking water services, and 30% had access to basic services, while in rural areas, access to safely managed services decreases to 4.6% (UNICEF, 2017).

Afar, one of the 11 regional states in Ethiopia, is the focus of this research because the hot arid climate, lack of year-round surface water, and deep aquifers have made the region a focus of interventions by national and international development institutions - including the drilling of motorized boreholes to supply water to communities. The deep mechanized borehole schemes in the region are designed to reduce the dependence of communities on expensive water trucking operations during drought emergencies, which aligns with the federal government's Climate Resilient WASH initiative of the One WASH National Program (Butterworth et al., 2018).

Ninety percent of the population in Afar practices pastoralism, where communities raise livestock and herd animals over long distances to access water and grazing (Nassef and Belayhun, 2012). Pastoralists are unique water consumers, and water usage patterns in communities vary widely depending on livestock herd compositions, time of year, recent rainfall, and degree of villagization (Degefu et al., 2020). For decades, there has been misalignment between traditional clan-based water and grazing rights management by Afar pastoralists and the water institutions, namely community-based management of drilled boreholes,

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established by aid agency and non-governmental organization development interventions (Nassef and Belayhun, 2012; Alexander et al., 2015; Behailu et al., 2016).

Afar is also a site of protracted ethnic conflict. The conflict has often been viewed as a land dispute along the Afar/Somali Region border (OCHA, 2021). However, the causes of the continuation of the century-long violence can more readily be tied to cultural identity than land rights issues (Alemu, 2017). This self-perpetuating conflict substantially affects water security, as 29,000 people were recently displaced and fighting often forces people to move their settlements to areas with sub-optimal water access (OCHA, 2021).

A review on the relationships between water security and well-being found strong connections for physical and psychosocial health and human-environment interactions (Kangmennaang and Elliott, 2021). In addition, Ethiopian pastoralists face many risks due to climate change, including droughts, loss of human and animal life, damage from winds and floods, and reduced economic productivity (Chinasho et al., 2017). Experiencing water stress, violence, and deprivation create a triple-vulnerability among these already-marginalized populations, lowering their ability to cope with current and future setbacks like the increasing frequency and severity of climate impacts (Ebi and Bowen, 2016; Vins et al., 2015). Therefore, expanding climate-resilient water supply is an appropriate mitigation strategy, balanced with the consideration that over-concentration of water sources will encourage sedentarization and rangeland degradation in pastoralist regions (Cooper et al., 2019; Nassef and Belayhun, 2012).

In this study, high water security is classified when water users experience satisfaction around their ability to access water that is reliable, affordable, adequate, and safe, i.e., free from contamination (Jepson et al., 2017). Water security through quality water management and service delivery is associated with improved well-being and good health, but there is little empirical data on whether interventions to provide water supply have ongoing effects on water security, especially in challenging contexts (Miller et al., 2020).

We designed this study to test whether the installation of motorized boreholes in this context contribute to improved water security and emotional well-being. With an improved understanding of the drivers of household water insecurity and water-related emotional well-being or distress, water supply installations for pastoralist populations may be designed around an improved understanding of water users' needs and wants (Whitley et al., 2019). Instead of the traditional infrastructure-first approach, we advocate for water security as a dynamic state where water users can "engage with and benefit from the sustained hydro-social processes that support water flows, water quality, and water services in support of human capabilities and well-being" after Jepson et al. (2017).

2. Materials and methods

2.1. Study setting

The study site is Mile Woreda in Afar Region, Ethiopia (see Fig. 1). Mile Woreda was estimated to contain 117,960 people in 2017, with an average household size of eight (Ethiopia, 2012). Mile Woreda is located 64 km from the regional capital of Semera and 530 km from Addis Ababa. The Woreda is divided into ten rural kebeles (78% of the population) and two small towns (22% of the population). Communities are semi-mobile and male pastoralists follow seasonal nomadic migration routes for water and pasture for their herds, but often congregate at permanent water sources during the dry season, if they exist (Whitley et al., 2019).

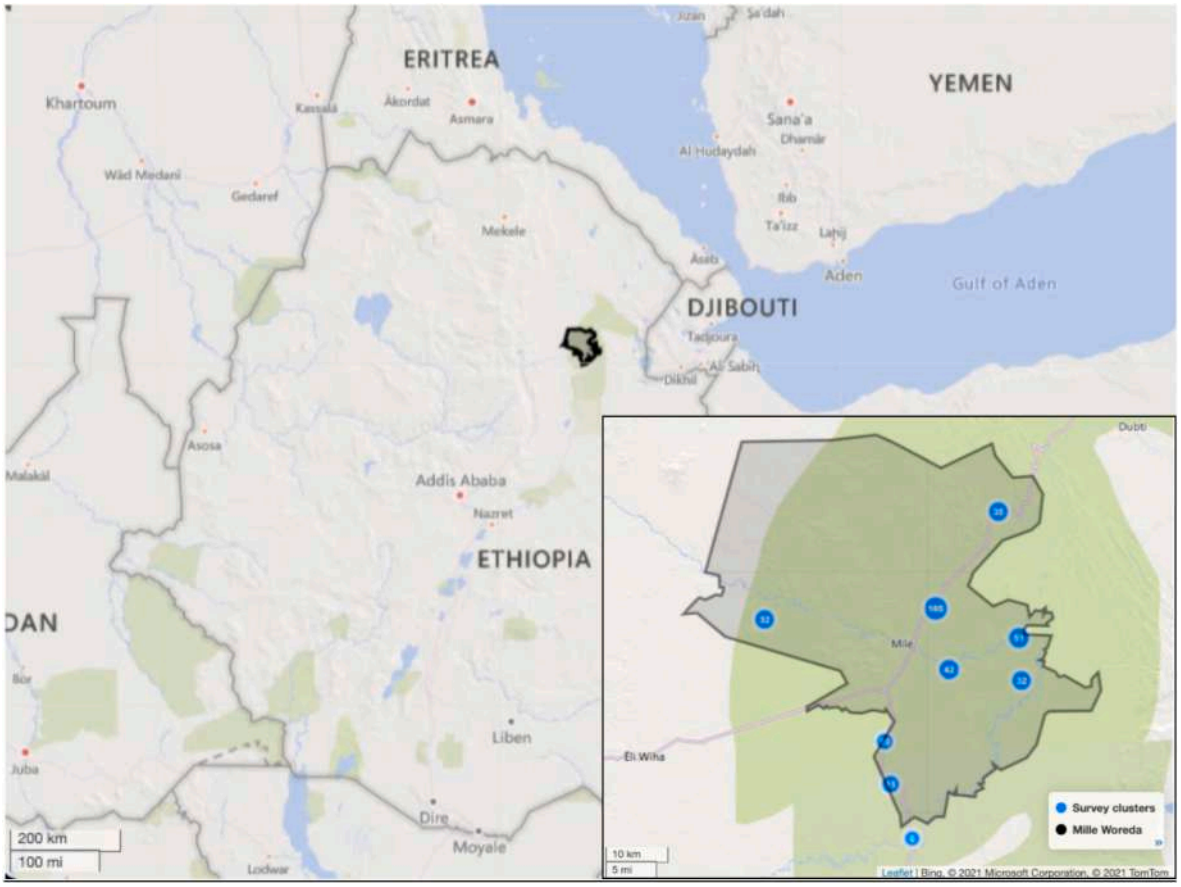

Fig. 1. Map of Ethiopia with inset of Mille Woreda boundary and sampling clusters.

2.2. Survey objective

This research uses a mixed qualitative and quantitative approach toward evaluating water supply interventions designed to improve the effectiveness and long-term sustainability of investments in rural water infrastructure in the resource-constrained context of Mile woreda, Ethiopia. This cross-sectional household survey is designed to characterize the multi-dimensional relationships between measured groundwater borehole functionality and usage, household water insecurity, and emotional distress in rural pastoralist communities.

Where available, a quantitative measurement of community water usage is used to divide households into groups of high and low borehole use. Borehole use is measured remotely as a continuous variable in hours by electronic sensors installed on motorized boreholes in the region since 2017 by the USAID Lowland WASH Activity (Thomas et al., 2021). Five out of ten clusters had a working sensor monitoring near-real-time borehole usage to report breakdowns to the Afar Regional Water Irrigation and Energy Bureau with a true positive rate of 82% and a true negative rate of 48% (Thomas et al., 2021). After correcting for sensor accuracy, the boreholes of Afar Region are measured to be 81% functional on average, and only 50% are turned on and used every day (Butterworth et al., 2021).

This study examines differences in the proportion of households experiencing water insecurity and WRED according to demographic and infrastructure predictors of water service levels. Development interventions assume water security and well-being improvements are associated with high-quality services that guarantee adequate water quantity and reliability, but this has not been proven empirically.

There are three main survey hypotheses examined:

1. We hypothesize a positive correlation between household water insecurity experiences and the respondent's WRED;
2. We hypothesize higher individual scores for water insecurity and WRED are associated with lower measured water household service levels and more vulnerable/less resilient household demographics;
3. We hypothesize that low reported household water insecurity correlates to high sensor-measured use of nearby electrical water pumps (>6 h per day).

2.3. Sample size and randomization

The population of Mile Woreda is an estimated 127,400 considering population growth since the last census, with an average of eight persons per household (Ethiopia, 2012). For a conservative 50% baseline proportion of outcome variables and a desired confidence level of 95%, the minimum sample size needed for this cross-sectional descriptive study is 375 households.

In May 2021, the height of the dry season, a total of 409 households and 469 individuals were sampled, of which 60 were spouses of the head of the household. The woreda is 78% rural and 22% urban, so to avoid over-representing the urban population, we divide the population into urban and rural strata for probability proportional one-stage cluster sampling.

The urban and rural strata of woreda population are futher divided into clusters located near each suitable motorized borehole to identify households using the water schemes of interest. The survey team selected nine rural clusters and one urban cluster for their proximity to one or more working boreholes, ease of access (there was conflict in the Eastern half of the woreda), and community size. Nine of the chosen boreholes are located in rural kebeles, and three are in Mille town (see Fig. 1).

There was no further randomization possible in the rural clusters. This is typically the case when sampling nomadic communities due to the lack of density and distance of male pastoralists from their settlements during the day (Hutchings et al., 2022). For the urban schemes in Mille town, the team consulted with the water utility to check which sections of the town are served by each borehole selected for inclusion as a sampling block. Every other household was sampled from a transect walked across a gridded area until the sample size for the urban cluster was fulfilled.

2.4. Ethics review

This study received approval from the University of Colorado Institutional Review Board (Protocol #20–0693; Boulder, Colorado, United States) and the Afar National Regional State Health Bureau (Ref No. 3011/3777; Afar, Ethiopia). All participants were adults over 18 years of age. Oral informed consent was obtained from all participants before each interview and the option to end the survey was repeated, given the sensitive nature of some questions.

2.5. Measurement methods

The household survey questions were broken into sections on material conditions and demographics, livestock ownership, water service levels, HWISE, and WRED. Respondents were asked about aspects of water security and water point reliability in the current dry season and to recall the previous rainy season in the rural areas only, with respondents providing their own interpretation of what constitutes the rainy/dry seasons, given the increasing unpredictability of rainfall in this drought-prone region. The complete list of survey questions are available in the Supplementary Materials.

2.5.1. Water service level

Household water service levels were evaluated according to standardized Joint Monitoring Programme (JMP) methodology (WHO, 2018). The length of time to collect, the container volumes, and the frequency of water collection was also recorded for the dry and rainy seasons. Households were asked about water storage, treatment, and coping practices with water shortages. Several water quality measurements of E. coli presence/absence using the Aquagenx CBT also informed service level calculations at six rural boreholes, one surface water source, and two town tap stands.

2.5.2. Household water security

The HWISE section includes questions with a four-week recall on experiences that respondents have around their daily tasks, household needs, and water usage. The 12 HWISE questions ask how often respondents experience feeling worry, water supply interruptions, inability to wash clothes, interruptions to plans, challenges with food preparation, hand washing, body washing, feeling thirst, feeling anger, sleeping thirsty, being completely out of water, or feeling shame or stigma due to their water situation. All questions, guidance for enumerators, and suggested avenues of probing are available in the HWISE manual (Network, 2019).

Household water insecurity experiences (HWISE) scale scores are calculated by summing responses to each question. Four response categories are used: never (0 times) is scored as zero; rarely (1–2 times), is scored as one; sometimes (3–10 times), is scored as two; often and always (more than 10 times) are both scored as three. Scores range from 0 to 36, where higher scores indicate greater water insecurity. A score was not generated for the household if a participant responded with "I don't know" or "not applicable" to any item. Households with an HWISE scale score of 12 or higher are considered water insecure. The final HWISE water insecurity score and the cut-off point of 12 are cross-compatible worldwide, as verified in a published protocol on the scale development (Young et al., 2019).

2.5.3. Water-related emotional distress

Water-related emotional distress (WRED) is a proxy for water insecurity due to the relationships between mental health, stress, and other emotions to water insecurity (Cooper et al., 2019). Rather than

observing water use, these questions are designed to capture how people respond to their water situation emotionally in the present day and when they recall the last season (dry or rainy). First, respondents assess their life, water, and security situation satisfaction levels using a Likert scale. Respondents were then asked to pick three emotional words from a list of 12 to describe their feelings about for each of eight questions on aspects of their water situation, including their ability to cope in times of drought, having sufficient quantities of water for domestic needs, and for livestock in the dry and rainy seasons, and the distance traveled to collect water in the dry and rainy seasons. These questions were only asked in the rural clusters because of the lack of livestock ownership or seasonal differences in water supply in the town.

Next, respondents were asked whether they have experienced conflict over their water situation in the last six months and the location of that conflict in the home, village, clan, region, or outside of their region.

The frequency of responses determines the analytical outcome for WRED measurement as a proportion. Here we compare emotional categories (quadrants of the circumplex in Fig. 2) to capture emotional experiences of a person's water situation. Twelve emotion words representing strong or weak (active/passive) and positive or negative emotional states, were chosen from the 19 most frequently used emotion words in focus group discussions and interviews in nearby Dulessa Woreda, Afar Region (Cooper et al., 2019). Previous studies have scored the questions individually (Hutchings et al., 2022), but the contribution we present here is an aggregated score for higher-level monitoring of an intervention. In this study, the responses to each question are scored from one to four based on the emotional circumplex quadrants, summed, and divided by 24. A final score of four is the maximum emotional distress (100% negative active responses), and one is the minimum emotional distress (100% positive active responses). The weighted WRED score = $(4*negative - active + 3*negative - passive + 2*positive - passive + positive - active)/24$.

Where spouses were available and consented to be surveyed, they were also asked the HWISE and WRED questions to gather more responses from women. Additionally, several informal interviews with the Mille town water utility and village water committees were conducted to gather preliminary data to inform sampling design and question suitability.

2.6. Analysis methods

Outcome variables correlation testing: Testing the correlation between the HWISE and the WRED scores demonstrates whether these accurately represent the pastoralist water context in Afar and are valid measures of household-level water insecurity cross-comparable globally. Correlation sample estimates >0.5 and a p-value of <0.01 are considered "moderately correlated" with high statistical significance. Pearson's correlation coefficient was chosen as the best method for calculating the correlation for this sample size.

Regression modeling: Linear regression is used to describe the extent, direction, and strength of the relationship of the predictor variables to HWISE and WRED scores. Predictor variables include household demographics and water service levels. Mixed linear and logistic models with different combinations of fixed and random effects were tested using the lme4 R package to account for the clustered sampling design (Bates et al., 2021). Data were pre-processed to scale/code variables for model convergence. All numerical predictors are center mean-scaled, and categorical variables are either treatment coded when 2-level (gender, urban or rural) or deviation coded when multiple levels (JMP category, life satisfaction, or experience of conflict). Model goodness-of-fit testing and dropping of superfluous variables using likelihood ratio testing was used to pick the best models for HWISE and WRED outcome variables.

Runtime cutoffs for water security: Because of the availability of sensor data for five out of the ten clusters, measured pump runtime is compared to the HWISE and WRED scores of the households located in each of the sensor-equipped borehole clusters whether they reported using borehole water or not. One-sided hypothesis testing was used to test for a difference in HWISE and WRED scores for households associated with low versus high-runtime boreholes with a cutoff point of >6 h of pumping per day on average over the previous 30 days. This cutoff point was pre-selected to match the definition of high groundwater use from Fankhauser et al. (2022) using the same model of sensors reporting borehole usage in Northern Kenya (Fankhauser et al., 2022).

3. Results

3.1. Participant demographics and service levels

Table 1 presents the results for respondent demographics and JMP water service levels as mean values with standard deviations in parentheses or as percentages for categorical variables. The units are per household for all but the last two rows, which incorporate responses from male and female adults.

