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Association between greenspace and lung function in Italian children-adolescents

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ABSTRACT

Background: Few studies have examined the impact of urban greenspace exposure on lung function in children-adolescents, and the available evidence is still inconsistent. The aim of the current study was to verify the hypothesis that the effects of greenspace exposure vary with differing levels of lung function of children-adolescents.
Methods: Between November 2005 and May 2006, 2150 children-adolescents (age-range: 10–15 years) living in the city of Palermo were enrolled in a cross-sectional survey. Parents were interviewed through a modified version of the Italian Studies on Respiratory Disorders in Children and the Environment (SIDRIA) questionnaire. All children-adolescents performed spirometry and were tested for allergic sensitization. Exposures to greenspace and grey space at the home addresses were measured using the CORINE land-cover classes. Parametric quantile regression models were applied for assessing the association between greenspace exposure and spirometry parameters, accounting for possible confounders and effect modifiers. A p-value lower than 0.05 was considered statistically significant.
Results: From the 1st to the 21st percentile, children-adolescents living within greenspace had higher FEV_1 than those living within grey space. In particular, the estimated effects were: 1st ($\beta = 0.238$ L, $p = 0.01$), 5th ($\beta = 0.140$ L, $p = 0.01$), 10th ($\beta = 0.097$ L, $p = 0.015$), and 15th ($\beta = 0.073$ L, $p = 0.025$).
Similarly, from the 1st to the 29th percentile, children-adolescents living within greenspace had higher FVC than those living within grey space. In particular, the estimated effects were: 1st ($\beta = 0.367$ L, $p = 0.0003$), 5th ($\beta = 0.215$ L, $p = 0.0003$), 10th ($\beta = 0.150$ L, $p = 0.0004$), and 15th ($\beta = 0.112$ L, $p = 0.001$). No significant associations were found for FEV_1/FVC, FEF_{25-75} and FEF_{25-75}/FVC.
Conclusion: Quantile regression techniques may provide new insights into the evaluation of the association between greenspace exposure and lung function in children-adolescents, showing substantially heterogeneous effects from lower to higher quantiles of spirometry parameters. These results may help implementing policies for planning sustainable housing and surrounding greenspaces.

1. Introduction

In the last decade, a worldwide increase in the prevalence of respiratory and allergic diseases has been observed in children-adolescents. Environmental exposures play an important role in the increased prevalence of respiratory and allergic diseases (Sly et al., 2016), especially in children-adolescents.

Access or exposure to greenspaces within the urban context may influence human health through many different pathways which may promote healthy lifestyle and decrease environmental hazards, contributing to reduce the risk of adverse health outcomes (Markevych et al., 2017). In particular, a low exposure to greenness, as measured by normalized difference vegetation index (NDVI), i.e. ≤0.15 (1st quartile), has been associated with a higher risk of nasal symptoms in children living in an

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urban environment (Cilluffo et al., 2018). Similarly, a protective effect of greenness exposure within 100 m from residential address has been observed for lifetime wheezing (Eldeirawi et al., 2019). Differently, results from nine European cohorts provided evidence that exposure to greenspaces is associated with increased respiratory disease in children (Parmes et al., 2020). Thus, the role of greenness exposure on allergic respiratory diseases in children is still controversial (Ferrante et al., 2020; Lambert et al., 2017) and there is very limited understanding of the impact of greenspace on atopic sensitization (Lambert et al., 2018).

Few studies have examined the impact of urban greenspace exposure on lung function in children-adolescents, and the available evidence is also inconsistent. Recently, Kuiper et al. reported that greenness exposures (both lifetime and during 10–18 years exposure) increased the risk of low FEV_1 and FVC (Kuiper et al., 2021). In addition, some studies reported no effect (Agier et al., 2019; Boeyen et al., 2017), whilst other studies described a protective effect (Fuertes et al., 2020; Paciência et al., 2019; Squillacioti et al., 2020; Zhou et al., 2021) of greenness on lung function parameters.

The aforementioned studies investigated the association between greenspace and lung function using conventional regression techniques, assuming that greenness exposure has a homogeneous effect across the distribution of lung function parameters. However, this assumption may not be valid if the same exposure has heterogeneous effects, e.g. a relatively larger effect in children-adolescents with lower lung function, but no effect or even a negative effect in children-adolescents with normal or higher lung function.

Possible heterogeneity in the effects of greenspace on lung function parameters has not been investigated so far. To fill this research gap, we used quantile regression models to assess the effect of greenspace that might vary across the distribution of lung function parameters measured by spirometry. Quantile regression models have already provided new insights in pulmonary function research (Bottai et al., 2011; Zhang et al., 2015).

The aim of the current study was to verify the hypothesis that the effects of greenspace exposure vary with differing levels of lung function of school children-adolescents in the city of Palermo, Italy.

2. Materials and methods

Between November 2005 and May 2006, a sample of children-adolescents (age-range: 10–15 years) living in the city of Palermo were enrolled in a cross-sectional survey (Cibella et al., 2011). Palermo is a Mediterranean area of Southern Italy, located in the northwest part of the Sicily island, on the Gulf of Palermo in the Tyrrhenian Sea (38°06′56″N 13°21′41″E). At the survey time, Palermo had about 670,820 inhabitants, according to the 2006 registry office. It has a Mediterranean climate characterized by hot and dry summers with mild temperatures for the rest of the year. The municipality was subdivided in three geographical zones (coastal, downtown, and hilly) and 16 schools with 9922 children-adolescents were selected. From them, 2481 children-adolescents were randomly selected (one every four). 2150 of them were able to perform a valid spirometry. The study was approved by the Institutional Ethical Committee. All parents signed written informed consent.

2.1. Questionnaire

Parents were interviewed through a modified version of the Italian Studies on Respiratory Disorders in Children and the Environment (SIDRIA) questionnaire (Asher et al., 1995; Galassi et al., 2006).

Lifetime asthma was defined as a positive answer to the question ‘Have you ever had asthma?’. Lifetime rhinoconjunctivitis was defined as a positive answer to both the questions ‘Have you ever had a problem with sneezing, or runny, or blocked nose apart from common cold or flu?’ and ‘Has this nose problem been accompanied by itching and/or watering eyes?’ (Asher et al., 1995). Based on these criteria, we identified three subgroups: children-adolescents with lifetime asthma (A), children-adolescents with lifetime rhinoconjunctivitis (RC); and children-adolescents with no history of asthma or rhinoconjunctivitis (nAnRC). The three subgrups were divided in any allergic disease (A or RC) vs no allergic disease (nAnRC). Eczema (E) was defined as positive answers to both the questions ‘In the last 12 months, have you had an itchy rash which was coming and going for at least six months?’ and ‘Has this itchy rash at any time affected any of the following places: folds of elbows, behind the knees, in front of the ankles, under the buttocks, or around the neck, ears, or eyes?’.