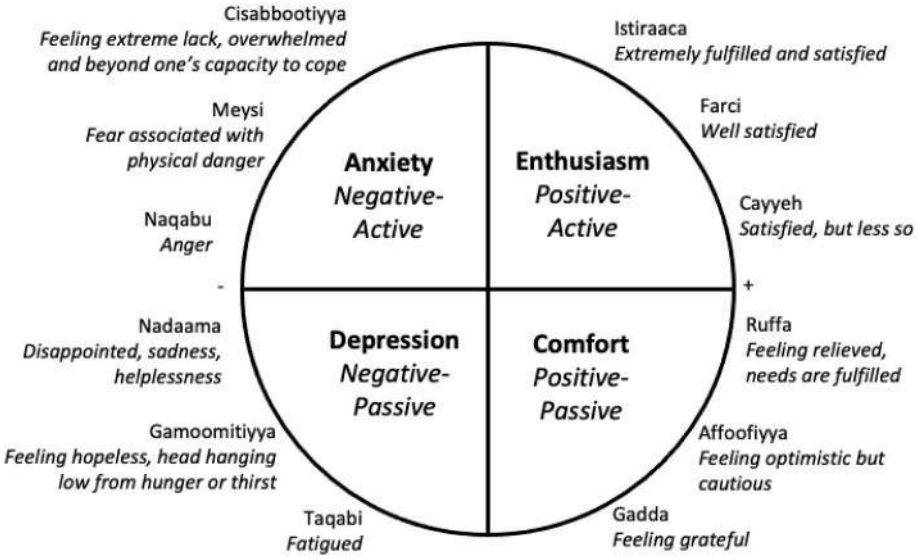

Fig. 2. Emotional quadrants in the Afarigna language are divided by enthusiasm, comfort, depression, and anxiety. Originally published by Cooper et al. (2019). Alternate spellings and expanded definitions are available in the supplementary materials Table A.6.

Table 1
Demographics, water service levels, and life satisfaction. Numerical values are mean and standard deviation. Time to collect water was not collected for the town responses, making us unable to differentiate between limited and basic or safely managed JMP water service levels.

Household	Rural N = 313	Town N = 96
Respondent age	35 (12)	37 (10)
Household size	7 (10)	5 (3)
Household primary income	68.9% Sales of animals & animal products	65.1% Employment wages
	22.6% Employment wages	17.9% Daily labor
	3.9% Daily labor	4.7% Rent income
	3.9% Sales of firewood & charcoal	3.8% Sales of animals & animal products
	0.9% Other	8.5% Other
Est. household spending (ETB/month)	3148 (1718)	4706 (3464)
Camels	2 (5)	0 (0)
Cattle	2 (5)	0 (1)
Goats	19 (17)	2 (3)
Sheep	4 (6)	0 (2)
Water production, liters/capita/day	37 (259)	75 (249)
JMP water service levels	0% unimproved	1.1% unimproved
	37.5% limited	18.2% basic or limited
	43.5% basic	80.7% safely managed or limited
	18.7% safely managed	
Individual	Rural N = 363	Town N = 106
Life satisfaction	77% neutral or satisfied (female)	71% neutral or satisfied (female)
	81% neutral or satisfied (male)	76% neutral or satisfied (male)
Personal experience of conflict or lack of security over water in the last six months	12% in the household	33% in the household
	21% in the village	22% in the town
	9.0% another village	15% another village
	6.0% another clan	14% another clan
	17% another tribe	16% another tribe

A seasonal component was also assessed for the reliability of the primary drinking water source, seen in Fig. 3. Participants rated the water point on reliability and predictability for the dry (current) season, and recalling the last rainy season. Sources used in the dry season were most likely to be rated as reliable year round. When recalling the rainy season, there was a tie between reliable year-round sources and unreliable/unpredicTable sources. Fewer responded that the water point was currently out of service or unreliable but predictable, suggesting an all-or-nothing split between functional and non-functional services in the rural clusters.

3.2. Water security and emotional distress

Table 2 presents the HWISE and WRED scale scores. The HWISE scores range from 0 to 36, where a score $>=12$ is considered water insecure. The mean HWISE score in this survey was 13.7, meaning the average household experienced water insecurity over the previous month. The means are significantly different when grouped by urban/rural ($p<0.01$). The rural mean HWISE score was 11.9, and the town mean HWISE score was 20.0. There are 284 total responses $>=12$; therefore, 61% of all individuals experienced water insecurity.

The aggregated and weighted WRED scores range from 1 to 4. A WRED score $>=1.84$ is in the 50th percentile for emotional distress, corresponding to 150 households or 32% of respondents.

Both score distributions are strongly right-skewed, with many scores

Table 2
HWISE and WRED scale score means, standard deviations, and correlations with confidence intervals. Values in square brackets indicate the 95% confidence interval. ** indicates $p < 0.01$.

Variable	Range, cut-off point	Mean	Median	Standard Deviation	Correlation
HWISE (urban)	0-36, >12	20.0	18	9.14	
HWISE (rural)	0-36, >12	11.9	12	10.39	0.57**
WRED (rural)	1-4, >1.84	1.84	1.42	0.71	[0.49, 0.64]

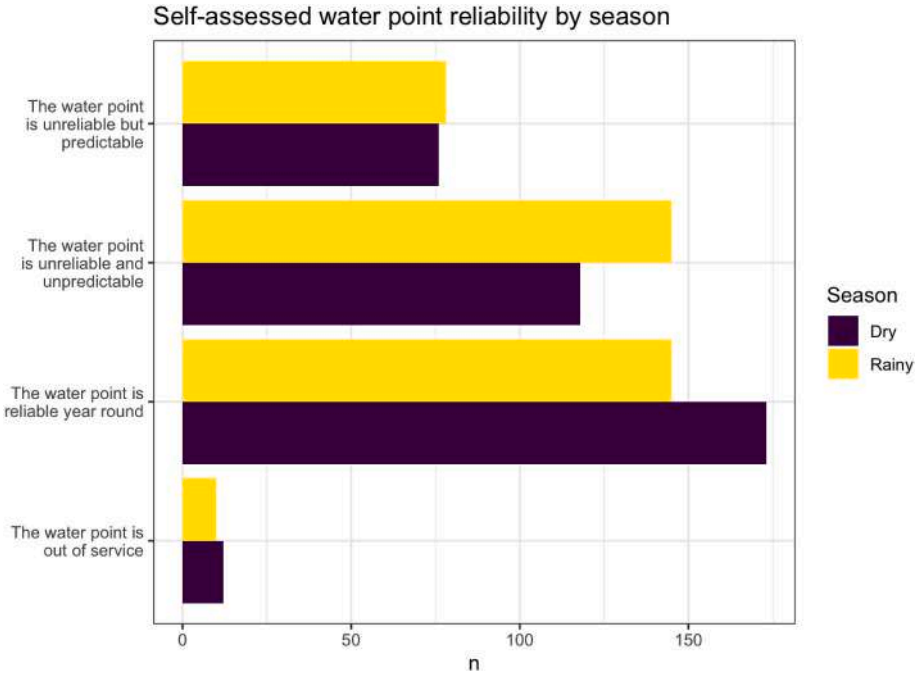

Fig. 3. Categorical assessment of primary water source reliability by season among rural respondents.

of one or zero. Despite the different scale ranges and weights, the HWISE and WRED scores are not independent and are moderately correlated with high statistical significance (0.57, $p<0.01$). We did not find a difference between genders for the HWISE or WRED scores ($p>0.5$).

3.3. Linear regression

The logistic regressions with no random effects are not appropriate as the WRED one did not converge, and the HWISE one has only significant effects from the cluster variable - which does not provide helpful information. The best-fitting models were the linear mixed-effects models with cluster random intercept effects where the outcome variables are treated as continuous variables.

In the HWISE model in Table 3, the JMP water service level, specifically whether the service was classified as limited or safely managed, and the household income source, specifically whether households said their primary source of income was sales of firewood and charcoal, had significant effects.

A limited JMP level, where time to collect took longer than 30 min, was the strongest predictor of a higher HWISE score, which makes sense given the frequency scoring method. Despite the researchers' assumptions that firewood sellers would have lower water insecurity because of access to equines and carts to transport water as well as firewood, in this sample only one owned an equine. This relationship can be better explained by the location of the firewood sellers along main roads at the most crowded waterpoints, earning most of them a limited JMP service level.

In the WRED model in Table 4, which only covered the rural kebele clusters, a limited or safely managed JMP service level, the respondent's gender, and a primary source of household income from sales of animals and animal products were the significant predictors. The technical predictors have effects as expected, where a JMP level of "limited" led to higher emotional distress than baseline (basic), while safely managed services significantly lowered emotional distress.

The WRED score picked up on social predictors of water insecurity better than HWISE, such as how a female-headed household was a strong predictor of higher emotional distress. This can be qualified however, by the lower numbers of females surveyed and the possibility of different openness to expressing emotions among genders. Employment wages, sales of animals, crops, firewood, and charcoal increased emotional distress, while household income from the government safety net was associated with lower WRED scores. Experiencing water-related conflict in the last six months, whether in the household, village, clan or with another clan or tribe/region, was accoiated with higher WRED scores.

Table 3
HWISE linear mixed effects model.

Term	Beta	95% CI	t	df	p
Intercept	11.09	[5.86, 16.31]	4.16	21.92	<0.001
JMP level limited	7.43	[5.57, 9.29]	7.81	430.61	<0.001
JMP level safely managed	−3.51	[-6.07, −0.95]	−2.69	431.27	0.007
JMP level unimproved	−6.53	[-21.87, 8.80]	−0.84	428.22	0.404
Primary source of household income:					
Employment wages	−0.54	[-3.65, 2.56]	−0.34	427.85	0.733
Financial support from government	−1.77	[-17.52, 13.98]	−0.22	429.05	0.826
Financial support from relatives	0.68	[-10.43, 11.79]	0.12	427.65	0.905
Other	7.17	[-8.56, 22.90]	0.89	429.59	0.372
Pension allowance	−1.82	[-12.93, 9.29]	−0.32	427.65	0.748
Rent	−2.07	[-11.29, 7.15]	−0.44	427.6	0.66
Sales of animals & animal products	−0.41	[-3.82, 3.01]	−0.23	434.48	0.815
Sales of crops	6.78	[-4.27, 17.83]	1.2	427.23	0.23
Sales of firewood & charcoal	5.93	[0.79, 11.07]	2.26	429.15	0.024
Sales of handicraft	3.44	[-11.95, 18.82]	0.44	427.29	0.662

Table 4
WRED Linear Mixed Effects Model (rural only).

Term	Beta	95% CI	t	df	p
Intercept	31.64	[21.37, 41.92]	6.04	104.06	<.001
JMP level limited	7.75	[3.94, 11.57]	3.98	340.56	<.001
JMP level safely managed	−12.79	[-19.09, −6.49]	−3.98	271.35	<.001
Gender: Female	−5.59	[-9.07, −2.12]	−3.15	340.42	0.002
Household spending	0	[0.00, 0.00]	−1.65	341.19	0.099
Conflict Yes	3.45	[-0.50, 7.40]	1.71	342.15	0.088
Primary source of household income:					
Employment wages	3.79	[-5.14, 12.71]	0.83	337.35	0.406
Financial support from government	−3.91	[-36.02, 28.20]	−0.24	339.09	0.812
Other	11.35	[-20.82, 43.51]	0.69	340.54	0.49
Sales of animals & animal products	9.09	[0.12, 18.06]	1.99	342.74	0.048
Sales of crops	4.16	[-27.24, 35.56]	0.26	335.35	0.795
Sales of firewood & charcoal	8.23	[-3.67, 20.13]	1.35	338.29	0.176

3.4. Borehole runtime cutoffs for water security

Only one cluster has an average of greater than 6 h a day of pump runtime: rural cluster 5. There is a difference in HWISE ($p<0.01$) and WRED ($p<0.5$) scores between households in this cluster and the four low-runtime rural borehole clusters using a one-sided *t*-test. The difference is significant for HWISE, where the high-runtime group had a mean of 8.7, or not water-insecure, while the low-runtime group had a mean of 12.3, making that group water-insecure on average. The difference between high and low-runtime groups' WRED scores was insignificant, although WRED decreased on average from the low to the high runtime groups. The average daily borehole usage over the past month prior to survey data collection for each cluster with a working sensor is presented below in Table 5 and the cluster contexts described in order of increasing pump runtime.

Rural cluster 1 had a non-functional generator and the system was not providing water, as reflected in the lack of runtime over the prior month. As the site is close to the conflict between the Afar and the Issa and there was no functional WASHCO, minimal attention was given to repairing the system, and it is still non-functional. Accordingly, HWISE and WRED scores were the highest of the rural clusters as respondents indicated that their daily water experiences were highly impacted by the

Table 5
Borehole runtime in clusters with sensors and water security scores (* HWISE and WRED scores are considered water insecure/distressed).

Cluster	Sample Size	Mean Runtime (hours)	HWISE	WRED
Rural 1	36	0.00	21.6*	2.41*
Rural 2	45	0.785	11.0	1.48
Rural 3	40	1.26	13.8*	1.84*
Rural 4	29	3.40	1.03	1.40
Urban 1	29	4.52	21.7*	–
Rural 5	43	7.43	8.77	1.73

broken water system and the security situation. As a response, many households had to make difficult decisions to relocate for better water and pasture while avoiding areas with conflict.

Rural cluster 2 had an infrequently used but functional water system with an average runtime of 47 min a day. Several challenges to reliable operation and service provision were identified by a WASHCO member. Many households access the water via backyard connections, but the leakage rate is very high, and many pipes are corroded due to high salinity. Respondents complained about the taste and worried about the health effects of the poor water quality. A shortage of funds to buy diesel for the generator, and a broken fiberglass storage tank were other reasons for the low use. The HWISE and WRED scores for this cluster were close to but underneath the cut-off points.

Rural cluster 3's borehole was used only 75 min per day over the prior month, but the sensor was offline the day the community was sampled, making the runtime data less reliable. HWISE and WRED scores were both over the thresholds for water insecurity/emotional distress.

Rural cluster 4, located close to Mille River, has a motorized borehole with drinking water taps and a livestock drinking trough. Most users have an on-plot tapstand, although there are many broken taps and water points. The solar-powered pump is often left running until the reservoir overflows and few users contribute to maintenance or pay for water.

Urban cluster 1 serves the upper part of Mille town. Most residents have unpredicTable and unreliable water supply, with many households purchasing water from merchants. None of the ten public tap stands were working and the utility manager reported many household connections are non-functional, do not reach all areas, or have insufficient pressure to deliver water.

Rural cluster 5 is worth expanding on. This water system is solar-powered and leads to a large concrete reservoir, livestock drinking trough, and three fenced tap stand installations open 24 h/day. Due to heavy rainfall three weeks prior to the survey, many households switched to consuming surface water instead of borehole water. Cloudy days also can affect the performance of the solar pump, but the system was in good condition and had no problems with functionality over the prior month. No E. coli was detected on the day of sampling and users are happy with the borehole water quality. The reasons cited for using surface water were the convenience and accessibility as many households are located far from the water points. The water tariff is 20 Birr per month but not all users pay. The community has a functional water and sanitation committee (WASHCO) which has successfully arranged for minor repairs to the distribution line, but a major repair of the solar panels after wind damage required intervention from an NGO.

4. Discussion

The data collection in May 2021 occurred in the dry season when daily temperatures ranged from 37 to 42 °C. But in the prior to three to four weeks there was rain in most of the sampled kebeles and in the neighboring highlands, making surface water more accessible. Due to the nature of a one-time cross-sectional survey, assessing any seasonal effects or the established effects of rainfall on borehole usage due to source-switching in this context was difficult (Thomas et al., 2019). Asking respondents about 6+ month recall periods about the last rainy period also introduced some unreliability.

The way well-being is measured in this study is heavily affected by conflict and a similar population with identical water use patterns might not have the same WRED scores. Conflict was ongoing during sampling to the East towards the Somali border in Adaytu kebele. However, rather than being a drawback of the methodology, we believe the sensitivity to emotional distress caused by conflict is relevant to water in this context.

We expected to see poor performance of the standard 12-question HWISE scale in the context of pastoralist water usage due to source switching and the semi-nomadic context, however, the HWISE score had a significant relationship to borehole runtime while the correlated WRED score did not. Regardless, the WRED score may still be considered a stronger measurement of water stress in this context because the different predictors in the mixed effects models highlight a sensitivity to social factors that HWISE did not pick up. Both scores were likely to increase if a household was classified as a limited JMP service level and decreased if the service level was safely managed.

Another unexpected result was that while urban water customers have access to higher quality services on paper due to the presence of piped water, they actually have higher household water insecurity than those in the rural areas of Mille woreda. This can be explained by the trade-off in paying more for water or spending more time and effort acquiring it. For town residents not reached by the piped system or in cases of network failure, the only option was to purchase water from private merchants who charge steep prices. Meanwhile, pastoralists in Afar respond to changing seasons, grazing conditions, and outbreaks of conflict by moving their herds and settlements.

Conducting a household survey during the global COVID-19 pandemic led to some extra challenges, namely the delays in survey implementation two years after preparatory fieldwork and the lack of sufficient question and translation testing. Some questions were dropped once it became clear that the enumerators could not gather quality data that addressed the intended objective. The lead researcher also could not be physically present in Ethiopia and managed the survey team remotely.

5. Conclusion

Pastoralists in the Afar region of Northern Ethiopia have been poorly served by improved water supplies that operate infrequently and breakdown 1–2 times per year, a problem caused by a general lack of maintenance and management (Libey et al., 2022). Traditional monitoring methods in the water sector frequently label a population covered when there is access to infrastructure, but fail to account for possible long-term effects unique to fragile rangeland ecosystems. Interventions in Afar must consider overcrowding, the preference for surface water when available, and the increasing severity of drought due to climate change.