Gender information was provided by school official documents. The gender was only categorized as male and female. Height (in cm) and weight (in kg) were measured in standing position without shoes, using a stadiometer. Obesity was defined according to the WHO classification (Onis et al., 2007): weight-for-height greater than 2 standard deviations above WHO Child Growth Standards median. Information on environmental exposures was also collected. Lifetime mold exposure was evaluated through the question: ‘Have you ever seen mold/dampness/fungi on the walls or on the ceiling of your bedroom?’. Current passive smoking exposure was assessed through the question ‘Are there smokers at home?’. Self-reported traffic exposure was recorded as the frequency of vehicles passing on the street of residence on weekdays (never/-rarely/frequently/constantly): children-adolescents were considered exposed if they answered ‘frequently’ or ‘constantly’. A crowding index was defined as the number of co-residents per household, divided by the number of rooms, excluding the kitchens (except kitchens included in dining rooms) and bathrooms (Audino et al., 2014). Physical activity was defined as a positive answer to the question: Do you perform physical activity for at least 3 times per week? Parental history of asthma was defined as at least one parent with personal history of asthma. Long-term residence was defined as $\geq$8 years at the same address (Pénard-Morand et al., 2010).

2.2. Skin prick tests

Allergic sensitization was defined as at least one positive (wheal $\geq$3 mm) skin prick test for a panel of common aeroallergens (*dermatophagoides* mix, cat, dog, *blattella germanica*, grass mix, *parietaria judaica*, olive, alternaria). Allergens were grouped into outdoor (grass mix, *parietaria judaica*, olive), indoor (*dermatophagoides*, dog and cat dander, and *blattella germanica*) allergens, and spores (alternaria).

2.3. Spirometry

Spirometry was performed through a portable spirometer (Micro-Loop, Micro Medical, Chatham Maritime, Kent, UK) in accordance with ATS/ERS guidelines (Miller et al., 2005). Individual spirograms are ‘‘acceptable’’ if they are free from artefacts, they have good starts (extrapolated volume $\leq$5% of FVC or 0.15 L) and they show satisfactory exhalation (for $\geq$ 3–6 s according to age and volume–time curve, no change in volume for $\geq$1 s - plateau criteria).

Out of three acceptable tests, the best forced vital capacity (FVC, i.e. the maximal volume of air exhaled with maximally forced effort from a position of full inspiration) and the maximal expiratory volume in the first second (FEV_1, i.e. the maximal volume of air exhaled in the first second of a forced expiration from a position of full inspiration) were retained, from which the FEV_1/FVC ratio was computed as a marker of airway obstruction. The forced mid-expiratory flow ($FEF_{25–75\%}$, i.e. the mean forced expiratory flow between 25% and 75% of the FVC) was selected from the manoeuvre with the largest sum of FEV_1 and FVC. At last, FEF_{25-75}/FVC ratio was also computed as marker of dysanapsis (Parker and McCool, 2002).

2.4. Geocoding

The addresses at the survey time were obtained using the questionnaire filled by parents. We geocoded the addresses of the children-

adolescents using ArcGIS for Desktop 10.3. Only 3.2% of the children-adolescents did not have valid coordinates and they were excluded from the analyses.

2.5. CORINE land-cover classes

The CORINE (Coordination of information on the environment) framework is a Europe-wide satellite-based inventory of land-cover developed by the European Environmental Agency in order to create a Geographical Information System (GIS) for providing information on the environment. The CORINE programme categorized land-cover into 44 classes at a scale of 1:100,000, updated in 2006. CORINE land-cover classes (CLC) are organized into three hierarchical levels (Level 1: five categories; Level 2: fifteen categories; Level 3: forty-four categories) based on the area unit definition. For each home address, a class was assigned based on the forty-four categories of Level 3 with a 100 m resolution. In our study, the following classes were identified: grey space (1.1.1 Continuous urban fabric, 1.1.2 Discontinuous urban fabric, 1.2.3 Port areas) and greenspace (1.4.1 Green urban areas, 2.1.1 Arable land, 3.1.1 Broad-leaved forest, 3.2.1 Natural grassland, 3.2.3 Sclerophyllous vegetation). More detail is reported in supplementary material.

2.6. Statistical analysis

Data were presented as absolute and percentage frequencies or as mean and standard deviation. Differences of categorical variables were assessed through the Chi-squared test. Quantitative variables were compared through the Kruskal Wallis test to avoid distributional assumptions. Missing values were imputed through a nonparametric technique based on Random Forest, as implemented in the "missForest" R package (Stekhoven, 2015).

In order to explore the distribution of the outcome of interest, outlier detection was carried out using the "mvoutlier" R package (Filzmoser and Gregorich, 2020). Since defining outliers by using a fixed threshold is rather subjective, two different methods were used. The first one marks observations as outliers if they exceed a certain quantile of the chi-squared distribution. The second "adjusted quantile" is an adaptive procedure searching for outliers specifically in the tails of the distribution, beginning at a certain χ2-quantile (Filzmoser et al., 2005).

Parametric quantile regression (PQR) models (Frumento and Bottai, 2016) were applied for assessing the association between greenspace exposure and spirometry parameters. The quantile function of PQR is defined as:

$$Q(p|x) = x^T\beta(p)$$

with

$$\beta(p) = \theta b(p)$$

where $Q(p|x)$ is the quantile (p) function of a response variable of interest (spirometry parameters) conditional on a q-dimensional vector of observed covariates (x), $b(p)$ is a basis functions of p, and θ is a q $\times$ k matrix with entries θ_{jk}. Specifically, in our example we have:

$$FEV_1(p|greenspace,\ z) = \text{greenspace}^*\beta(p) + z^T\eta(p)$$

with

$$\beta(p) = \eta(p) = \theta \log(p)$$

This statistical approach simultaneously regresses all the percentiles of the outcome distribution, and is particularly useful when information provided by the mean is affected by the presence of outliers. Moreover, extreme quantiles such as the 1st, 5th, 10th and 15th, may be of greater interest than the mean for clinical practitioners, as individual measures comprised within these quantiles may suggest abnormalities in the lung function.

The outcomes of interest were FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{25-75}/FVC. Outcomes were expressed in L, in L/sec or dimensionless (observed values) since GLI reference equations did not perfectly fit the population-based samples of schoolchildren from Southern Italy (Fasola et al., 2017).