A methodological contribution is the validation of the HWISE household water security scale against the Afarigna-specific WRED score. We introduce a cut-off point and scoring method for the WRED method to compare to other numerical indicators. The two outcome variables are moderately correlated with high significance (0.57, $p<0.01$), meaning that emotional distress from a household's water situation is related to the frequency of activity interruptions from water deprivation experienced - making both scales good choices to survey population water security and well-being efficiently.

Although the outcome variables are correlated, the different predictors in the mixed effects models highlight a sensitivity to social factors in the WRED method that HWISE did not pick up. Both scores were likely to increase if a household was classified as a limited JMP service level and decreased if the service level was safely managed. Urban water customers, while having access to higher quality services on paper due to the presence of piped water, actually have higher household water insecurity than those in the rural areas of Mille woreda. Town residents earn their income from employment and villagization, making them more reliant on a single source of water, while pastoralists in Afar respond to changing seasons, grazing conditions, and outbreaks of conflict by moving their herds and settlements.

Counter-intuitively, income from livestock was most likely to improve a household's HWISE score but income from livestock lowered WRED scores. Traditional water and grazing land management techniques are highly sensitive to rainfall and water availability (Nassef and Belayhun, 2012). Although higher household wealth in livestock can mean higher water security experiences, the negative relationship to emotional distress means that owners of larger herds worry about

drought and its effect on their livelihoods more than those who have alternate sources of income. Additionally, gender was not a significant predictor of HWISE scores, although the labor of water collection in Afar is highly gendered and other studies have found significant burdens on women (Hutchings et al., 2022).

Although cross-sectional studies of rural water uses and security are numerous, none have integrated borehole pump usage over time to definitively declare what level of water source reliability is necessary before improvements are seen in household water security and water-related emotional well-being. More than 6 h of motorized borehole usage per day for a community of around 400 people is associated with greater household water security ($p<0.01$) and emotional well-being ($p<0.5$) in this study setting.

Adding additional water points to the dry lowlands of Afar have led to overcrowding and rangeland degradation in the past. However, we see that there are substantial improvements in water security and emotional well-being among households with access to safely managed and functioning water supplies. Water security and improved well-being have substantial effects on household health and wealth, especially for pastoralists. Our findings suggest that increasing the reliability and daily usage of existing piped systems over the short-sighted expansion of sources is worth the investment in services it will take to reach even the most far-flung communities.

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Appendix A. Appendix

Table A.6
Afar emotion words and alternate spellings

Spelling Used	Alternate Spelling	Emotion Translation
Istiraaca	Estrahina	Extremely fulfilled and satisfied
Farci	Ferhi	Well satisfied
Cayyeh	Haye	Satisfied, but less so
Ruffa	Rufa	Feeling relieved, needs are fulfilled
Affoofiyya	Alfe	Feeling optimistic but cautious
Gadda	Gada	Feeling grateful
Cisabbootiyya	Hisabona	Feeling extreme lack, overwhelmed, and beyond one's ability to cope
Meysi	Meysi	Fear associated with physical danger
Naqabu	Naqubu	Anger
Taqabi	Tabaqi	Disappointed, sadness, helplessness
Gamoomitiyya	Gemoma	Feeling hopeless, head hanging low from hunger or thirst
Nadaama	Nedama	Fatigued

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The mediating role of the gut microbiome in the association between ambient air pollution and autistic traits

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ABSTRACT

Air pollution has been reported to be an environmental risk factor for autism spectrum disorder. However, the gut microbiome's role as a potential mediator has not been investigated. We aimed to clarify whether particulate matter with an aerodynamic diameter ≤10 μm (PM_{10}) and nitrogen dioxide (NO_2) exposure impact autistic traits through the gut microbiome. Using 170 mother–child pairs, PM_{10} and NO_2 exposure levels during pregnancy (1st, 2nd, and 3rd trimesters) and annual residential PM_{10} levels at age 2, 4, and 6 years were estimated. Autistic traits and gut microbiome were assessed at age 6 years. The associations of PM_{10} or NO_2 exposure, gut microbiome composition, and autistic traits were explored, and mediation analyses of statistically significant findings were also conducted. Exposure to PM_{10} during the 1st trimester of pregnancy was associated with increased autistic traits (10.6% change per interquartile range (IQR) increase, 95% confidence interval [CI]: 1.1, 21.0) and with *Proteobacteria* relative abundance at age 6 years (66.9% change per IQR increase, 95% CI: 21.3, 129.8). First trimester NO_2 exposure was associated with autistic traits (12.1% change, 95% CI: 0.1, 25.5) and *Proteobacteria* relative abundance at age 6 years (48.1% change, 95% CI: −0.1, 119.6). *Proteobacteria* relative abundance was related to autistic traits (4.4% change per 2-fold increase, 95% CI: 1.3, 7.5). Relations between PM_{10} or NO_2 exposure during the 1st trimester and autistic traits at age 6 years were partially mediated by *Proteobacteria* (proportion mediated 23.2%, $p = 0.01$ and 16.7%, $p = 0.06$; respectively). PM_{10} and possibly NO_2 exposure during early pregnancy may affect autistic traits at age 6 years through the alteration of *Proteobacteria* abundance.

1. Introduction

Autism spectrum disorder (ASD) affects one in 44 children in the United States (Maenner et al., 2021), and is marked by deficits in social communication, restricted interests, and repetitive behavior (American Psychiatric Association, 2013). Autistic traits are detectable between 6 and 18 months (Barbaro and Dissanayake, 2009), indicating that critical windows to genetic and environmental factors occur during prenatal and early postnatal periods. Although the high heritability of ASD suggests that genetics is a key factor (Tick et al., 2016), previous studies have estimated that non-heritable factors account for >50% of the neurobiology of ASD (Mayer et al., 2014).

Traffic-related air pollutants such as particulate matter (PM) and nitrogen dioxide (NO_2) have been suggested as environmental risk factors

Abbreviations: ASD, autism spectrum disorder; PM, particulate matter; NO_2, nitrogen dioxide; SCQ, social communication questionnaire; OTU, operational taxonomic unit; DAG, directed acyclic graph; IQR, interquartile range; RDA, redundancy analysis.

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for ASD (Dutheil et al., 2021; Volk et al., 2013). While NO_2 is mainly emitted from automobile exhaust and combustion of fossil fuels (Shang et al., 2020), PM is a mixture of toxic substances with various particle sizes and chemical properties, including sulfates, nitrates, ammonia, black carbon, dust, polycyclic aromatic hydrocarbons, metallic carbon, and volatile organic compounds (Zhang et al., 2021). Both air pollutants show high annual exposure levels in South Korea and are under active regulation by the Korean government. In 2019, the annual mean PM_{10} and NO_2 levels (42 and 52.6 μg/m^3) in Seoul, the capital of South Korea, were higher than in metropolitan cities such as Los Angeles (29 and 43.2 μg/m^3), Tokyo (16 and 26.3 μg/m^3) and London (18 and 32 μg/m^3) (airkorea.or.kr, http://www.epa.gov, http://www.kankyo.metro.tokyo.jp, http://uk-air.defra.gov.uk). Although results on the association between traffic-related air pollution and ASD have been inconsistent, previous research has suggested that PM with an aerodynamic diameter ≤10 μm (PM_{10}) and NO_2 are related to an increased risk of ASD (Flores-Pajot et al., 2016; Chen et al., 2018; Wang et al., 2021). However, research on the mechanism underlying the association between air pollution and autistic traits is scarce.

Many individuals with ASD report comorbid gastrointestinal symptoms—constipation, abdominal pain, diarrhea, gas, and vomiting (Vuong and Hsiao, 2017)—as well as deficient gut epithelium integrity and increased intestinal permeability (Emanuele et al., 2010). The gut microbiota regulates central nervous system activities through various pathways (Liu et al., 2019a), such as regulating the hypothalamic–pituitary–adrenal axis (Sudo, 2012) and producing short-chain fatty acids (SCFA) that affect brain function (Ray, 2017). A previous meta-analysis found dysbiotic microbial compositions in children with ASD (Iglesias-Vázquez et al., 2020); however, a distinct microbial signature for ASD has not been defined yet (Vuong and Hsiao, 2017).

Air pollution exposure can alter the composition of the gut microbiome (Bailey et al., 2020). Mucociliary clearage of inhaled air pollutants in the lung and contaminated food/drinking water are major routes that PM enters the gastrointestinal tract (Salim et al., 2014). PM can either support or inhibit the growth of specific microbes, causing alteration in the composition and function of the gut microbiota (Gao et al., 2017; Korpela et al., 2019; Adams et al., 2015). Moreover, $PM_{2.5}$ and PM_1 exposures showed negative associations with alpha diversity indices and the relative abundance of most *Firmicutes*, *Proteobacteria*, and *Verrucomicrobia* bacteria (Liu et al., 2019b). NO_2 was associated with alternation in the gut microbiome profile in young adults, including increased *Firmicutes* abundance at the phylum level and *Coriobacteriaceae*, *Ruminococcaceae*, and *Adidobacteriaceae* abundance at the family level (Fouladi et al., 2020).

The microbiome is associated with both air pollution and autistic traits; however, this complex relationship has not been investigated yet. Furthermore, it can potentially mediate environmental risk factors in ASD (Vuong and Hsiao, 2017). The microbiota has bi-directional relationships with both genetics and environment; host genetics affect its composition and function, while environmental factors, including age, infections, diet, and xenobiotics, further shape the microbial profile (Falony et al., 2016). Moreover, early-life alterations in the microbiota can have long-term consequences for health and disease (Kumar et al., 2014). This study aimed to examine whether pre- and postnatal PM_{10} and NO_2 exposures impact autistic traits at 6 years of age through the alteration of the gut microbiome among the children in an ongoing birth cohort. It also aimed to explore the association of PM_{10} and NO_2 exposure (1st, 2nd, and 3rd trimesters of pregnancy; ages 2, 4, and 6 years) with autistic traits at age 6 years, the relationship of PM_{10} and NO_2 exposure with the gut microbiome composition at age 6 years, and the association between microbiome profiles and autistic traits. Mediation analyses of statistically significant findings were also conducted to confirm the "air pollutant exposure–gut microbiome–autistic traits" pathway.

2. Methods and materials

2.1. Study design and participants

Data from an ongoing prospective cohort study—the Environment and Development of Children (EDC) study—were used (Kim et al., 2018). From the 726 pregnant women recruited from hospitals in Seoul, the capital city of South Korea, and two nearby regions (Incheon and Kyoung-gi) from August 2008 to July 2010, we collected information on the mothers' socio-demographical characteristics during the second trimester of pregnancy (between 14 and 27 weeks of gestation). We then contacted the mothers and enrolled 425 children of the mothers at age 2 and additionally 301 at age 4 at the Seoul National University Hospital, Seoul, South Korea. Their children were followed up every 2 years; 425, 645, and 574 children at age 2, 4 and 6 years, respectively. At age 6, we started to collect one fecal sample from each child in 2016 and analyzed the gut microbiome of 173 randomly selected children, due to limited budget, out of the 243 children who were not exposed to antibiotics at the time of sample collection. After excluding those with missing air pollution data or autistic trait scores, 170 of the children were included in the prenatal exposure analyses. For postnatal exposure analyses, 132 children with information on air pollution exposure levels at all 3 ages (age 2, 4, and 6 years) were included. The sample size for prenatal exposure analyses and childhood exposure analyses for the *Verrucomicrobia* was 108 and 84, after excluding the participants with zero relative abundance for *Verrucomicrobia*. For comparison, we explored the relation between air pollution and autistic traits in the main cohort after excluding those with missing data (n = 568).

Informed consent was obtained from all guardians. The study protocol was reviewed by the Institutional Review Board of Seoul National University Hospital (IRB No. 1201-010-392) and followed the principles of the Declaration of Helsinki.

2.2. Estimation of PM_{10} and NO_2 exposure levels

Air pollution exposure levels were extracted from air quality monitoring data recorded by 300 air quality monitoring systems of the Ministry of Environment (Seoul, South Korea: https://www.airkorea.or.kr). Air pollutant concentrations, including PM_{10} (in micrograms per cubic meter μg/m^3) and NO_2 (in μg/m^3) were recorded by the hour. The mean was calculated using 75% of the contributing values. Finally, 24-h mean concentrations were calculated for each monitoring site. According to the participants' addresses, individuals were linked to the air pollution levels measured at the nearest monitoring station based on Euclidean distance (between 100 m and 10 km) using ArcGIS (version 10.1; ESRI Inc., Redlands, CA, USA). Levels of PM_{10} and NO_2 exposure during pregnancy (1st, 2nd, and 3rd trimester) and annual residential levels at ages 2, 4, and 6 years were estimated.

2.3. Assessment of autistic traits

We used the parent-rated Social Communication Questionnaire (SCQ) at age 6 years to quantify autistic traits. The SCQ is a 40-item questionnaire that evaluates ASD symptoms like communication abilities, social skills, and repetitive behaviors during the previous 3 months. The first item asks about minimal verbal skills, and the sum of the remaining 39 binary items (1: yes, 0: no) equals the total SCQ score (Snow, 2013). Individuals with higher scores were considered more autistic.

2.4. 16s rRNA sequencing

Fecal material of the participants was collected at age 6 years and frozen at −80 °C until DNA extraction with a DNeasyPowerSoil Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and quantification using Quant-IT PicoGreen (Invitrogen,

Waltham, MA, USA).

The sequencing libraries were processed using the Illumina 16S Metagenomic Sequencing Library protocols to amplify the V3 and V4 regions (Supplemental Methods).

2.5. Operational taxonomic unit (OTU) analysis

We formed the original library and single long reads by assembling paired-end sequences generated sequencing both directions of the library with FLASH (v1.2.11) (Magoč and Salzberg, 2011). (Supplemental Methods). Quality control data was presented in Table S1.

The alpha diversity of the microbiome (Chao, Shannon, and Inverted Simpson index) was calculated to evaluate species diversity and evenness using the "phyloseq" package of R version 4.0.2 (The Comprehensive R Archive Network, Vienna, Austria; http://cran-r-project.org). We excluded rare phyla, which were not commonly detected in the children, such as Cyanobacteria, Fusobacteria, Syndergistetes, and Tenericutes (detection rates were 2.3%, 13.7%, 0.6%, and 4.0%, respectively). In the main anlaysis, we examined four phyla that were detected in all participants (n = 170): *Actinobacteria*, *Bacteriodetes*, *Proteobacteria*, and *Firmicutes*. In addition, we examined Verrucomicrobia that was detected among 108 and 84 children in the prenatal exposure and postnatal exposure analyses, respectively. The $\log_2$-transformed values of the relative abundances were used to normalize the phylum distribution (Fig. S1).

2.6. Definition of covariates

The list of potential covariates was created after review of previous literature (Kim et al., 2021; Yi et al., 2021). The potential covariates were maternal age at pregnancy (years), maternal education ($<$ or $\geq$ college education), family income status (monthly family income $<$ or $\geq$ $3500), diabetes mellitus (DM) during pregnancy (yes or no), pre-pregnancy body mass index (BMI, kg/m^2), child's age (in months), sex, multiple gestation birth (singleton or twin/triplet), child's BMI, birth order ($<$ or $\geq$ 2nd), delivery mode (vaginal delivery or cesarean section), prematurity ($<$ or $\geq$ 37 weeks), low birth weight ($<$ or $\geq$ 2.5 kg), breastfeeding status (exclusive breastfeeding, mixed feeding, or exclusive formula feeding), and season of birth (spring, summer, autumn, or winter). Different covariates were selected for the prenatal and postnatal air pollution analyses. We excluded potential mediators for the prenatal analyses, which were DM during pregnancy, prematurity, and low birth weight. Some potential covariates, including breastfeeding, delivery mode, child's BMI, and season of birth, could not have confounded prenatal air pollutant exposure levels and were only addressed in postnatal analyses. Based on exploratory analyses, potential covariates that were related to SCQ scores, air pollutant levels, or relative abundances of phyla were found (Table S2, S3, S4, and S5). The following final covariates were selected based on the definition of confounders: variables associated with both exposure and outcome, but are not in the causal pathway between exposure and outcome (Hernán et al., 2002). We depicted the relationships between variables by building a data-driven directed acyclic graph (DAG; http://www.dagitty.net/) based on the statistical associations between the involved variables and potential covariates (Fig. S2): age, sex, multiple gestation births, and family income for the prenatal models and age, sex, multiple gestation births, family income, the season of birth, low birth weight, delivery mode, and breastfeeding for the postnatal models.

2.7. Statistical analysis

Pearson correlation coefficients for the correlations between PM_{10} and NO_2 levels during pregnancy and childhood were calculated. The differences in PM_{10} and NO_2 over time were investigated using intraclass correlation coefficients (ICCs; two-way mixed models, single rater, absolute agreement option: ICC(3,1)).

We compared the characteristics of the main (n = 568) and subset cohorts (n = 170) using independent t-tests (for continuous variables) or chi-square tests (for categorical variables).

Due to the right-skewness of SCQ scores at age 6 years (Fig. S3), we implemented Poisson regression for subsequent analyses. The associations between potential covariates and SCQ scores were explored with univariate Poisson regression models. The association between covariates and air pollutant exposure levels and between covariates and phyla relative abundance were examined in linear regression models.