The independent variable of interest was greenspace (Y vs N). Moreover, models were adjusted for the following confounders known to possibly affect the evaluated outcome variables: age (continuous variable), gender (Male vs Female), height (continuous variable), obesity (Y vs N), smoke exposure (Y vs N), mold exposure (Y vs N), parental history of asthma (Y vs N), indoor sensitization (Y vs N), outdoor sensitization (Y vs N), crowding index (continuous variable), physical activity ($\geq$3 times per week) (Y vs N), eczema (E), subgroups of children-adolescents (any allergic disease vs no allergic disease) and high vehicular traffic (Y vs N).

The Kolmogorov-Smirnov statistic was used to test the goodness of fit of the model for assessing different b(p). The Kolmogorov-Smirnov statistic allows assessing goodness of fit by testing the null hypothesis that the cumulative density function (CDF) values follow a standard uniform distribution. A uniform distribution for the CDF indicates that the model is correctly specified. Since the CDF values depend on estimated parameters, the distribution of the test statistic is not known. To evaluate it, the model is fitted using Monte Carlo simulations. QR was estimated using the "qrcm" R package (Frumento, 2017). A sensitivity analysis excluding children-adolescents with A, RC and/or E (thus, including only nAnRCnE) was carried out. In order to examine the effects of the long-term exposure, a model restricted to children living $\geq$8 years at the same address was also estimated.

Analyses were performed with R 4.0.0 software. A p-value lower than 0.05 was considered statistically significant.

3. Results

3.1. Characteristics of the study sample

Over a total of 2150 children-adolescents, it was not possible to geocode the home addresses for 78 children-adolescents. Thus, 2082 (96.8%) children-adolescents with valid addresses were retained in the study.

Only 0.6% of values regarding anthropometric characteristics, host and environmental factors, and spirometry were missing: thus, they were imputed. Fig. S1 illustrates the missing percentage for each variable (represented by a vertical line) of each child (represented by a horizontal line).

Fig. 1 illustrates the land cover map of the area derived from the CORINE database. Black points indicate the residences of children-adolescents, while colored polygons identify the CORINE land cover categories, as shown in the legend of the Figure. Spatial distribution of home addresses was quite uniform.

Table 1 reports the characteristics of the study sample by grey space and greenspace exposures. Children-adolescents living within greenspace were slightly younger, reported more frequently eczema in the last 12 months, and were less exposed to high vehicular traffic than those living within grey space.

The distributions of spirometric parameters are reported in Fig. 2. No difference was found between children-adolescents living within grey space and those living within greenspace, but some parameters (FEV_1, FVC and FEF_{25-75}) were characterized by long tails.

3.2. Outlier detection

Fig. S2 highlights the presence of outliers in the outcomes. The first panel (A) shows the data, the second panel (B) reports the distribution function of χ^2 and the two vertical lines correspond to the 97.5 χ^2-quantile and to the adjusted quantile. The third panel (C) shows the outliers detected by the 97.5 χ^2-quantile distribution (n = 426), and the

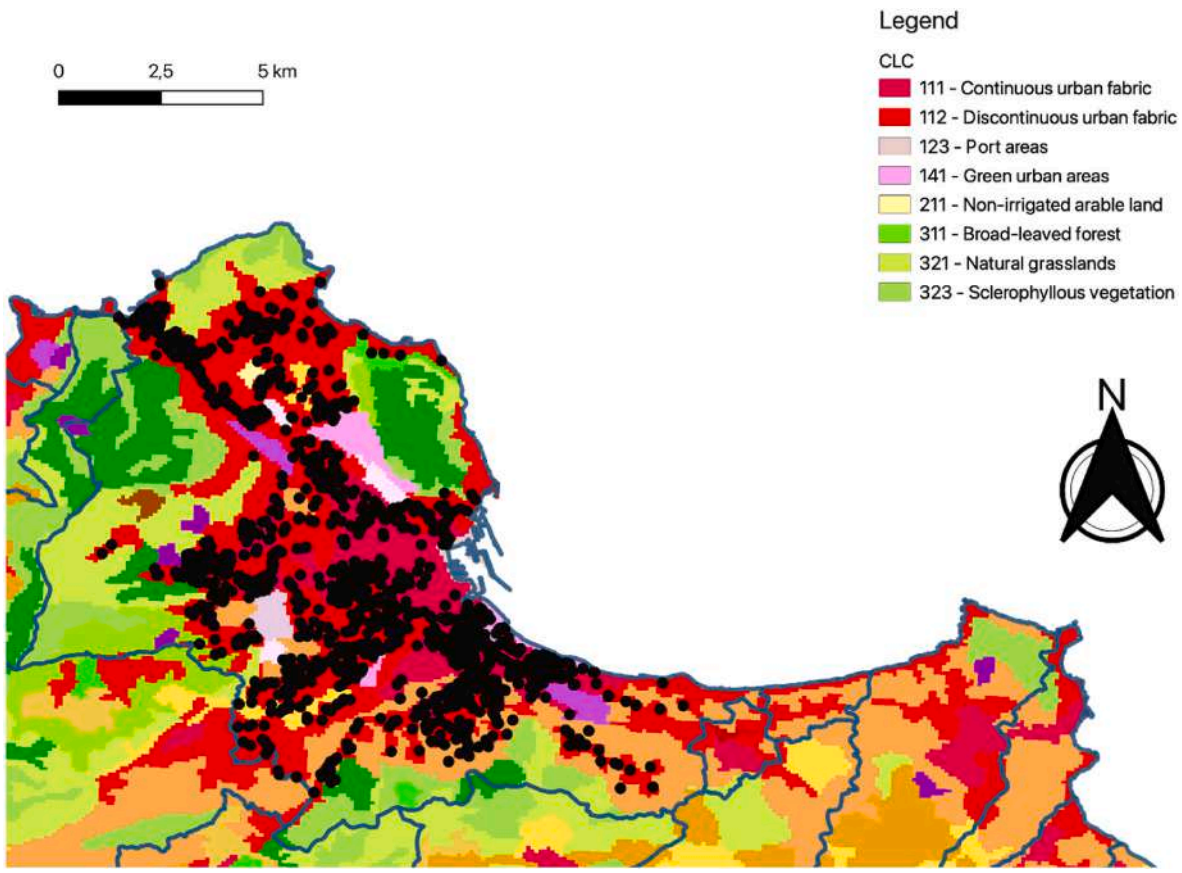

Fig. 1. Corine Land Cover map of geo-coded children-adolescents in the city of Palermo (38°06′56″N 13°21′41″E). Points indicate the residences of children-adolescents.

fourth panel (D) shows the detected outliers by the adjusted quantile (n = 377). Since quantile regression is not influenced by outliers, no observation was excluded from the analyses. On the other hand, the presence of large number of outliers rules out the possibility to use an ordinary least square model.