2.8. Association between air pollution and SCQ scores

The association between PM_{10} or NO_2 exposure in each exposure period and SCQ scores at age 6 years was examined using multivariable Poisson regression models in the main (n = 568) and subset (n = 170 for prenatal, n = 132 for postnatal) cohorts. The statistically significant associations were visualized using smoothing splines. As we assumed a Poisson distribution of SCQ scores using a log-link function, the risk of higher SCQ scores associated with an interquartile range (IQR) increase in air pollution level was expressed as a percent change (%) using the following formula: (e^(β*IQR)-1)*100%, where β was an estimate from the Poisson regression model, and IQR is interquartile range of air pollution.

2.9. Associations between air pollution and microbiome profile

The association of air pollutants with alpha diversity indices was explored using multivariable linear regression. Levels of PM_{10} and NO_2 were included as explanatory variables in the redundancy analysis (RDA) computed squared-root-transformed unweighted UniFrac distances, conducted by the "vegan" package of R. We estimated the significance of variation in the microbiome data explained by explanatory variables by the Monte Carlo permutation test (1000 permutations). We used partial RDA models to determine the amount of variation in microbiome community composition explained solely by PM_{10} or NO_2 exposure after controlling for covariates. The associations between exposure to air pollutants and the relative abundance of the four phyla were also tested using multiple linear regression models. As the relative abundance was $\log_2$-transformed, the risk of higher relative abundance associated with an IQR increase in air pollution level was expressed as a percent change (%) using the following formula: (2^(β*IQR)-1)*100%, where β was an estimate from the regression model, and IQR is interquartile range of air pollution.

2.10. Association between microbiome profile and SCQ scores

The associations of the alpha diversity indices/relative abundance of the phyla found statistically significant in the aforementioned analyses and SCQ scores were examined using multivariable Poisson regression.

2.11. Mediation analyses

The "air pollution–microbiome–autistic traits" pathway was tested in cases where all three pairs of associations among air pollution exposure, microbiome, and SCQ scores were statistically significant. We tested the indirect association between air pollution and autistic traits through changes in the microbiome composition by using nonparametric estimation model-based mediation analyses. A predetermined pathway was established, in which air pollution influences a mediator (gut microbiome), which then affects autistic traits. No unmeasured confounding or effect modification was anticipated among the included components. The proportion mediation represents the average amount of indirect association between air pollution and autistic traits via changes in the microbiome composition relative to the average total association.

For comparison, mediation models were constructed for various time windows of exposure (1st, 2nd, and 3rd trimester of pregnancy, age at 2,

4, 6 years) to air pollutants (PM_{10} and NO_2) and microbiota at phyla levels (*Actinobacteria*, *Bacteriodetes*, *Proteobacteria*, *Firmicutes*, and *Verrucomicrobia*). As the microbiome profile and SCQ scores were measured cross-sectionally, the different direction of mediation effects (i.e., air pollution–autistic traits–gut microbiome) was examined. The "mediation" package in R was used to precise p-values of the estimates of the total, direct, and median effects using nonparametric bootstrapping with 20,000 simulations (Tingley et al., 2014).

All statistical analyses were performed using IBM SPSS Statistics for Windows version 22 (IBM Corp., Armonk, N.Y., USA) and R version 4.0.2. Statistical significance was defined as $p < 0.05$ (two-tailed).

3. Results

3.1. General characteristics of the participants

The characteristics of the participants in the main (n = 568) and subset (n = 170) cohorts were similar (Table 1). In the subset cohort, the mean maternal age at pregnancy was 31.4 ± 3.5 years. The majority of mothers were college graduates (85.9%) and most children came from higher-income families (71.8%). Regarding the children's characteristics, 51.2% were boys, 32.4% were born by cesarean section, and 5.3% were born with a low birth weight or prematurely.

Table 1
Characteristics of the participants in the main and subset cohort at age 6.

Characteristics	Variables	Main cohort (n = 568)	Subset cohort (n = 170)	P-value
Maternal	Maternal age at pregnancy, years, mean (SD)	31.4 (3.6)	31.4 (3.5)	0.99
	Maternal Education, N (%)			0.48
	< College education	92 (16.2)	24 (14.1)	
	≥ College graduate	476 (83.8)	146 (85.9)	
	Monthly household income, N (%)			0.63
	< $3500, N (%)	173 (30.5)	48 (28.2)	
	≥$3500, N (%)	395 (69.5)	122 (71.8)	
	Smoking during pregnancy, N (%)			
	Non-smoker	554 (100)	151 (100)	
	Smoker	0 (0)	0 (0)	
	DM during pregnancy, yes, N (%)	22 (3.9)	9 (5.3)	0.42
	Prepregnancy BMI, mean (SD)	20.9 (2.7)	20.8 (2.3)	0.63
Child	Sex, boys, N (%)	296 (52.1)	87 (51.2)	0.86
	BMI, kg/m^2, mean (SD)	15.8 (1.8)	15.6 (1.7)	0.19
	Season of birth, N (%)			**0.03**
	Spring	146 (25.7)	33 (19.4)	
	Summer	175 (30.8)	51 (30.0)	
	Autumn	163 (28.7)	45 (26.5)	
	Winter	84 (14.8)	41 (24.1)	
	Delivery mode, N (%)			0.36
	Vaginal delivery	362 (63.7)	115 (67.6)	
	Cesarean section	206 (36.3)	55 (32.4)	
	Low birth weight, yes, N (%)	40 (7.0)	9 (5.3)	0.42
	Prematurity, yes, N (%)	44 (7.7)	9 (5.3)	0.28
	Breastfeeding			0.37
	Exclusive breastfeeding	172 (30.3)	58 (34.1)	
	Mixed feeding	374 (65.8)	109 (64.1)	
	Formula feeding	20 (3.5)	3 (1.8)	
	Twin, yes, N (%)	50 (8.8)	15 (8.8)	0.99
	Birth order, first child, N (%)	325 (57.2)	94 (55.3)	0.66
	SCQ score, age 6, mean (SD)	3.6 (2.7)	3.4 (2.9)	0.42

Abbreviations: SD, standard deviation; DM, diabetes mellitus; BMI, body mass index; SCQ, social communication questionnaire.
P-value for difference of characteristics between main and subset cohort (chi-square test or Fisher's exact test for categorical variables and *t*-test for continuous variables).

3.2. PM_{10} and NO_2 exposure levels

The distribution of PM_{10} and NO_2 levels at exposure periods is presented in Table S6. The mean levels of PM_{10} exposure were 55.5 ± 10.3, 54.1 ± 11.7, 53.8 ± 12.8, 54.5 ± 7.4, 46.0 ± 4.7, 50.7 ± 5.6, and 49.2 ± 5.2 μg/m^3 during the 1st, 2nd, 3rd, and 1–3rd trimester of pregnancy, and at age 2, 4, and 6 years, respectively. The mean exposure levels for NO_2 were 63.2 ± 9.5, 62.0 ± 11.2, 61.6 ± 11.4, 62.2 ± 7.9, 58.3 ± 7.7, 60.2 ± 7.0, and 54.9 ± 7.5 μg/m^3, during the 1st, 2nd, 3rd, 1–3rd trimester of pregnancy, and at age 2, 4, and 6 years, respectively. The ICC for PM_{10} was 0.91 for the trimesters of pregnancy and 0.46 for age 2–6 years. The ICC for NO_2 was 0.38 for the trimesters of pregnancy and 0.38 for age 2–6 years. The correlation coefficients of both PM_{10} pairs (0.3 and 0.41 for pregnancy, 0.65 and 0.55 for childhood) and NO_2 (0.5 and 0.53 for pregnancy, 0.39 and 0.55 for childhood) for adjacent periods were small to moderate. There were moderate and weak correlations between PM_{10} and NO_2 measured in the same pregnancy trimesters (range 0.48–0.60) and during childhood (range −0.14–0.06), respectively (Fig. S4).

3.3. Association between PM_{10} and NO_2 exposure and autistic traits

The association between PM_{10} and NO_2 exposure and SCQ scores at age 6 years are shown in Table 2 for the subset cohort (n = 170 and n = 108) and Table S7 for the main cohort (n = 568). In the subset cohort (n = 170), an IQR increase of PM_{10} exposure during the 1st trimester and at ages 2 and 4 years was associated with increased SCQ scores at 6 years of age (10.6% change, 95% confidence interval [CI]: 1.1, 21.0; 16.8% change, 95% CI: 2.3, 33.3; 15.7% change, 95% CI: 1.9, 31.4, respectively; Fig. S5). When the sample size was reduced to n = 108, only PM_{10} exposure at age 4 was associated with SCQ scores at age 6 (19.1% change, 95% CI: 1.4, 40.1), and NO2 exposure during the 1st trimester of pregnancy was associated with increased SCQ scores at age 6 (18.3% change, 95% CI: 4.4, 34.1). Similarly, in the main cohort, the exposure windows that showed statistically significant associations between PM_{10} exposure and SCQ scores were the 1st trimester, and the age 2 and 4 periods (9.4% change, 95% CI: 4.4, 14.5; 14.6% change, 95% CI: 6.3, 23.6; 17.4% change, 95% CI: 8.1, 27.6). The effect sizes of associations were similar in the subset and main cohorts.

In the subset cohort, NO_2 exposure during the 1st trimester was statistically significantly associated with increased SCQ scores at age 6 years (12.1% change per IQR increase, 95% CI: 0.1, 25.5). However, in the main cohort, NO_2 exposure was not related to increased SCQ scores in any exposure windows. Rather, NO_2 exposure at ages 4 and 6 years was negatively associated with SCQ scores at age 6 years (−8.8% change, 95% CI: −13.6, −3.7; −6.6% change, 95% CI: −12.6, −0.1).

3.4. Association between PM_{10} or NO_2 exposure and gut microbiome

The range of alpha diversity indices according to sex is shown in Fig. S6. The distribution of the relative phyla abundance is presented in Fig. S7, which shows that *Bacteroides* was the most dominant phylum, followed by *Firmicutes*, *Actinobacteria*, and *Proteobacteria and Verrucomicrobia*. Considering alpha indices, only NO_2 exposure during the 3rd trimester was associated with the Chao index at age 6 years (4.9 increase per IQR increase, 95% CI: 0.14, 9.67; Table S8). In the RDA analysis, PM_{10} exposure during the 1st trimester showed a statistically significant association with the composition variation of the gut microbiome at age 6 years (R^2 = 1.6%, p = 0.03; R^2 = 1.4%, p = 0.01 for the order and family level, respectively; Fig. 1, Table S9).

When examining the phylum level, PM_{10} exposure during the 1st trimester was associated with increased *Proteobacteria* abundance (66.9% increase per IQR increase of PM_{10}, 95% CI: 21.3, 129.8). PM_{10}

Table 2
Associations between exposure to air pollution and SCQ scores at age 6, according to exposure windows.

Pollutant and Exposure windows	Crude[a] (n = 170)		Adjusted[b] (n = 170)		Crude[a] (n = 108)		Adjusted[b] (n = 108)	
	(% change [95% CI])[c]	p-value	(% change [95% CI])[c]	p-value	(% change [95% CI])[c]	p-value	(% change [95% CI])[c]	p-value
PM_{10}								
1st trimester[d]	**11.9 (2.4, 22.1)**	**0.01**	**10.6 (1.1, 21.0)**	**0.03**	4.6 (−6.5, 17.0)	0.40	5.8 (−5.6, 18.5)	0.37
2nd trimester[d]	3.0 (−7.4, 14.6)	0.59	−1.5 (−12.2, 10.6)	0.73	−5.7 (−17.2, 7.3)	0.40	−11.1 (−22.8, 2.4)	0.11
3rd trimester[d]	6.3 (−7.2, 21.9)	0.31	4.2 (−9.1, 19.4)	0.47	0.8 (−15.9, 20.8)	0.93	−2.0 (−18.6, 17.9)	0.83
1st-3rd trimesters[d]	**11.1 (1.7, 21.4)**	**0.02**	8.4 (−1.3, 19.1)	0.09	2.5 (−8.2, 14.3)	0.65	0.1 (−11.0, 12.6)	0.98
Age 2[d]	**16.0 (3.5, 30.1)**	**0.01**	**16.8 (2.3, 33.3)**	**0.03**	**19.7 (3.7, 38.2)**	**0.01**	13.9 (−79.2, 524.3)	0.14
Age 4[d]	**18.3 (5.0, 33.2)**	**<0.01**	**15.7 (1.9, 31.4)**	**0.02**	**27.2 (9.5, 47.9)**	**<0.01**	**19.1 (1.4, 40.1)**	**0.03**
Age 6[d]	4.2 (−10.5, 21.5)	0.62	7.8 (−8.3, 26.6)	0.38	7.6 (−11.7, 31.0)	0.46	0.8 (−18.0, 24.0)	0.93
NO_2								
1st trimester[d]	**15.0 (2.9, 28.5)**	**0.01**	**12.1 (0.1, 25.5)**	**0.04**	**19.6 (5.8, 35.3)**	**<0.01**	**18.3 (4.4, 34.1)**	**0.01**
2nd trimester[d]	6.2 (−15.4, 33.3)	0.60	−3.2 (−14.9, 10.1)	0.57	6.1 (−7.6, 21.8)	0.43	−1.5 (−15.2, 14.5)	0.87
3rd trimester[d]	17.2 (−5.5, 5.4)	0.15	4.8 (−6.7, 17.7)	0.40	17.8 (−2.8, 42.9)	0.11	10.8 (−9.0, 34.9)	0.29
1st-3rd trimesters[d]	**26.9 (1.9, 58.0)**	**0.03**	3.1 (−4.5, 22.3)	0.20	**15.7 (3.7, 29.1)**	**0.01**	12.0 (−0.2, 25.8)	0.06
Age 2[d]	11.2 (−4.7, 29.6)	0.18	4.9 (−4.2, 14.9)	0.27	10.4 (−1.4, 23.6)	0.09	11.1 (−2.3, 26.4)	0.11
Age 4[d]	**−17.2 (-29.6, -2.6)**	**0.02**	−7.6 (−15.6, 1.1)	0.10	**−17.9 (-26.9, -7.8)**	**<0.01**	−10.4 (−21.4, 2.2)	0.10
Age 6[d]	3.1 (−15.0, 25.1)	0.75	2.6 (−7.9, 14.3)	0.59	−4.9 (−16.1, 7.8)	0.44	−0.8 (−12.9, 13.0)	0.90

Abbreviations: PM_{10}, particulate matter with an aerodynamic diameter ≤10 μm; NO_2, nitrogen dioxide; SCQ, social communication questionnaire; CI, confidence interval; IQR, interquartile range.
Adjusted for child's age, sex, twin, family income, season of birth, low birthweight, delivery mode and breastfeeding for exposure windows during childhood.
[a] Adjusted for age and sex.
[b] Adjusted for child's age, sex, twin, family income for exposure windows during pregnancy.
[c] Per IQR increase; Statistically significant results shown in bold.
[d] Sample size: n = 170 for pregnancy exposure (1st – 3rd trimesters), n = 132 for childhood exposure (age 2–6).

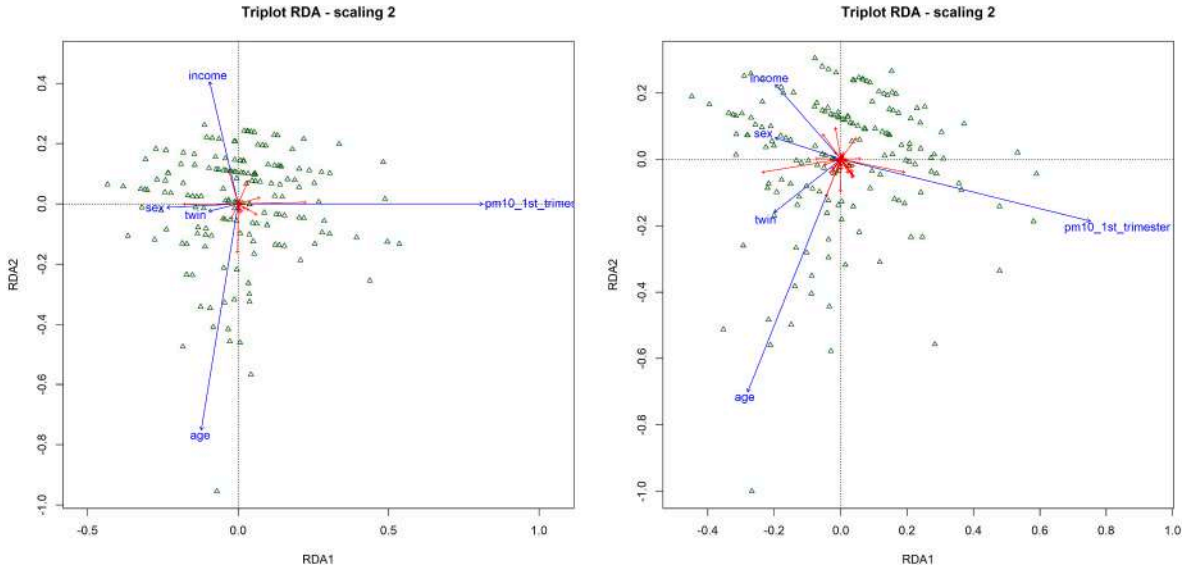

Fig. 1. RDA analysis on the association between PM_{10} exposure in the 1st trimester of pregnancy and distribution of gut microbiome (order level and family level), (a) A 3.2% of the total variance was explained by the model. The first and second axes explained 1.7% and 0.9% of the variance, (b) A 3.5% of the total variance was explained by the model. The first and second axes explained 1.8% and 0.9% of the variance., Abbreviations: RDA, redundancy analysis; PM_{10}, particulate matter with an aerodynamic diameter ≤10 μm; NO_2, nitrogen dioxide.

exposure at age 2 years was also related to an increase in *Proteobacteria* relative abundance (74.1% change, 95% CI: 6.0, 188.6; Table 3). The association between NO_2 exposure during the 1st trimester and *Proteobacteria* relative abundance was marginally statistically significant (48.1% change, 95% CI: −0.1, 119.6). NO_2 exposure at age 2 years was associated with a decrease in *Proteobacteria* relative abundance (−29.2% change, 95% CI: −47.2, −5.0). NO_2 exposure at age 2 was associated with increased *Bacteroidetes* relative abundance at age 6 (21.6% increase per IQR increase of NO_2, 95% CI: 4.1, 42.0). PM_{10} exposure during the 1st trimester was associated with decreased *Verrucomicrobia* abundance (45.4% decrease per IQR increase of PM_{10}, 95% CI: −67.5, −8.4).

3.5. Association between gut microbiome and autistic traits

The relative abundance of *Proteobacteria* was statistically significantly associated with SCQ scores (4.4% change per 2-fold increase in relative abundance, 95% CI: 1.3, 7.5) in the prenatal model (n = 170). However, there was no statistically significant relationship between *Proteobacteria* relative abundance and SCQ scores in the postnatal exposure group (n = 132; Table 4).

Table 3
Associations between exposure to air pollution and relative abundance at the phylum level, by exposure windows.

Exposure windows	*Bacteroidetes*[a]		*Actinobacteria*[a]		*Proteobacteria*[a]		*Firmicutes*[a]		*Verrucomicrobia*[a]	
	((% change [95% CI])[b]	p-value	(% change [95% CI])[b]	p-value	(% change [95% CI])[b]	p-value	(% change [95% CI])[b]	p-value	(% change [95% CI])[b]	p-value
PM_{10}										
1st trimester*	−13.7 (−29.2, 5.2)	0.15	−1.5 (−20.4, 21.8)	0.88	**66.9 (21.3, 129.8)**	**<0.01**	3.2 (−4.4, 11.3)	0.45	**−45.4 (-67.5, -8.4)**	**0.02**
2nd trimester*	−15.0 (−43.5, 10.1)	0.22	22.6 (−7.3, 62.2)	0.16	25.1 (−17.7, 90.3)	0.29	2.1 (−9.5, 15.1)	0.73	−32.1 (−64.2, 28.6)	0.24
3rd trimester*	8.9 (−19.8, 47.9)	0.60	4.4 (−25.3, 45.8)	0.81	−10.7 (−45.9, 47.3)	0.63	−2.8 (−13.0, 8.7)	0.59	−9.5 (−60.7, 108.8)	0.80
1-3 trimesters*	−8.6 (−26.6, 13.9)	0.41	7.6 (−14.6, 35.5)	0.55	38.5 (−1.5, 94.8)	0.06	0.6 (−7.9, 9.8)	0.89	−34.4 (−61.3, 11.3)	0.12
Age 2*	−11.7 (−33.1, 16.6)	0.39	2.2 (−27.7, 44.3)	0.91	**74.1 (6.0, 188.6)**	**0.03**	2.6 (−8.8, 15.5)	0.69	−30.0 (−69.3, 59.8)	0.40
Age 4*	3.1 (−20.3, 33.4)	0.83	−14.5, (−37.8, 17.4)	0.33	58.5 (−0.6, 152.6)	0.06	0.1 (−10.6, 12.1)	0.99	−13.6 (−60.2, 87.2)	0.72
Age 6*	16.1 (−16.3, 61.1)	0.37	1.2 (−32.6, 51.8)	0.96	31.8 (−27.5, 139.6)	0.36	−3.4 (−16.6, 11.9)	0.64	−15.9 (−68.5, 124.4)	0.73
NO_2										
1st trimester*	−14.5 (−33.9, 10.5)	0.21	−17.5 (−37.3, 8.5)	0.17	48.1 (−0.1, 119.6)	0.05	5.8 (−4.3, 16.9)	0.28	15.0 (−40.0, 120.4)	0.68
2nd trimester*	−16.6 (−37.6, 11.4)	0.24	−3.4 (−29.3, 32.0)	0.84	−1.1 (−38.1, 57.9)	0.97	8.3 (−3.1, 21.0)	0.21	7.1 (−49.8, 128.4)	0.86
3rd trimester*	11.4 (−13.6, 43.7)	0.41	−15.9 (−36.1, 10.8)	0.21	−22.0 (−49.0, 19.1)	0.24	−1.1 (−11.0, 10.0)	0.85	16.3 (−41.0, 129.2)	0.66
1-3 trimesters*	−10.2 (−31.6, 17.8)	0.43	−16.9 (−38.5, 12.4)	0.23	3.9 (−32.9, 60.9)	0.85	6.3 (−4.3, 18.2)	0.29	16.6 (−42.6, 136.9)	0.67
Age 2*	**21.6 (4.1, 42.0)**	**0.02**	−6.8 (−24.0, 14.2)	0.49	**−29.2 (-47.2, -5.0)**	**0.02**	−0.8 (−7.8, 6.7)	0.83	−4.5 (−42.4, 58.4)	0.86
Age 4*	11.6 (−7.6, 34.7)	0.24	−0.9 (−21.5, 25.1)	0.92	−13.2 (−38.3, 22.0)	0.41	7.1 (−1.2, 16.1)	0.11	40.3 (−21.0, 149.0)	0.25
Age 6*	18.6 (−3.6, 46.0)	0.11	−11.6 (−31.5, 13.9)	0.33	−15.2 (−41.4, 22.7)	0.38	0.6 (−8.3, 10.3)	0.87	20.7 (−35.3, 125.3)	0.56

*Sample size: n = 170 for pregnancy exposure, n = 132 for childhood exposure (age 2–6) for *Bacteroidetes, Actinobacteria, Proteobacteria* and *Firmicutes*. n = 108 for pregnancy exposure, n = 84 for childhood exposure (age 2–6) for *Verrucomicrobia*.
Abbreviations: PM_{10}, particulate matter with an aerodynamic diameter ≤10 μm; NO_2, nitrogen dioxide; CI, confidence interval; IQR, interquartile range.
Adjusted for child's age, sex, twin, family income, season of birth, low birthweight, delivery mode and breastfeeding for exposure windows during childhood.
[a] Adjusted for child's age, sex, twin, family income for exposure windows during pregnancy.
[b] Per IQR increase; Statistically significant results shown in bold.

Table 4
Associations between gut microbiome and SCQ scores at age 6.

Microbiome	Crude[a]		Adjusted[b]	
	(% change [95% CI])[c]	p-value	(% change [95% CI])[c]	p-value
Proteobacteria (n = 170)	**4.7 (1.7, 7.9)**	**<0.01**	**4.4 (1.3, 7.5)**	**<0.01**
Proteobacteria (n = 132)	**4.3 (0.9, 7.8)**	**0.01**	2.6 (−26.6, 43.5)	0.13

Abbreviations: SCQ, social communication questionnaire; CI, confidence interval.
Adjusted for child's age, sex, twin, family income, season of birth, low birthweight, delivery mode and breastfeeding for n = 132 sample.
[a] Adjusted for age and sex.
[b] Adjusted for child's age, sex, twin, family income for exposure windows for n = 170 sample.
[c] Per 1-unit increase for *Bacteroidetes*, per 2-fold increase for *Proteobacteria*; Statistically significant results shown in bold.

3.6. Mediation effect of microbiome on the association between air pollution and autistic traits

In the mediation analysis of *Proteobacteria* relative abundance for the association between PM_{10} exposure during the 1st trimester and SCQ scores at age 6 years, both the indirect and direct effects were statistically significant, indicating that the association between PM_{10} exposure during the 1st trimester and autistic traits was partially mediated by changes in *Proteobacteria* relative abundance (mediation proportion: 25.1%, p = 0.01). The mediation analysis also showed that a marginally significant proportion of the association between NO_2 exposure during the 1st trimester and autistic traits is attributed to changes in *Proteobacteria* relative abundance (proportion mediated: 16.5%, p = 0.06; Fig. 2 and Table 5).

3.7. Comparison with other mediation models

There were no other mediation models wherein the indirect, direct, and total effects were all statistically significant, nor were there models wherein the mediated proportion was statistically significant (Table S10). No other time window of air pollution exposure showed significant mediation. The indirect effect of *Proteobacteria* abundance on the association between PM_{10} exposure at age 2 years and SCQ scores at age 6 years was marginally significant (proportion mediated: 13.5%, p = 0.07). *Proteobacteria* abundance also marginally mediated the association between PM_{10} exposure at age 4 years and SCQ scores at age 6 years (proportion mediated: 15.3%, p = 0.06). In other models, the indirect effect of *Proteobacteria* abundance on the association between NO_2 at age 2 years and SCQ at age 6 years was statistically significant (−4.9% change per IQR increase, 95% CI: −9.5, −0.001); however, the association showed a negative direction, in contrast to the direct effect (15.7% change, 95% CI: 5.8, 26.5). Therefore, the total effect was not statistically significant (10.1% change, 95% CI: −4.5, 27.0).

When the mediation effects of autistic traits on the association between air pollution (PM_{10} or NO_2) levels during the 1st trimester of pregnancy and *Proteobacteria* abundance were examined, the indirect effect was no longer statistically significant, and the proportions

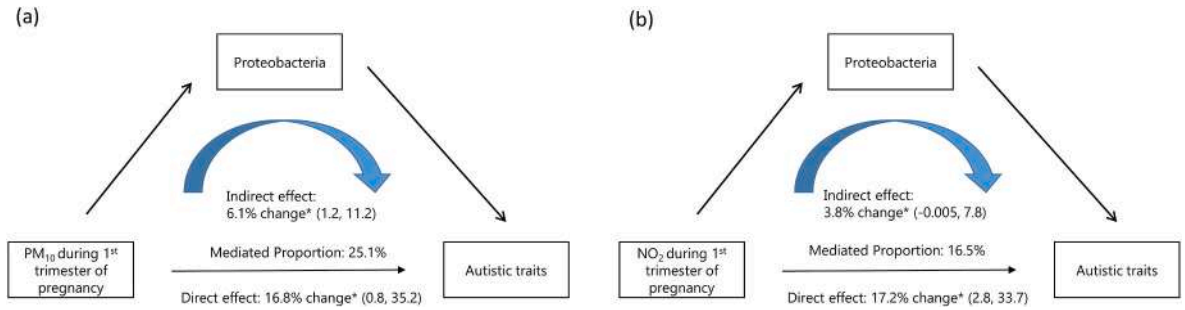

Fig. 2. Mediation analysis of the association between air pollution and autistic traits through gut microbiome changes, (a) Mediation of *Proteobacteria* relative abundance on the association between PM_{10} during the1st trimester and autistic traits, (b) Mediation of *Proteobacteria* relative abundance on the association between NO_2 during the1st trimester and autistic traits, *: % change per interquartile range, Abbreviations: PM_{10}, particulate matter with an aerodynamic diameter ≤10 μm; NO_2, nitrogen dioxide.

Table 5
Mediating role of proteobacteria on the association between air pollution and SCQ scores.

Path	Indirect effect		Direct effect		Total effect		Mediated proportion (%)	p-value
	% change [95% CI])[a]	p-value	% change [95% CI])[a]	p-value	% change [95% CI]))[a]	p-value		
1st trimester PM_{10} – proteo – SCQ	**6.1 (1.2, 11.2)**	**<0.01**	**16.8 (0.8, 35.2)**	**0.04**	**23.8 (10.6, 38.7)**	**<0.01**	25.1	**0.01**
1st trimester NO_2 – proteo – SCQ	3.8 (−0.005, 7.8)	0.05	**17.2 (2.8, 33.7)**	**0.03**	**21.7 (9.4, 35.3)**	**0.01**	16.5	0.06

Abbreviations: SCQ, social communication questionnaire; CI, confidence interval; PM_{10}, particulate matter with an aerodynamic diameter ≤10 μm; NO_2, nitrogen dioxide; IQR, interquartile range, statistically significant results shown in bold.
Sample size: n = 170 and n = 132 for the 1st trimester and age 2 analyses, respectively.
[a] Per IQR-increase in air pollutant exposure.

mediated were 3.1% and 11.0%, respectively (p-values > 0.05; Table S11).

4. Discussion

To our knowledge, this study is the first to investigate the mediating role of the microbiome in the association between environmental toxins and autistic traits. Interestingly, the microbiota profile, specifically *Proteobacteria* at the phylum level, showed 25.1% and 16.5% mediation effects on the associations between both 1st trimester PM_{10} and NO_2 exposure, respectively, and autistic traits in children. These findings suggest a plausible mechanism underlying the relation between air pollution and ASD and add evidence to the existing literature on the gut–brain axis suggested in ASD.

Despite the proposed role of the microbiome as a mediator of genetic and environmental risk factors, research has been scarce, specifically on ASD. The majority of earlier evidence comes from animal studies that indirectly investigated the effect of the microbiome. For example, oral treatment of *B. fragilis* or *B. thetalotaomicron* to the offspring of the rodent model of maternal immune activation, a model that resembles infection during pregnancy, improved the gut microbial composition and permeability while reducing ASD-related behavior (Hsiao et al., 2013; Kentner et al., 2019). Similarly, modeling maternal exposure to valproic acid, an anticonvulsant drug associated with an increased risk for ASD (C.G. de Theije et al., 2014), resulting in offspring with altered gut microbiota composition, neuro-inflammation, and ASD-associated behavioral abnormalities (C.G.M. de Theije et al., 2014). Meanwhile, two human studies have investigated the mediating role of the microbiome linking air pollution with liver function or glucose levels (Yi et al., 2021; Alderete et al., 2018); however, this study is the first study to have autistic traits as the outcome variable.

Prenatal (1st trimester of pregnancy) and postnatal (at age 2 and 4 years) exposure to air pollutants was associated with autistic traits at age 6 years, suggesting long-lasting effects. According to the Developmental Origins of Health and Disease, exposure to environmental agents results in long-lasting human physiology and behavior alterations (Barker, 2007). These long-term effects may be partly due to the developing epigenetic code (Suter et al., 2010, 2013) and microbiome (Chu et al., 2016; Chu and Aagaard, 2016), since the gut microbiome can be transferred across the placenta during fetal development (Braniste et al., 2014; Jašarević et al., 2016), leading to varied effects in the offspring. Moreover, alterations in the maternal microbiome due to environmental risk exposure can be passed on, since mammals acquire their initial microbiome via birthing. The microbiota may convey lasting effects on health and disease through the epigenetic modification of the host genome (Kumar et al., 2014; Cortese et al., 2016). Moreover, epigenetic changes caused by microbiota can influence host transcriptional patterns. For example, fatty acid butyrate—a SCFA produced by the microbiome—can inhibit the action of histone deacetylase inhibitor, and result in disruption in cell cycle progression, gene silencing, differentiation, and genotoxic reactions (Waldecker et al., 2008). As this study did not include methylation data, further studies that incorporate both methylome and microbiome data could provide an integrated multi-omics description on the pathway linking air pollution and autistic traits.

The 1st trimester of pregnancy as well as ages 2 and 4 years were susceptible exposure periods to the neurotoxic effects of PM_{10}. These exposure periods have been suggested to be susceptible to environmental risk factors in previous studies. Brain development most rapidly occurs during series of time-sensitive periods when neuroplasticity is heightened (Ismail et al., 2017; Meredith, 2015). The early fetal period is marked by prominent neurogenesis, the 2nd trimester of pregnancy and first 2 years of early life is characterized by synaptogenesis (Johnston et al., 2009), while ages 2 to 10 is the period when synaptic pruning rapidly occurs (Huttenlocher and Dabholkar, 1997). The susceptible periods of air pollution exposure related to autistic traits overlap with the critical periods of neurodevelopment, which also coincide with the developmental periods of the gut microbiome. Gut microbiome changes appear to occur most dynamically during the first 3 years of life (Yatsunenko et al., 2012; Derrien et al., 2019). Therefore, it can be

speculated that the microbiome would be more prone to environmentally toxic materials up to age 3, when changes are more rapid. However, only microbiome data at age 6 years were obtained, and thus, it is unclear in which period our participants' microbiome was most susceptible. Considering the relative stability of the gut microbiome after the age of 3 years, the microbiome profile at age 6 years might reflect that of an earlier stage. However, recent studies have also found differences in the gut microbiome of 7–12-year-olds compared to adults, suggesting that complete maturation of the gut microbiome may take longer than previously suggested (Zhong et al., 2019). Thus, further studies using microbiome data from periods of early life are warranted to confirm this study's hypothesis.

Proterobacteria abundance was associated with both PM_{10} and NO_2 exposure during the 1st trimester of pregnancy. Exposure to mixed vehicle emissions increased the abundance of lung *Proterobacteria* in mice (Daniel et al., 2021). Exposure to $PM_{2.5}$ was associated with increased *Proterobacteria* abundance in buccal mucosa microbacteria (Wu et al., 2021). A previous study involving adult patients with schizophrenia found that NO_2 exposure and PM_{10} in the preceding year explained 3.7% and 7.5% of the gut microbiome composition, respectively (Yi et al., 2021). Another study reported that NO_2 explained 4.4% of the variance in gut microbiome composition in overweight to obese adults (Fouladi et al., 2020). In our study, 1st trimester PM_{10} and NO_2 explained 1.6% and 0.7% of the gut microbiome at age 6 years, respectively, which is a smaller effect size compared to previous studies. However, direct comparison between these studies is not recommended since this study investigated children's microbiota profiles, whereas previous studies targeted adult populations. Moreover, the distribution and concentration of air pollutants from different countries or regions may vary.