3.3. Quantile regression models

Based on the goodness of fit, the models were not correctly specified with linear, second and third order polynomials, thus we chose the log (p) specification for b(p) (Table S1). Fig. 3 illustrates the greenspace effect on FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{25-75}/FVC over all the percentiles. When the black dashed line crosses the confidence bands, the effect of greenspace on spirometry parameters is not significant. Blue dashed lines represent the effect estimated using ordinary least squares (OLS), red dashed lines are the confidence intervals of the OLS estimates. From the 1st to the 19th percentile, children-adolescents living within greenspace had higher FEV_1 than those living within grey space. In particular, the estimated effects were: 1st (β = 0.238 L, p = 0.01), 5th (β = 0.140 L, p = 0.01), 10th (β = 0.097 L, p = 0.015), and 15th (β = 0.073 L, p = 0.025). Similarly, from the 1st to the 29th percentile, children-adolescents living within greenspace had higher FVC than those living within grey space. In particular, the estimated effects were: 1st (β = 0.367 L, p = 0.0003), 5th (β = 0.215 L, p = 0.0003), 10th (β = 0.150 L, p = 0.0004), and 15th (β = 0.112 L, p = 0.001). No significant association was found for FEV_1/FVC, FEF_{25-75} and FEF_{25-75}/FVC.

3.4. Sensitivity analysis

Fig. S3 illustrates the greenspace effect on FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{25-75}/FVC throughout all the percentiles after exclusion of children-adolescents with A, RC and/or E. From the 1st (β = 0.28 L) to the 28th (β = 0.06 L) percentile, children-adolescents living within to greenspace had higher FEV_1 than those living within grey space. In particular, the estimated effects were: 1st (β = 0.279 L, p = 0.013), 5th (β = 0.172 L, p = 0.008), 10th (β = 0.125 L, p = 0.007), and 15th (β = 0.098 L, p = 0.009). Similarly, children-adolescents living within greenspace had higher FVC than those living within grey space from the 1st to the 30th percentile. In particular, the estimated effects were: 1st (β = 0.395 L, p = 0.008), 5th (β = 0.236 L, p = 0.001), 10th (β = 0.168 L, p = 0.001), and 15th (β = 0.128 L, p = 0.001). No association was found for FEV_1/FVC, FEF_{25-75} and FEF_{25-75}/FVC. Fig. S4 illustrates the greenspace effect on FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{25-75}/FVC throughout all the percentiles, when restricted to those children-adolescents with long-term exposure: associations did not change substantially.

4. Discussion

This study provides new insight into the relationship between greenspace exposure and lung function in children-adolescents. Quantile regression models allowed a more comprehensive assessment of the effect of greenspace exposure across the whole lung function parameters distributions. After adjusting for potential confounders, we found significant differences in lung function between children-adolescents living within and not living within greenspace. Interestingly, these differences varied in magnitude across quantiles of the lung function parameters distributions; in particular, the effects were larger for lower spirometry quantiles (lower tail). In addition, sensitivity analyses, carried out on a subset of children-adolescents without asthma, rhinoconjunctivitis and eczema, confirmed these findings.

Only few studies investigated the association between greenspace exposure and spirometry parameters in children-adolescents. Since

Table 1
Characteristics of the study sample by grey space and greenspace exposure.

	All	Grey space	Greenspace	p-value
	n = 2082	n = 1875	n = 207	
Gender: Male, mean (SD)	1025 (49.23)	928 (49.49)	97 (46.86)	0.518
Age, years, mean (SD)	12.58 (0.94)	12.59 (0.94)	12.45 (0.96)	**0.033**
Weight, kg, mean (SD)	51.01 (12.93)	51.01 (12.95)	51.01 (12.83)	0.899
Height, cm, mean (SD)	154.18 (8.38)	154.16 (8.42)	154.34 (8.07)	0.635
BMI, kg/m^2, mean (SD)	21.27 (4.32)	21.28 (4.32)	21.21 (4.26)	0.870
Obesity, n (%)	83 (3.99)	76 (4.05)	7 (3.38)	0.778
Subgroups, n (%)				0.939
nAnRC	1517 (72.86)	1365 (72.80)	152 (73.43)	
RC	318 (15.27)	286 (15.25)	32 (15.46)	
A	247 (11.86)	224 (11.95)	23 (11.11)	
Eczema, n (%)	111 (5.33)	92 (4.91)	19 (9.18)	**0.015**
Atopy, n (%)	812 (39.00)	732 (39.04)	80 (38.65)	0.972
Indoor sensitization, n (%)	635 (30.50)	574 (30.61)	61 (29.47)	0.795
Outdoor sensitization, n (%)	486 (23.34)	440 (23.47)	46 (22.22)	0.753
Physical activity (≥3 times/week), n (%)	1052 (50.53)	948 (50.56)	104 (50.24)	0.989
Crowding index	1.06 (0.41)	1.06 (0.41)	1.05 (0.39)	0.838
Environmental exposure				
High vehicular traffic, n (%)	218 (10.47)	211 (11.25)	7 (3.38)	**<0.001**
Mold exposure ever, n (%)	304 (14.60)	264 (14.08)	40 (19.32)	0.054
Current passive smoke exposure, n (%)	1173 (56.34)	1057 (56.37)	116 (56.04)	0.985
Long term exposure[a] (≥8 years)	1346 (64.65)	1216 (64.85)	130 (62.80)	0.611
Spirometry				
FEV_1, L, mean (SD)	2.68 (0.52)	2.68 (0.53)	2.66 (0.45)	0.935
Z-score, mean (SD)	−0.03 (1.02)	−0.03 (1.02)	−0.05 (0.94)	0.907
FVC, L, mean (SD)	2.97 (0.60)	2.98 (0.61)	2.97 (0.53)	0.901
Z-score, mean (SD)	−0.33 (1.02)	−0.33 (1.03)	−0.31 (0.92)	0.913
FEV_1/FVC, %, mean (SD)	0.90 (0.06)	0.90 (0.06)	0.90 (0.06)	0.284
Z-score, mean (SD)	0.58 (1.07)	0.59 (1.07)	0.51 (1.09)	0.259
FEF_{25-75}, L/s, mean (SD)	3.26 (0.84)	3.27 (0.85)	3.19 (0.75)	0.267
Z-score, mean (SD)	0.03 (0.98)	0.04 (0.98)	−0.05 (0.95)	0.216

[a] at residential address. p-values in bold are significant

quantile regression has never been applied to such data, our findings can only partially be compared with previous studies which investigated this relationship using standard approaches such as linear regression on the expected value of lung function. Indeed, Fuertes et al. found a significant positive association between greenspace exposure at 100 and 300 m buffers, measured through NDVI, and spirometry parameters (FEV_1 and FVC) in a population-based birth cohort, using measures of exposure to greenspaces and lung function data at 8, 15 and 24 years (Fuertes et al., 2020). Furthermore, a very recent study carried out on 6740 school-children reported that increasing exposure to greenness measured by NDVI within 500 m was significantly associated with higher FEV_1 and FVC values. Nonetheless, among children exposed to the highest compared with the lowest quartile of particulate matter, increasing NDVI was associated with lower FVC (Zhou et al., 2021).