Proterobacteria abundance was cross-sectionally associated with autistic traits at age 6 years. Although previous studies on microbiota differences in patients with ASD have been highly heterogeneous, most studies found that the overall microbiota composition of ASD cases differs from that of controls (Bundgaard-Nielsen et al., 2020). However, there were no specific bacteria consistently associated with ASD diagnosis or severity. Nevertheless, *Proteobacteria* abundance was found to be elevated in individuals with ASD compared with controls (Finegold et al., 2010; Williams et al., 2011). *Proteobacteria* is associated with host inflammation (Shin et al., 2015) and produces a potent toxic factor lipopolysaccharide (Liu et al., 2019a); exposure to this factor can reduce glutathione in the brain (Zhu et al., 2007; Chauhan and Chauhan, 2006), suggesting possible neurotoxic effects.

Among the Organization for Economic Cooperation and Development member countries, South Korea ranked first in terms of mean population exposure to $PM_{2.5}$ in 2019 (Lee et al., 2018), as most cities in South Korea were urbanized (Shin et al., 2022). The new air quality guideline by the World Health Organization (WHO) recommends that the annual mean PM_{10} value should not exceed 15 $\mu g/m^3$ and that the annual standard NO_2 concentration should not exceed 10 $\mu g/m^3$ (World Health Organization, 2021). The air quality guideline by the Ministry of Environment in South Korea has set higher limit levels: 50 $\mu g/m^3$ for the annual PM_{10} value and 0.03 ppm (56.4 $\mu g/m^3$) for the annual NO_2 value (Kumbhakar et al., 2021). As the mean concentrations of these pollutants in the present study were similar to the recommended values mentioned in the South Korea guideline and higher than those mentioned in the WHO guideline and neurotoxicity of air pollutants was observed at these values, we suggest a stricter policy to regulate air pollution levels.

This study has some limitations. We used data from a community-based cohort and none had undergone formal testing for ASD. Although the SCQ has a valid and reliable questionnaire (Corsello et al., 2007), its primary purpose is screening for ASD. Therefore, further studies using diagnostic interviews are needed to expand the results to clinical populations. As the relationship between the gut microbiome and autistic traits was cross-sectional, causal relations are not definite, and reverse causation is possible. We did not provide refined information such as genera or species, due to the lower detection rates compared to the phlya and substantial reduction in sample size.. Moreover, the small sample size limits the results' statistical power, and further replication in a larger population is warranted. We did not adjust for multiple testing and rather focused on the trend of the results; thus, the findings of this study should be interpreted cautiously. Although various covariates were accounted for, some confounding factors, including diet diversity and other endocrine-disrupting chemicals, were not considered (Yap et al., 2021). Furthermore, other individual data that may affect exposure to air pollution, such as indoor air pollution, physical activity, time spent outdoors, and occupational status, were lacking (Lim et al., 2021). Air pollutant exposure was obtained through data from monitoring stations and exposure misclassification cannot be ruled out. Moreover, we did not consider changes in exposure levels over time among individuals when we investigated exposure windows. This problem may not influence our study results significantly as we compared exposure levels within a 3-year period (e.g., exposure years in 2015–2017 for children at age 6), where exposure levels may not change significantly in the three years. Furthermore, we did not consider other air pollutants such as ultrafine particles, which may have greater capacity to reach the gut and brain (Akimoto, 2003; Oberdorster et al., 1994), due to lacking data. In addition, other air pollutants, including carbon monoxide, ozone and $PM_{2.5}$, were not included in the analyses as these pollutants were not associated with autistic scores in the study (Fig. S8). Lastly, the majority of participants resided in urban areas, and most were from highly educated and high income families; thus the results may not be generalizable to populations in rural regions.

Despite these limitations, this study was strengthened by its longitudinal design and repetitive assessment of $PM_{10,}$ NO_2 exposure and autistic traits. We identified multiple susceptible periods in early life and explored the mediating role of the gut microbiome in bridging environmental toxins and neurodevelopmental outcomes.

5. Conclusions

Air pollution during the 1st trimester of pregnancy may affect autistic traits at age 6 years through the alteration of *Proteobacteria* abundance. Future studies with larger sample sizes and microbiome samples at earlier ages are warranted. Moreover, research on whether correction of gut microbial dysbiosis could reduce the impact of PM_{10} exposure on autistic traits is needed.

Institutional review board statement

Informed consent was provided by all guardians. The study protocol was reviewed by the Institutional Review Board of Seoul National University Hospital (IRB No. 1201-010-392) and followed the principles of the Declaration of Helsinki.

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Declaration of competing interest

The authors declare they have no actual or potential competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.114047.

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Toxicological and Exposure Database Inventory: A review

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ABSTRACT

Background: Knowledge of hazards and exposure data about chemical pollutants are essential for epidemiological studies for assessing health risks and preventing the development of diseases. Access to this type of information is clearly needed and although numerous databases (DBs) have been compiled, they are often extremely difficult to find and navigate.

Objectives: This paper presents the creation of an online inventory of toxicological and exposure DBs to support human health risk assessment resulting from the chemical exposome.

Methods: A free searchable online inventory, called TEDI (Toxicological and Exposure Database Inventory), was set up to collect meta-data on toxicological and exposure DBs resulting from a review of the literature conducted on PubMed. Only chemical agents, including drugs, natural and manufactured/synthetic chemicals and air pollutants, were considered. Regarding exposure DBs, only human exposure to chemicals were included. Because of time and resource constraints, only an inventory was performed, and no effort was made to rate, assess or rank DBs on quality criteria.

Results: A total of 715 DBs were identified, catalogued, described and made available on TEDI. The number of chemicals ranged from one single chemical to 90 million for toxicological DBs and from 1 to 103,817 chemicals for exposure DBs. Some DBs were restricted to specific chemicals, with for instance 71 exposure DBs reporting polycyclic aromatic hydrocarbon measurements. Toxicological DBs (n = 362) provided a broad range of different information such as toxicity data (57%), physicochemical properties (38%), experimental study results (28%) or prediction data (16%). A total of 382 exposure DBs were found, including exposure measurements DBs (71%), web application/tool (21%), exposure and risk assessment models (19%) and epidemiological DBs (13%).

Discussion: TEDI is of value to the broader community and could support human health risk assessment to chemicals in various contexts. This inventory remains open for further additions, to enlarge its coverage, increase the meta-data collected and include newly developed DBs.

1. Introduction

The introduction of the exposome concept emphasizes on the multiplicity, interaction and complexity of health risks. The exposome can be defined as the study of the impact of all potential stressors on human health (including chemical, biological, physical, psychological, social, etc.) from conception onwards (Dennis et al., 2017; Kim and Hong 2017; Siroux et al., 2016; Wild 2005, 2006). This concept, first formulated in 2005, was developed to highlight the need for better and more complete exposure data related to all life-stages for various populations (Siroux et al., 2016; Wild 2005).

Because chemicals are among the major environmental threats to human health, knowledge of hazards and exposure data about chemical pollutants (chemical exposome) are essential for assessing health risks and preventing the development of diseases. Risk assessment can be viewed as a four-step process including hazard identification, dose-response assessment, exposure assessment, and risk characterization (Persad and Bobst 2020). Since the REACH regulation, knowledge about hazards has increased, but there is still a scarcity of data pertaining to thousands of chemicals, in particular for chronic hazards, chemical mixtures and the condition of use of many chemicals (ECHA 2015; Wignall et al., 2018). Regarding exposure data, the knowledge is much more limited and highly variable. Some chemicals have been intensively monitored, such as asbestos, while others have been well characterized in various media (water, sediment, food) but poorly in humans.

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Abbreviations

ADME	absorption, distribution, metabolism, excretion
DB	database
EU	European Union
GBD	Global Burden of Disease
GHS	Globally Harmonized System of Classification and Labeling of Chemicals
JEM	job-exposure matrices
LB	lower bound
MS	Microsoft
OMEGA-NET	Network on the Coordination and Harmonization of European Occupational Cohorts
PAHs	polycyclic aromatic hydrocarbons
QSAR	structure-activity relationship
QSPR	quantitative structure-property relationship
REACH	Registration, Evaluation, Authorization and restriction of CHemicals
SAR	structure-activity relationship
TEDI	Toxicological and Exposure Database Inventory
UB	upper bound

However, for most chemicals, there is a paucity of knowledge and even occasionally no data, with only a small fraction of the chemical exposome covered (Vineis et al., 2017; Wignall et al., 2018).

Technological advancement has led to evolving exposure environments/conditions and to a drastic increase in the health data generated and captured. To be used for research purposes, the generated data have to be collected, recorded, stored and properly handled (Gaudet et al., 2011; Zheng et al., 2017). A great diversity of databases (DBs) exists, whose nature and scope, as well as the degree of complexity vary immensely (Andreu-Perez et al., 2015; Frelinger 2015; Sowe and Zettsu 2014; Steckling et al., 2018; Zheng et al., 2017). The true challenge is to identify what data are truly relevant/useful and concentrate efforts on gathering it. Access to this type of information is clearly needed and although numerous DBs have been compiled, they are often extremely difficult to find and navigate. A concerted effort is needed to catalogue available toxicological and exposure DBs that can support human health risk assessment of the chemical exposome.

This paper attempts to catalogue relevant DBs that might benefit human health risk assessment resulting from the chemical exposome into an easily accessible format. This inventory could be a useful tool/resource for epidemiologist, risk assessors, risk managers, practitioners, industry practitioners, decision makers and sanitary agencies to obtain and find relevant information from available resources when conducting risk assessment to chemicals.

2. Material & methods

2.1. Scope of the study

The scope of this study was to create an online inventory to collect meta-data on various toxicological and exposure DBs into an easily findable, accessible, interoperable, and re-useable format. No attempt to conduct an analysis of the scientific content of the identified records was undertaken because it was outside the scope of this study. In addition, no data quality analysis (qualitative or quantitative) of the DBs compiled from this work was made because it was also outside the scope of this work.

For this study, a toxicological DB was defined as a collection of data which included at least one of the following information: physico-chemical properties, results of experimental studies, reference/limit values (e.g., cancer slop factor), prediction data, prediction models, dose response models or other toxicity information. An exposure DB was defined as a collection of data which included at least one of the following information: results of the analysis/monitoring of one or several chemicals in at least one media (e.g., air, urine, food), information on the number of people exposed to at least one chemical, exposure models used to rank or estimate chemical exposure levels or health risk assessment tools (e.g., software, web application, methods or numerical analysis models) used to qualify or quantify the risk posed to human health resulting from chemical exposure.

For this work, only chemical agents, including drugs, natural and manufactured/synthetic chemicals (including unwanted by-products) and air pollutants, were considered as chemical exposome stressors and referred to as chemicals in the remaining of this paper. Biological agents (e.g., mycotoxins, virus), physical agents (e.g., radiation), and active smoking were outside the scope of this inventory. Regarding exposure DBs, only human exposure to chemicals were considered.

2.2. Literature search

For the purpose of this study, a review of the literature, conducted by one author, was undertaken using PubMed. All identified papers addressing the subject of toxicological or human exposure DBs regarding chemicals that were published in peer-reviewed journals were considered for inclusion. Edited books and book chapters were also considered. Gray literature was also examined. No records that were deemed relevant to the scope of this study were purposely excluded from this review. Using PubMed, the following search strategy/query was used: *(toxicological OR toxicology OR exposure OR exposome) AND (database OR dataset OR databank)*. The search was carried out on February 7th, 2022 and was limited by language: 'English' and 'French' and by date: from 1950 to the end of January 2022.

When possible, the main information (e.g., DB name, topic, country, number of data observations, references) and other meta-data regarding the identified DBs were retrieved. The meta-data of interest to be retrieved during the literature search are listed in Table 1. The characteristics/meta-data of each DB were collected using only information that was available from the screened records. Descriptors of

Table 1
List of meta-data of interest to collect from the literature search.

Database type	Database descriptor	Example
Toxicological and exposure databases	Name	ACROPOLIS
	Creator	Institution
	Owner	Institution
	Contact person	Name and email address
	Status	Still active
	Data availability	Open
	Country/Scale	The Netherlands
	Time period	2001–2010
	Number of agents	97
	Topic/Agents	Chemicals
	Full list of chemicals	Arsenic, cadmium, lead
	Reference	Bopp et al. (2018)
Toxicological database only	Data type	Physicochemical properties, experimental data about acute toxicity
Exposure database only	Data origin	Original study
	Exposure setting	Environmental exposure
	Media	Air
	Monitoring strategy	Airborne monitoring (area) from 8405 air stations located in 38 countries
	Number of data	166
	Data type	Samples

interest that were never available or almost never reported in the screened records were excluded. Because of time and resource constraints, only an inventory was performed, and no effort was made to rate, assess or rank DBs on quality criteria. In addition, no effort was made to reach out directly to the owners of the DBs to request additional details.

2.3. Creation of an online tool – TEDI

To make the inventory of toxicological and exposure DBs easily findable, accessible, interoperable, and re-useable, a user friendly and interactive web application/tool, named TEDI (Toxicological and Exposure Database Inventory), was created. It was built using the Shiny package in the R programming language (Chang et al., 2018). TEDI can be accessed on any device with an internet browser and requires no programming knowledge to use. TEDI is freely available to anyone who wants to use it and is available at: https://exporisk-timc.imag.fr/TEDI/.

Data analysis, all calculation and the creation of TEDI were performed with R software 4.1.2® (R Core Team, Vienna, Austria) for Windows 10©.

3. Results

3.1. Literature search

The literature search yielded 33,328 records that had been published before the end of January 2022. A total of 113 additional records were identified through other sources (gray literature). Irrelevant and duplicate records (32,820) were excluded based on the examination of titles, abstracts, and full-texts, after which 621 records were kept (Fig. 1). Most of the records screened were articles (81%), followed by websites (7.4%), reviews (5.8%), reports (4.2%) and other resources (1.7%). Most references were published after 2005 (88%), and almost two-third of them (58%) were published since 2015 (Fig. 2).

3.2. Database inventory

A total of 715 toxicological and exposure DBs were identified through the literature search (Fig. 1). Some records described more than one DB, which explains why there were more DBs identified than records screened (715 *vs.* 621). There were 333 DBs (46.6%) solely classified as toxicological DBs, 353 DBs (49.4%) solely classified as exposure DBs and 29 DBs (4.1%) that were classified in both categories. The geographical coverage showed that most DBs were developed in North America and Europe (Fig. 3). No DB from Africa was found while only a few DBs from South America, Central America, Asia, Australia and Oceania were identified.

A total of seven descriptors of interest were excluded and not recorded on TEDI because they were either not available or almost never reported in the screened records. These descriptors were the owner, the creator, the contact person, the data availability, the full list of chemicals, the time period and the status of the DB. As a results, to address

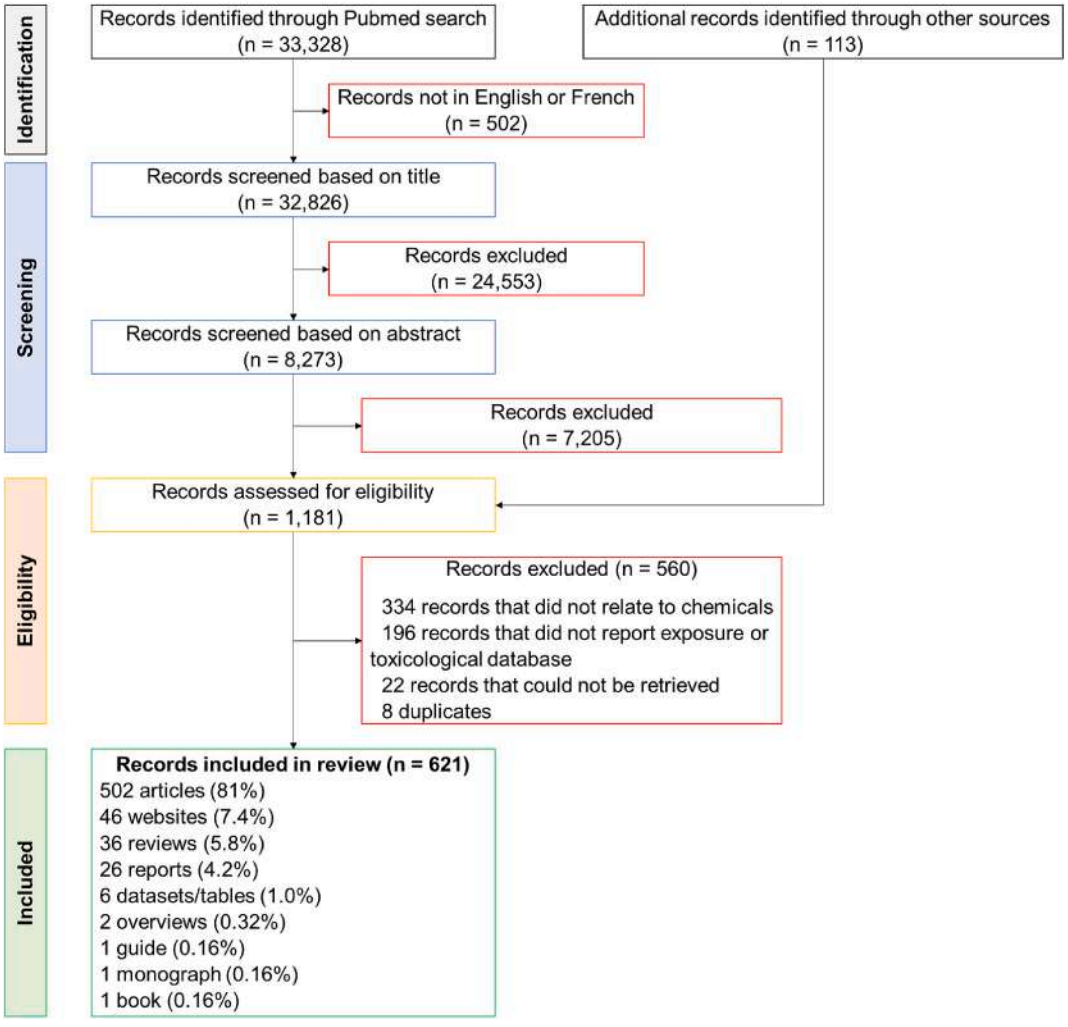

Fig. 1. Flow chart depicting the literature search and the evaluation process for finding relevant records.

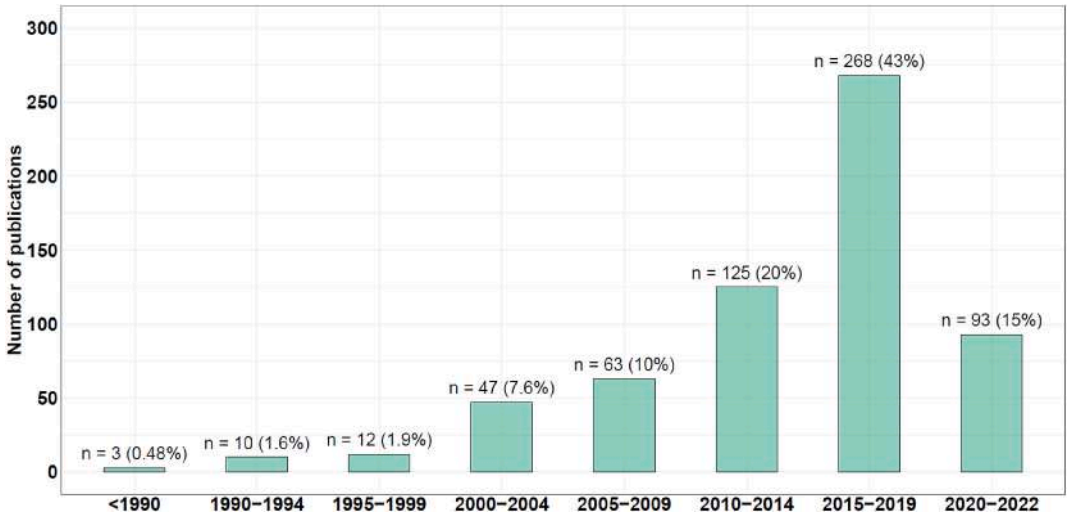

Fig. 2. Number of records per 5-year period.
Regarding the bar on the right side of the graph (2020–2022), the number of records corresponds only to the total number of records from 2020, 2021 and January 2022.

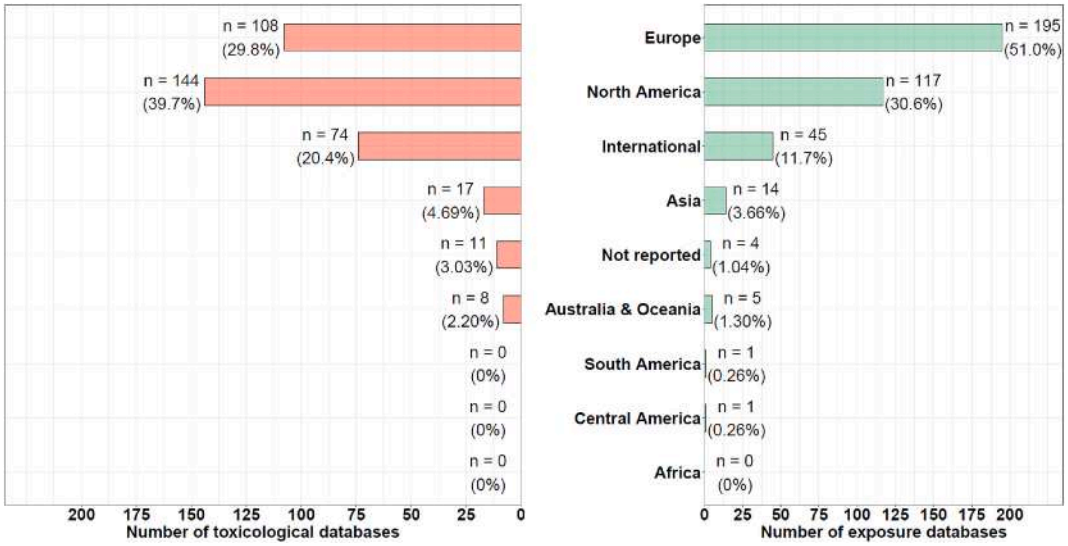

Fig. 3. Number of databases by continent and database type.

this shortcoming, a proof of concept was undertaken using the screened records to investigate the possibility of collecting more detailed information about the missing descriptors of interest. This proof of concept focused only on one descriptor (the full list of chemicals) and was restricted to exposure DBs reporting results of airborne and/or biological monitoring to polycyclic aromatic hydrocarbons (PAHs), which is one of the most studied family of carcinogens (Mallah et al., 2022; Yang et al., 2021). The results of the proof of concept are detailed below in subsection *3.2.3*.

3.2.1. Toxicological databases

Among the 715 DBs identified, 362 (50.6%) were classified as toxicological DBs. Most of the time, they were made up of data retrieved through literature reviews, data collation and/or data collection. Data on a wide variety of chemicals were available. The number of chemicals found in the identified toxicological DBs ranged from one single chemical to 90 million. They were 83 (23%) DBs that investigated specific topics or classes of chemicals, in particular nanomaterials (n = 20), pesticides (n = 19), chemicals in food (n = 13), chemical mixtures (n = 11), endocrine disrupting chemicals (n = 9), carcinogens (n = 6) and PAHs (n = 4).

A broad range of data types were available (Fig. 4). A total of 208 (57%) toxicological DBs provided toxicological information while 16 reported ecotoxicological data. There were many types of toxicity information reported such as data on target organ, mode of action, adverse effects, as well as toxicity profile, toxicity thresholds and dose-response relationship of a chemical in different organisms and/or the environment. Some of these DBs also provided information on pharmacokinetic data (n = 10), in particular ADME (absorption, distribution, metabolism, and excretion), but also on adverse outcome pathway (n = 7).

A total of 139 (38%) DBs provided chemical structure and physicochemical properties. Most of these DBs were usually freely accessible and had their own search engine. Information regarding the chemical identity (e.g., name, synonym, formula, structure, identification number) were often provided. A broad range of physicochemical properties could be found such as vapor pressure, solubility, partition coefficients, molar mass, molar volume, pH and critical temperature. Chemotype and activity annotations were sometimes also available.

A total of 100 (28%) DBs gathered results from experimental studies. Most experimental data came from animal and *in vitro* studies. DBs with

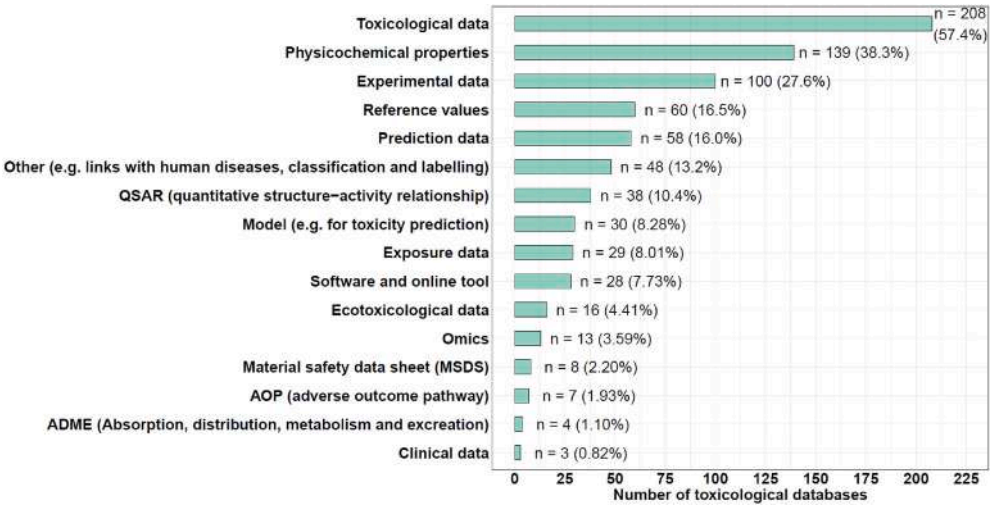

Fig. 4. Number of toxicological databases by data type.

experimental data provided results for 1 to 1,563 different species. There were many types of assays that were reported, in particular carcinogenicity, mutagenicity, genotoxicity, hepatotoxicity, acute toxicity, developmental toxicity and skin sensitization tests.

Several toxicological DBs provided prediction data (16%) as well as different models (8%) that can mostly be used to predict toxicity. Toxicity prediction was usually performed with quantitative structure-activity relationship (QSAR) (n = 38), read-across, structure-activity relationship (SAR), or quantitative structure-property relationship (QSPR) models.

A total of 60 (17%) of the DBs gathered reference values. There were also 12 DBs that reported threshold of toxicological concern. A broad range of reference values were recorded, such as cancer slope factors, unit risks or tolerable daily intake. Most of the time, for each reference value, the chemical, the route of exposure, the targeted population, the adverse effect, the target organ, the type of reference value (e.g., acute, chronic) and the year of establishment was provided.

Other information was also available. There were 15 DBs (4%) that recorded safety data, such as material safety data sheets (n = 8). A total of 13 (4%) DBs reported omics data, with 9 DBs that gave information on toxicogenomics, 3 on metabolomics and 1 on chemogenomics. A total of 12 DBs recorded classification and labeling information according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS) and European Union (EU) rules. There were 3 DBs that recorded links between chemicals and human diseases or conditions.

3.2.2. *Exposure databases*

Exposure DBs are useful for estimating exposure levels and profiles, estimating the number of individuals exposed, identifying exposure determinants, tracking co-exposures/multiple exposures, triggering and guiding medical surveillance, estimating spatial and temporal exposure traceability, etc. Among the 715 DBs identified, 382 (53.4%) were classified as chemical exposure DBs. There were many types of exposure DBs, such as exposure measurement DBs (71%), epidemiological DBs (13%), job-exposure matrices (JEM) (9%), exposure information systems (9%), national registers (3%), clinical DBs (3%) and occupational health surveillance systems (1%) (Fig. 5). In addition, around 20% of the exposure DBs were web applications/tools or provided different statistical models that were used, for example, to estimate and/or rank exposure levels or to conduct health risk assessment. A wide variety of exposures to chemicals were covered, with between 1 and 103,817 chemicals reported. Most data originated from original studies (48%)

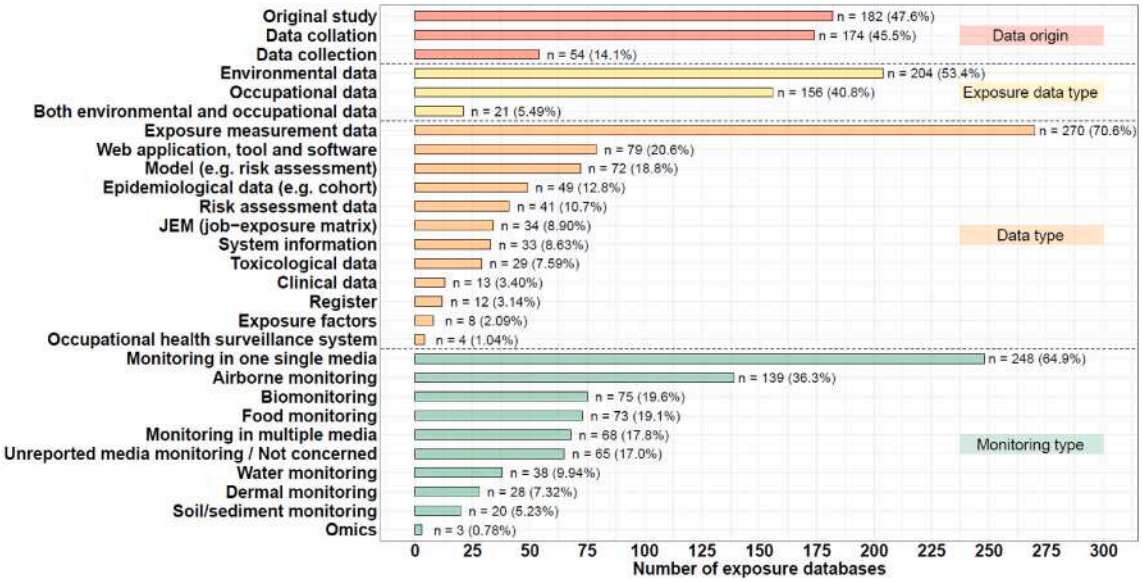

Fig. 5. Number of exposure databases by data type.

and data collation – i.e., compilation of data directly from the source (e.g., companies, physicians, hygienists, agencies) - (46%). Some exposure DBs pertained solely to environmental exposures (53%) or to occupational exposures (41%) while 6% of them related to both. Most exposure DBs had a national stature. Occupational exposure DBs usually represented multiple industries, occupations, and workplaces over multiple years and sometimes decades.

National registers provided information on the number of exposed people (mostly workers) and their exposure to given chemicals (usually carcinogens). In these registers, only a few chemicals were recorded. Exposure information systems also provided information about the number of people exposed, the levels of exposure, the places where the exposure occurred and sometimes emission inventories. Clinical DBs gathered cases, exposure information and factors seeking to explain disease occurrence or prognosis, while epidemiological DBs recorded data to study exposure determinants that play a role in the occurrence of a given disease in a particular human population. Regarding JEMs, they were commonly used to assign exposure estimates, within a given industry, department or for a specific job title, to subjects in epidemiological studies. JEMs were usually created for occupational exposure but also existed for the general population.

Regarding exposure measurement DBs, some of them had administrative purposes and were made for insurance, control, compliance or regulatory purposes. This was in particular true for occupational exposure DBs. Within industry, chemical exposure measurements were typically carried out for the purpose of compliance with occupational safety and health regulations or under specific circumstances (e.g., incidents, turnarounds). When reported, there were between 50 to more than 2 million samples recorded in exposure measurement DBs. DBs with a high number of samples were usually made from data collation and/or literature search (data collection). Most samples were measured into one single media (65%). The monitoring strategies the most used were airborne monitoring (37%), biomonitoring (19%) (e.g., urine, blood) and contaminant monitoring in food (19%) (Fig. 5). Airborne monitoring consisted of measuring the concentrations of chemicals in the air. Biomonitoring consisted of measuring the concentrations of chemicals and/or their biomarkers in body fluids (e.g., blood, urine, and breast milk) or tissues (e.g., hair, nails, fat, and bone). In contrast to targeted analyses of single compounds, exposomic biomonitoring, which are untargeted analyses of many analytes using omics technologies (e.g., metabolomics), were still rare (only 3 DBs identified). Some exposure measurement DBs contained measurements of numerous chemicals, while others focused on one family of compounds.

3.2.3. *Proof of concept – PAH exposure measurement databases*

Among the 270 exposure measurement DBs identified, a total of 71 (26%) contained information about exposure to PAHs. Occupational exposures to PAHs were investigated alone in 49% of the DBs and in parallel to environmental exposures in 10% of them. For most DBs (73%), it was possible to collect the full list of PAHs and/or PAH biomarkers. A total of 28% DBs reported measurements of PAH substitutes (products containing or emitting PAHs) and 18% did not report which PAH congeners were measured. Most DBs recorded airborne samples (73%), with between 1 and 39 PAHs measured. Only 8% of the DBs reported both airborne monitoring and biomonitoring. When biomonitoring was conducted (31% of the time), between 1 and 38 biomarkers were measured, with 1-hydroxypyrene levels reported most of the time (82%).

3.3. *Online inventory – TEDI*

The results of the inventory were implemented online. Fig. 6 presents a screenshot of the TEDI tool. On top of the web page (on the left), there are 8 tabs, written in orange fonts, that can be clicked on. The active tab has an orange background and a bold black font. The first three tabs (starting from the left) display as interactive tables the toxicological, exposure and PAH exposure measurement DBs, respectively. The "*Reference*" tab provides a table listing all of the references mentioned in the reference column of the three aforementioned tables. The "*News & Articles*" tabs will report articles describing, using or citing TEDI. The "*Help/Info*" tab provides general information regarding TEDI, such as the goal, strengths and limitations of the tool as well as information regarding table interactivity, information pertaining to condition of use, data privacy, data protection and legal mentions and how to report bugs or mistakes. The "*Contact*" tab provides contact information details while the "*Mentions légales*" tab stipulates the legal mentions in French.