A study on 187 children (10–13 years old), carried out in Turin, Italy, found a significantly positive association between an extreme value of NDVI (the 3rd vs the 2nd tertiles) and FEF_{25-75}, indicating a particular susceptibility of small airways to the effect of greenness (Squillacioti et al., 2020). Moreover, these authors did not find significant associations on FEV_1 and FVC. In our study, no association between greenspace and FEF_{25-75} was found; a possible explanation for this discordant result may be linked to the different metrics used for greenspace assessment or to the different sample sizes.

Other studies reported no association between greenspace and lung function parameters. In particular, data from the Human Early-Life Exposome (HELIX) study, a European cohort of 1033 children aged 6–12 years, reported that greenspace exposure was not linked to lung function (Agier et al., 2019). Such a study was different from ours regarding sample size and age-ranges. In particular, the different age-distribution may partly explain variations in the observed findings, since the HELIX sample composed by younger children leads to non-comparable times of greenspace exposure with our sample. Indeed, long-term exposure is usually defined as living ≥8 years at the same address (Pénard-Morand et al., 2010), whereas, for example, children <9 years old represented 75% of the HELIX cohort. Our study was characterized by children-adolescents who were older (68% ≥ 13 years old) than HELIX cohort; in addition, most of the children-adolescents, corresponding to 63% of the total, lived at the same residential address for ≥8 years. However, the role of duration of exposure is not clarified yet, indeed, a recent study of Kuiper et al. (2021) found that greenness exposures increased the risk of low FEV_1 in lifetime exposure. Therefore, further longitudinal studies are required in order to clarify the role of the duration of exposure to greenspaces on lung function in pediatric age.

The current study provides novel evidence about the use of advanced statistical analyses applied as a tool to verify the hypothesis that the effects of greenspace exposure vary with differing levels of lung function, thus overcoming usual approaches. In the current literature, linear and logistic regressions are commonly applied, limiting the impact of risk factors only to the conditional mean of the response variable. On the other hand, quantile regression can be considered a complementary tool of the mean regression: it provides a more flexible way to understand the role of potential factors on the dependent variable. The use of quantile regression in epidemiology is gradually emerging, showing that inference on percentiles, rather than just the mean, may help to better understand the effect of various risk factors on health outcomes (Bottai et al., 2011; Bottai and Cilluffo, 2020).

With regard to the pathophysiological interpretation of the findings, there are some possible pathways through which greenspace exposure may influence lung function. First, it is likely that children-adolescents living within greenspaces are less exposed to traffic. However, when our models were adjusted for high vehicular traffic exposure, the observed associations did not change. Moreover, we tested the interaction between high vehicular traffic and greenspaces and no significant differences were found (data not shown). However, high vehicular traffic exposure may still be a confounder by the structural definition, thus adjusting the model for high vehicular traffic is advisable. Second, greenspace might negatively influence lung function through pollen exposure. However, even if in our study it was not possible to distinguish