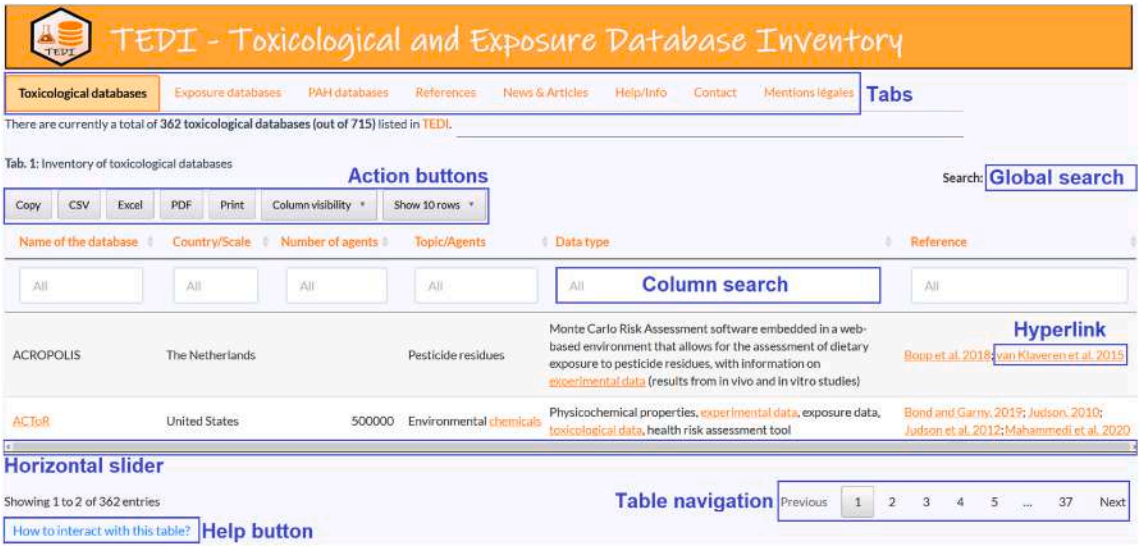

Fig. 6. Screenshot of the TEDI app

Orange fonts that are underlined refer to hyperlinks providing either the definitions and descriptions of terms or direct access to the mentioned references. Buttons allow user actions such as copying the table to clipboard, saving the table as CSV/XLS/PDF, printing the table and selecting columns to visualize. It is possible to make global (entire table) and column specific queries. Beware, when opening TEDI on a web browser there are several horizontal sliders that will be available, with one on the web page itself, one inside the toxicological DB table (at the bottom) and one inside the exposure DB table (at the bottom). To access all of the available information reported in these two tables, the user has to use the different horizontal sliders (moving it on the right or left) or to download the table.

All of the tables available on TEDI are interactives. There is a help button below each table, named *'How to interact with this table?'*, which explains how to interact with all the tables. On top of each table (on the left), there are 7 action buttons. The *"Copy"* button allows the user to copy the table to clipboard. The *"CSV"*, *"Excel"* and *"PDF"* buttons enable the user to export the table to CSV, XLS or PDF format, respectively. The *"Print"* button allows the user to print the table. The user can choose which columns to visualize using the *"Column visibility"* button. The last button, *"Show 10 rows"* allows the user to choose the number of rows to display. When the former button is not set on the "*Show all rows"* option, the user will have to click on the buttons under the table (on the right) to navigate through the table. Beware, for exporting or printing the entire table, the user needs to first click on the number of rows to display button (named *"Show 10 rows"* by default), then choose the "*Show all rows"* option and finally to click on one of the three export/download buttons.

The interactive tables allow the user to perform queries, either on a specific column or on the entire table. The global search is performed across all searchable columns and is starting when typing text on the *"Search"* rectangle/bar located on top of the table (on the right). Searching on individual columns can be performed by typing text on the white rectangle/bar below each column header. Queries are not case sensitive and provide on-the-fly filtering with immediate feedback to the user. For the global search, the order of the words in the query does not matter. Clicking on the cross symbol that appears on the right of the query will cancel the search. Columns containing numbers (e.g., number of data) can be filtered either using the rule that appears when clicking on the column search rectangle under the column header or by typing the following command in the search rectangle cell: *'LB … UB'*, where *LB* and *UB* are the lower and upper bounds, respectively.

It is possible to sort the table based on one column by clicking on a column double arrow head located on the right of each column header. Clicking on the arrowhead facing the top of the web page will sort the chosen column either alphabetically or in ascending numerical order, depending on the type of data the column contains (numerical values or texts). It is also possible to change the order of the columns of a table by clicking and dragging the column header to the left or to the right.

In each column, cells with terms underlined and written in orange font refer to special terms and abbreviations that can be clicked on to display the full definition/details. References and access links (last column of each table) are also underlined and written in orange font and can be clicked on to open the website where the paper/record can be viewed/obtained. Note that empty cells correspond to non-reported values.

4. Discussion

Toxicological and exposure DBs are complementary tools for conducting risk assessment that can help linking exposures to adverse outcomes, establish reference values, develop primary prevention and thus decrease disease incidence (Atwood et al., 2019). An online inventory collecting meta-data on various toxicological and exposure DBs was created. A total of 715 DBs were catalogued, described and are available on TEDI.

4.1. Remarks about databases identified

4.1.1. Data availability and geographical coverage

The understanding of, access to and acquisition of relevant information was often difficult. For many DBs, especially exposure DBs, the summary information reported was very limited. Several available resources (papers, reports, and websites) lacked necessary details to provide adequate descriptions of the DBs. In addition, some DBs had no name (7.2%) and it was difficult if not impossible to identify the proper contact person for potential correspondence and access to additional information. Based on the current inventory, DBs developed for Eastern Europe, Southern Europe, Africa, South America, Latin America and Asia were lacking.

4.1.2. Data accuracy and quality

The identified DBs contained a wealth of information that was relevant to risk assessment, but the value of these sources could not be considered equal. According to some authors, the quality and accuracy of available information greatly varies and is inconsistent from one DB to another, which has a direct impact on the quality and reproducibility of the resulting risk assessment that may be conducted (Bopp et al., 2018).

DBs were heterogeneous in nature, not only in terms of objectives and strategies but also in the accuracy and completeness of information available as well as in the general architecture and the semantics used. This heterogeneity mainly came from different economic, technical, practical and human choices that had been made by the founder(s)/creator(s) of each DB as well as the disparate purposes for which DBs were developed.

4.1.3. Toxicological databases

For most existing chemicals, there are no comprehensive toxicological data or some form of human health risk assessment has not been undertaken (Wignall et al., 2018). In addition, determining which available toxicological DB is the most suitable for addressing the goal(s) of a study depending on the desired aim might be potentially difficult (Laamanen et al., 2008). Indeed, the reported health effects could be different from one DB to another, depending on the methods, tests, experimental conditions, assumptions made, chemicals and species used. In addition, the degree of reliability and validity of the available data could highly vary, making it difficult to determine which relevant information to use (Beck et al., 2016).

4.1.4. Exposure databases

Most exposure DBs provided low temporal resolution information due to the complexity of gathering complete, rich long-term time-series data. Only a sample of the whole population of interest was usually studied, which may only represent a narrow picture of the development of a disease. In addition, most exposure DBs (52%) reported results from monitoring of chemicals in various media/matrices (e.g., food, water, sediments). While these types of monitoring are easier to undertake than monitoring chemicals in human body fluids or tissues, they do not provide information on individuals but only on media contamination, which makes the results sometimes difficult to interpret and not necessarily useful for the establishment of prevention and protection measures.

Regarding occupational measurement DBs, chemical exposure measurements were typically carried out for the purpose of compliance with occupational safety and health regulations or under specific circumstances (e.g., incidents, turnarounds), which explained why the focus was most often on carcinogens and high exposure tasks/events.

4.2. Limitations of this work

While a comprehensive literature search was conducted, the search was not exhaustive and not completely systematic. The search sentence may have not yielded every possible existing toxicological and exposure DBs, therefore results presented in this work only reflects this query. Hence, the identification of some type of DBs (e.g., JEMs) was not optimal. For instance, when screening records related to exposure data, it was found that some exposure models were either part of exposure DBs or provided exposure measurement data. Therefore, they were classified/considered as a category of exposure DBs in this work. If a literature search had been conducted using specific keywords related to exposure models, it would have likely allowed for the identification of more exposure models than what was found in this work. The same is true for other categories of toxicological and exposure DBs. This might

also explain why some DB categories, in particular exposure measurement DBs, were more identified than other exposure DB categories.

Only one author conducted the inventory and created the online inventory. Therefore, it is possible that some DBs may have been missed and the presence of some mistakes (e.g., typos) and bias cannot be excluded. Two independent reviewers may have decreased the chance of systemic error and bias. In addition, the literature search was conducted using only one source (PubMed). To conduct a more comprehensive and systematic work, other bibliographic DBs such as Web Of Science, Scopus or Dimensions could have been used. The information reported on TEDI came from screened records (mostly papers) that may not report the most up-to-date information regarding the DB(s). Thus, some information on TEDI is likely to be incorrect and is subjected to evolve if new data become available.

Potential misclassification of DBs is possible since the content of several DBs spanned multiple categories so that DBs often do not sit comfortably in a single category. This is not surprising because risk is a function of hazard and exposure and therefore some degree of exposure information can come into play during hazard identification and the establishment of dose-response relationship. This explained why some DBs (4.1%) were classified as both toxicological and exposure DBs. Another definition regarding toxicological and exposure DBs could have resulted in another DB classification.

TEDI provides access to 6 descriptors for toxicological DBs and to 12 descriptors for exposure DBs. The level of description of each DB reported in TEDI is heterogeneous and remains fairly general due to the broad scope of the literature search, which lead to a high number of data sources that differed in nature and content. Indeed, information reported in the records screened were found to be highly diverse and inconsistent across studies resulting in challenges summarizing relevant information from the reviewed records. Most of the time, many interesting and important information were not available, such as the database availability (e.g., public), the terms of use, the full list of chemicals, the number of variables (columns) and observations (rows) available or the database format (e.g., MS Excel sheet). Some information, such as the full list of chemicals are not available in TEDI, because this information was often unavailable and because it was not possible and feasible to list every chemical available in each DB due to the high number of chemicals sometimes available (up to several millions). Therefore, it is unfortunately not currently possible to pull up information on a specific chemical, with the exception of PAHs.

4.3. Perspectives from this work

As pointed out before, this work is far from being exhaustive, both in terms of DBs listed and in terms of information and meta-data retrieved. The inventory and its website remain open for new entries and a more global coverage would certainly be preferable to support the broader objectives to support human health risk assessment, which could help promote collaborative and harmonized research in this area.

A possible evolution of TEDI would be to integrate new core descriptors and meta-data from each DB, in particular the creator, the owner, the contact person, the data availability, the full list of chemicals, the coding system(s) used, the time period considered (e.g., data from 1995 to 2010) and the status of the DB (e.g., still maintained). These descriptors were initially considered but were most of the time missing from the records screened, and were therefore not collected and not recorded. The proof of concept showed that this was possible, at least for a specific type of DB, for a specific family of chemicals (PAHs) and for one of the missing descriptors. Another possible evolution of TEDI could be to focus on specific chemical families and to perform the same work than the one that was done for PAHs.

To collect the missing aforementioned meta-data and descriptors, another perspective from this work could be to send a questionnaire to each contact person of the identified DBs. Such initiative, which was beyond the scope of this work, would allow for the correction of potential mistakes and bias currently present in TEDI. It would also allow for the improvement of the level of description of each DB reported on TEDI.

4.4. Comparison with other international efforts to compile databases

To the best of the author's knowledge, to date, there is no review providing a comprehensive compilation of existing toxicological and exposure DBs, consolidated in a single resource that can support chemical risk assessment. Previous reviews on the subject in the past 15 years have only focused either on DBs that can assist computational, drug discovery and system toxicology (usually in the public domain) or on exposure DBs regarding carcinogens in the workplace. In addition, with the exception of one study (Pawar et al., 2019), existing reviews only listed a few DBs (no more than 41). TEDI, which listed 715 DBs focusing solely on chemicals, was complementary to other existing reviews and provided more information about existing toxicological and exposure DBs and their characteristics related to the chemical risk assessment. As a result, most DBs mentioned in TEDI (81%) were not listed in any other reviews.

Regarding toxicological DBs, one study listed 15 toxicological (*in vitro*, in vivo toxicity and ontology) public DBs that can be used for assisting computational toxicology (Judson 2010). Another study provided a comprehensive inventory of available data resources to support computational toxicology, which is complementary to TEDI (Pawar et al., 2019). In their work, they listed more than 900 chemistry, drug discovery, ADME, toxicological, clinical trials, biological, omics, protein-protein interactions, patents, pathways, animal alternative methods and nanomaterials DBs. However, 88% of the DBs listed in TEDI are not listed in their review. Another study reported 18 DBs on carcinogenicity and mutagenicity that are available in the public domain (Benigni et al., 2013). A review from 2014 described development of various bioinformatics tools that may be applied in the field of biodegradation (Arora and Bae 2014). This review also provided the list of 32 chemical DBs that enable classification identification and risk assessment of chemicals or that describe their environmental properties, toxicity and distribution. Another study published in 2014 described 14 toxicogenomics and toxicology DBs that can address question within systems toxicology (Fostel et al., 2014). A more recent study reported a list of 41 publicly available DBs that provides information on hazards and risks of industrial chemicals, and assessed the quality of each DB described (Bond and Garny 2019). An inventory of around 100 in silico resources (DBs, software, models and applications) that can be used for physiologically-based kinetic modeling and to predict physicochemical properties and chemical similarity was also published that year (Madden et al., 2019).

Regarding exposure DBs, a report from the European Risk Observatory provided the list of 26 carcinogen exposure DBs and evaluated these existing sources of information, identified major knowledge gaps and described some new approaches needed to assess and prevent occupational cancer risks (Liβner et al., 2014). A recent overview reported an online inventory on exposure assessment tools (https://occupationalexposuretools.net) (Peters et al., 2021). Meta-data on 36 general population JEMs, 11 exposure DBs, and 29 occupational coding systems from more than 10 countries were available. This inventory was created within the Network on the Coordination and Harmonization of European Occupational Cohorts (OMEGA-NET) framework, which aims to enable optimization of the use of industrial and general population cohorts across Europe to advance etiological research. This inventory reported less exposure DBs than TEDI, however, information recorded were more descriptive and accurate as they were obtained directly from DB owners.

Regarding health risk assessment tools, one study introduced and compared seven air pollution health risk assessment tools (Hassan Bhat et al., 2021). Other authors conducted a systematic review of risk assessment tools for contaminated sites (Mahammedi et al., 2020). For

each of the 31 identified tools the relevant risk assessment stages, harm type, hazard category, receptor type and pathways were reported.

There are other complementary resources worth mentioning that can provide with apposite and useful information. For instance, the *Nucleic Acids Research* journal has an online molecular biology DB collection that amounts to 1,737 DBs that falls into 15 categories such as metabolic and signaling pathways or microarray data and other gene expression DBs (Rigden and Fernandez 2018). The Global Burden of Disease (GBD), which is an international consortium, provides a tool to quantify health loss from more than 350 diseases, injuries, and risk factors, in 195 countries, by age and sex, from 1990 to the present. The GBD project collected, generated and analyzed over 1 billion data points (Shaffer et al., 2019). Finally, TEDI could also be expanded to include open exposure DBs from citizen science projects, which have not been found with the search strategy used in this work.

5. Conclusions

Toxicological and exposure DBs play an important role in determining the relationships between exposures and diseases. Access to this type of information is clearly needed and although numerous DBs have been compiled, they are often extremely difficult to find and navigate. This paper presented a searchable web-based inventory (TEDI) of 715 toxicological and exposure DBs that might benefit human health risk assessment resulting from the chemical exposome. Each DB had its own strengths and limitations, depending on the problem formulation, purpose of the assessment, regulatory context, relevance of the exposure, population, and chemical(s) considered in relation to the outcome(s) of interest. The available information regarding each DB was often very limited. In addition, a dearth of information remains, in particular for developing countries, for some life stages of an individual (e.g., pregnancy, childhood, late adulthood), mixtures of compounds, transformation products and new substances/emerging risks. Only a small fraction of chemicals, their life cycle and the chemical exposome were covered. Many challenges and data gaps remain, as outlined above. New studies to bridge the existing gaps are therefore needed.

TEDI is of value to the broader community and could support human health risk assessment to chemicals in various contexts. TEDI can help researchers, risk managers, risks assessors, practitioners, decision makers, stakeholders, sanitary agencies … in finding the available resources useful for assessing health risk. This is particularly true in the context of the launch of the European PARC (Partnership for the Assessment of Risks from Chemicals) project, which aims in particular to promote harmonization of data as well as tools and methods for conducting chemical risk assessment (Zare Jeddi et al., 2021). TEDI remains open for further additions, to enlarge its coverage and include newly developed DBs.

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Declarations of interest

None.

Author contributions - CRediT author statement

Pascal Petit: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing – Original Draft, Writing - Review & Editing, Visualization, Supervision, Project administration.

The work reported in the paper has been performed by the author, unless clearly specified in the text.

Data availability statement

The data that support the findings of this study are openly available on TEDI at https://exporisk-timc.imag.fr/TEDI/. Further information is available from the corresponding author upon request.

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