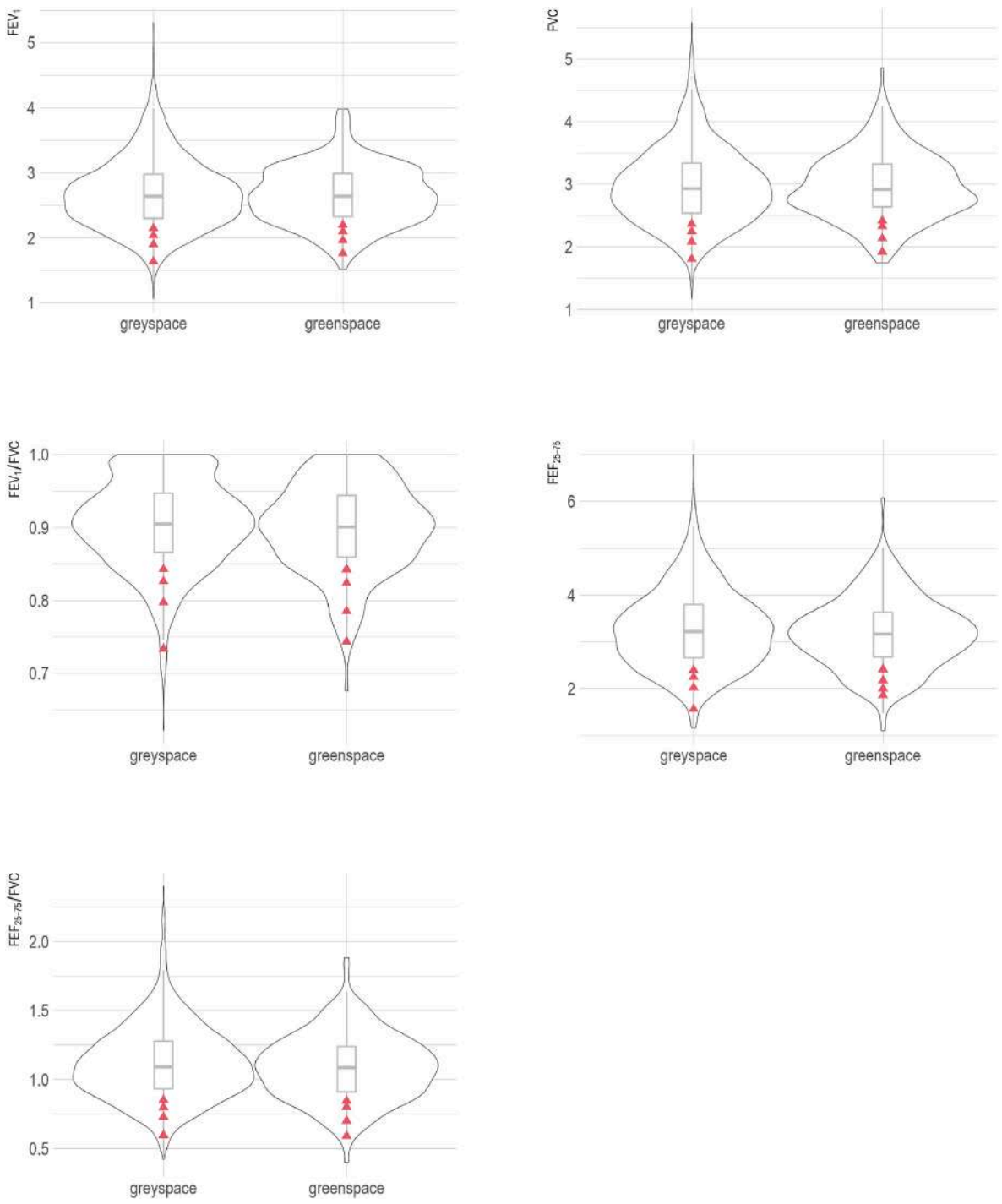

Fig. 2. Distributions of spirometry parameters between children-adolescents living within grey space and greenspace.

for the specific types of vegetation (i.e. pollen or non-pollen vegetation), the analyses were adjusted by indoor and outdoor allergic sensitization, thus the investigated associations were likely not confounded by this covariate. However, when we tested the interaction between greenspaces and atopic status, in order to assess if greenspaces exposure leads to pollen exposure which may reduce lung function mostly in those atopic, no significant differences were found. Since the relationship between lung function and greenness was not altered by atopic sensitization and high vehicular traffic, we can rule out any possibility of overadjustment in our analyses. Third, even if the combined influence of greenspace and human microbiome was not investigated in our study, it might be hypothesized that exposure to greenspace may influence airway's microbiome, which, in turn, could influence lung function development. Indeed, the "biodiversity hypothesis" proposes that biodiversity loss leads to immune dysfunction and chronic inflammatory diseases (Haahtela et al., 2019; Von Hertzen et al., 2011).

Our study has several strengths, such as the large sample of population-based schoolchildren included. Furthermore, the current study benefited from a comprehensive investigation of the association between greenspace and lung function using an advanced statistical approach such as quantile regression. Moreover, we accounted for several potential confounders, including personal and environmental factors. Lastly, we tested robustness of the results through sensitivity analyses.

Our study has also some limitations. First, the cross-sectional design may indicate associations, but it does not allow to make causal

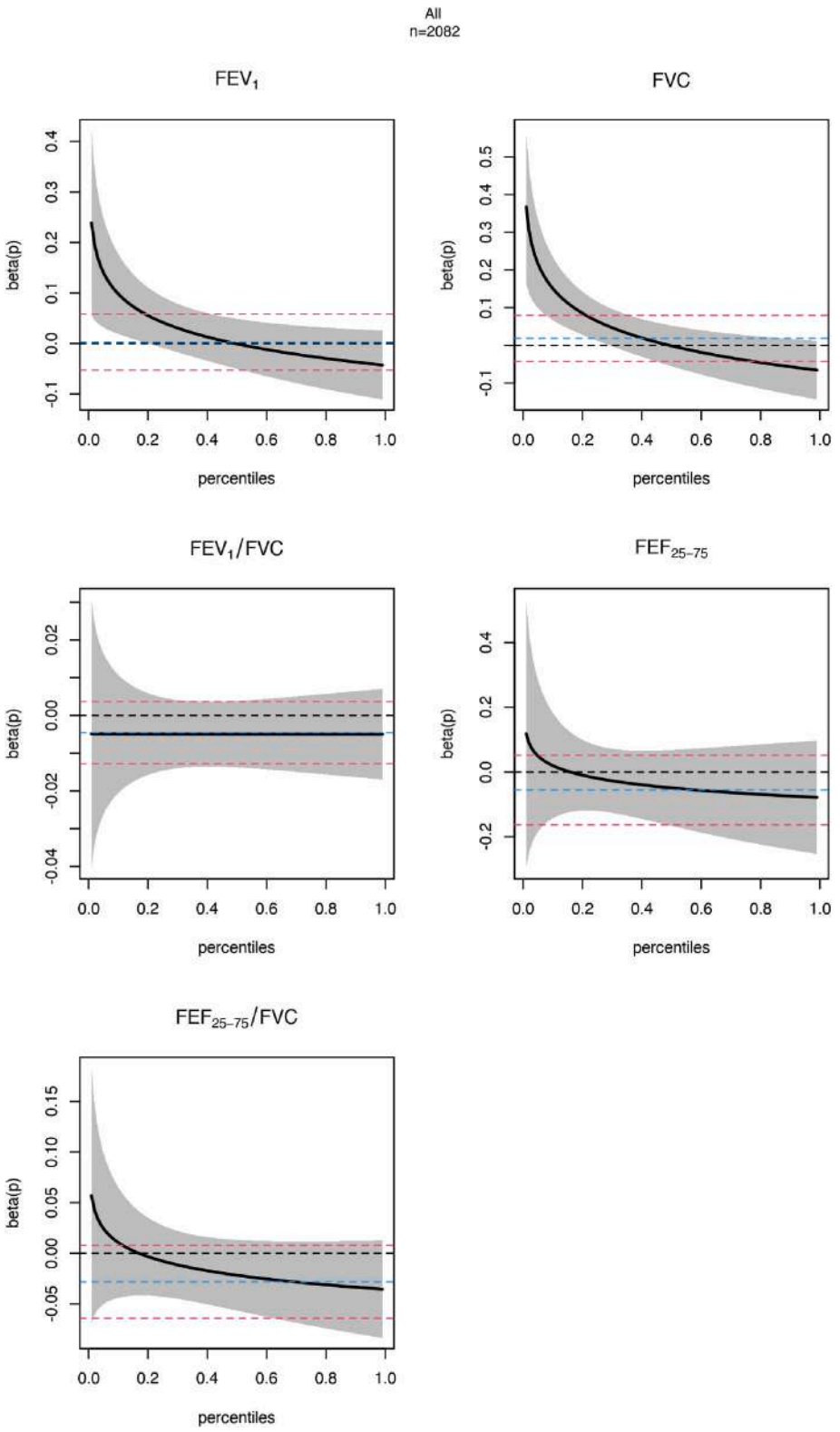

Fig. 3. Greenspace effect on FEV_1, FVC, FEV_1/FVC, FEF_{25-75} and FEF_{25-75}/FVC throughout all the percentiles. The dependent variables are the spirometry parameters, and the explanatory variables are age, gender, height, obesity, smoke exposure, mold exposure, parental history of asthma, indoor sensitization, outdoor sensitization, crowding index, physical activity (≥3 times per week), eczema, subgroups and high vehicular traffic. Confidence bands are highlighted in grey. When the black dashed line crosses the confidence bands, the effect of greenspace on spirometry parameters is not significant. Blue dashed line represents the effect estimated using an OLS, red dashed lines are the confidence intervals of the OLS estimates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

inferences. Information (i.e. mold exposure, lifetime asthma and rhinoconjunctivitis) was collected using a questionnaire, introducing a potential recall bias. However, the SIDRIA questionnaire (derived from the ISAAC one) is a standardized tool that has been widely used in previous published studies. Moreover, using self-reported asthma outcome may likely increase outcome misclassification. Indeed, including an asthmatic in the non-asthmatic group might introduce a bias due to measurement error both in the models adjusted for this factor and in the models of the sensitivity analysis. Another limitation concerns the metrics used for assessing exposure to greenspaces: other more informative metrics such as NDVI should be considered in further studies. Finally, we considered only the level of exposure at home address even if children-adolescents spend some hours in a school setting or in other places.

In conclusion, the application of quantile regression may provide new insights into the evaluation of the association between greenspace exposure and lung function, showing beneficial effects on children-adolescents with lower quantiles of spirometry parameters. Further studies with a longitudinal design, using either conventional or non-conventional regression methods (such as quantile regression) and including interactions between greenspaces exposure and factors such as traffic exposure and atopic status, are warranted. They would provide further evidence on the effect of residential greenspace on lung function and clarify the role of the duration of exposure to greenspaces on lung function in pediatric age. Such studies might help implementing nature-based solutions in the urban context, especially targeted to disadvantaged groups of children-adolescents at risk of impaired lung function.

Ethical approval

The study was approved by local Ethics Committee.

CRediT authorship contribution statement

Conceptualization, G.C.; methodology, G.C.; formal analysis, G.C.; investigation, G.F, D.G., S.R.; data curation, D.G; writing—original draft preparation, G.C.; writing—review and editing, G.F., S.F., F.C. G.V. and S.L.G.; supervision, S.L.G. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.113947.

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Associations of total urinary arsenic with total cholesterol and high-density lipoprotein among 12-17-year-old participants from the 2009–2016 NHANES cycles: A cross-sectional study

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ABSTRACT

Introduction: Hypertension and diabetes are highly prevalent among US adults. Arsenic exposure is associated with these cardiometabolic morbidities but the relationship between arsenic exposure and cholesterol markers of cardiometabolic disease has not been elucidated, especially at younger ages, when many chronic diseases may initiate. This study examined the association of total urinary arsenic with total cholesterol (TC) and high-density lipoprotein cholesterol (HDL) and explored effect modification by weight status.

Methods: The study sample consisted of 12-17-year-old participants with complete data from the 2009–2016 National Health and Nutrition Examination Survey cycles. The cross-sectional associations of creatinine-adjusted total urinary arsenic with TC and HDL were assessed using multivariable linear regression models with survey weights. Three models were built, adjusting for varying combinations of age, gender, race/ethnicity, weight status, survey cycle, family income to poverty ratio, reference person education level, arsenobetaine, and dimethylarsinic acid (DMA). Model adjustments for arsenobetaine approximated inorganic arsenic exposure, and further adjustment for DMA approximated unmethylated inorganic arsenic exposure. We also explored weight status (underweight/healthy, overweight, and obese) as a potential effect modifier of these relationships using stratified analyses and interaction tests.

Results: The final analytical sample consisted of 1,177 12–17-year-old participants. After adjusting for covariates and arsenobetaine, creatinine-adjusted arsenic was positively associated with HDL levels ($\beta = 0.063$; 95% CI: 0.007, 0.119). Upon further adjustment for DMA, creatinine-adjusted arsenic was positively associated with HDL levels ($\beta = 0.079$; 95% CI: 0.015, 0.143) and TC levels ($\beta = 0.258$; 95% CI: 0.002, 0.515). No effect modification by weight status was observed.

Conclusions: We found a positive association of approximated unmethylated inorganic arsenic exposure with TC, and contrary to our expectation, with HDL. There was no effect modification by weight status. Our findings should be confirmed by conducting longitudinal studies among adolescents exposed to low-level arsenic and focusing specifically on urinary inorganic arsenic concentrations.

1. Introduction

High cholesterol is a well-established risk factor for various cardiovascular diseases. Among adults, increased total cholesterol (TC) has been linked to a higher risk of coronary and ischemic heart disease and cerebrovascular disease (Zhang et al., 2003; Jeong et al., 2018). A higher triglyceride (TG)/high-density lipoprotein cholesterol (HDL) ratio is shown to be positively associated with both serum concentrations of TC and the prevalence of hypertension, hypercholesterolemia, and hypertriglyceridemia among adults (Li et al., 2016). Predictors of high-risk carotid intima-media thickness and atherosclerosis in adulthood include borderline low HDL, high low-density lipoprotein cholesterol

Abbreviations: TC, total cholesterol; HDL, high-density lipoprotein; TG, triglyceride; LDL, low-density lipoprotein; NHANES, National Health and Nutrition Examination Survey; As, arsenic; MMA, monomethylarsonic acid; DMA, dimethylarsinic acid; CHD, coronary heart disease; PAD, peripheral arterial disease; MEC, mobile examination center; HPLC, high performance liquid chromatography; CV, coefficient of variation; BMI, body mass index; CAPI, Computer-Assisted Personal Interviewing; SNAP, Supplemental Nutrition Assistance Program; 95%CI, 95% confidence interval.

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(LDL), hypertension, and overweight or obesity (Koskinen et al., 2018). Evidence of tracking TC (Fuentes et al., 2003; Osawa et al., 2021), LDL and TG levels (Harrabi et al., 2010) has been observed among both young children and adolescents. According to the 1999–2006 National Health and Nutrition Examination Survey (NHANES), the prevalence of high TC and LDL was 9.6–10.7% and 5.2–6.6% among children aged 12–17 years old, respectively (Ford et al., 2009).

Arsenic exposure has also been investigated as a risk factor for cardiovascular disease. Whereas organic arsenic and its species such as arsenobetaine and arsenocholine are considered less toxic, inorganic arsenic is highly toxic and is a human carcinogen (ATSDR 1991). In the human body, inorganic arsenic undergoes methylation to form monomethylarsonic acid (MMA), which has high toxicity, while a second methylation converts MMA to dimethylarsinic acid (DMA), a species thought to be less toxic (Basu et al., 2011). DMA is also formed as a result of the metabolism of organic arsenic compounds such as arsenolipids and arsenosugars (Raml et al., 2005; Schmeisser et al., 2006). Growing interest in arsenic exposure is due to the widespread exposure through common sources including drinking water and consumption of various foods (Signes-Pastor et al., 2017; Chen et al., 2019). Greater consumption of fish, grains, fruits, nuts, seeds, and rice was associated with elevated concentrations of MMA and DMA among people of all age groups (deCastro, Caldwell et al., 2014). High-chronic arsenic exposure through drinking water among adults has been associated with an increased risk of coronary heart disease (CHD) and peripheral arterial disease (PAD), carotid atherosclerosis, and a moderate risk of stroke (Wang et al., 2002; Moon et al., 2012; Tsuji et al., 2014; Xu et al., 2020). Mechanistically, inorganic arsenic exposure is hypothesized to disrupt the signal transduction pathways regulating glucose uptake and elevate other components of the metabolic syndrome including plasma lipids (Wang et al., 2007).

While the risk of cardiovascular disease has been strongly associated with cholesterol levels and increasingly with arsenic exposure among adults, the direct relationship between arsenic exposure and lipid levels remains unclear, particularly among children and adolescents. Additionally, findings from rat model studies indicated induced dyslipidemia and alterations to various types of lipids including TG following chronic and intergenerational exposure to low-level arsenic in drinking water (Afolabi et al., 2015; Wang et al., 2015; Rivas-Santiago et al., 2019). It is critical to investigate these associations further to determine the extent to which arsenic exposure contributes to chronic disease processes at earlier life stages, particularly given evidence of tracking high lipid levels among children.

The goal of this study was to investigate the association of total urinary arsenic levels with HDL and TC among adolescents participating in the NHANES cycles 2009–2016. The associations of inorganic arsenic exposure, approximated by adjusting the analytical models with arsenobetaine, and further with DMA, with HDL and TC were explored. An additional objective was to determine if weight status modified the aforementioned associations.

2. Methods

2.1. Study design

This nationally representative, cross-sectional study measured total urinary arsenic, serum HDL, and serum TC levels from participants in the NHANES 2009–2010, 2011–2012, 2013–2014, and 2015–2016 cycles. The NHANES sample represents the civilian, non-institutionalized US population of all ages. Participants are recruited annually through stratified, multistage probability sampling procedures (CDC 2017). All data collection procedures and study protocol were approved by the National Center for Health Statistics Research Ethics Review Board (CDC 2017).

2.2. Population

Participants aged 12–17 years old (N = 4130) from the 2009–2016 NHANES were eligible for this study and were included if they had complete information on total urinary arsenic, DMA, HDL, TC, arsenobetaine, creatinine, and body mass index (BMI). Adolescents who lacked complete information on these variables were excluded from analyses.

2.3. Assessments

2.3.1. Urine and blood sample collection

Per NHANES protocol, individuals aged 6 years or older from a random one-third subsample of all NHANES participants were eligible for urinary arsenic measurements. One spot urine sample was collected in the mobile examination center (MEC), whereas a second urine sample, a morning void, was collected at the participant's home. All participants aged 6 years and older were eligible for blood cholesterol tests. Non-fasting blood samples were collected during MEC appointments in all participants aged 1 year or older. These were drawn from a vein of the participant's arm by a phlebotomist. For tests that required blood specimens, the following individuals were excluded: people with hemophilia, individuals who received chemotherapy within the prior 4 weeks, and those with a presence of rashes, gauze dressings, casts, edema, paralysis, tubes, open sores or wounds, withered arm or limb, missing, damaged, sclerosed or occluded veins, allergies to cleansing reagents, burned or scarred tissue, or shunt or intravenous lines on both arms.

2.3.2. Laboratory assays

Total urinary arsenic concentrations were assessed from the spot urine samples using inductively coupled-plasma dynamic reaction cell-mass spectrometry (ICP-DRC-MS) at Centers for Disease Control and Prevention (CDC), Atlanta, GA (Tanner and Baranov 1999). Speciated arsenic levels in the urine were measured using high performance liquid chromatography (HPLC). The lower limits of detection for total urinary arsenic were 0.74 μg/L (2009–2010), 1.25 μg/L (2011–2012), and 0.26 μg/L (2013–2016) (NHANES 2011; NHANES 2013; NHANES 2016; NHANES 2018). The total urinary arsenic concentrations below the lower detection limit were imputed with the value equal to the detection limit divided by the square root of two. Urinary creatinine concentrations were also measured in the spot urine sample using the Roche/Hitachi Modular P Chemistry Analyzer (Roche Diagnostics 2016). Total urinary arsenic concentrations were adjusted for urinary creatinine concentrations (μg arsenic/g creatinine).

Serum samples were sent to the University of Minnesota, Minneapolis, MN for the determination of cholesterol levels. The analysis of HDL cholesterol consisted of adding a magnesium/dextran sulfate solution to the serum sample. A reagent was then added to convert HDL-cholesterol esters to HDL cholesterol. Hydrogen peroxide was produced when HDL cholesterol was acted upon by PEG-cholesterol oxidase, which then reacted once more to form a color mixture that was photometrically measured at 600 nm. The analytical process makes the HDL components more reactive and, thus, more readily measured. HDL analyses were run in duplicate with a coefficient of variation (CV) of 2–3.5%. For TC, esterified cholesterol was converted to cholesterol by cholesterol esterase which then reacted with cholesterol oxidase to form hydrogen peroxide and cholest-4-en-3-one. Then the former reacted with aminophenazone in the presence of peroxidase in a Trinder reaction, and the product was absorbed at 520 nm. The rate of absorption is directly proportional to the concentration of cholesterol in the serum. The Roche Modular P chemistry analyzer (Roche Diagnostics 2016) was used to measure TC in serum. Measurements were conducted in duplicate and the CV ranged 1.5–1.8%. LDL and triglycerides were measured in a sub-sample of NHANES participants, therefore, ~60% of our study participants had missing data on these variables. Hence, these measures

were not part of the current study.

2.3.3. Anthropometric examinations

Weight and standing height were measured for all participants 2 years or older in the MEC. BMI was calculated for all participants 2 years and older. For this study, BMI was categorized based on percentiles for a child's sex and age (in months) according to the CDC growth charts (Kuczmarski et al., 2002). Weight status categories were defined as follows: underweight (less than 5th percentile); healthy weight (5th to less than 85th percentile); overweight (85th to less than 95th percentile); and obese (95th percentile or greater).

2.3.4. Sociodemographic data

Sociodemographic information was collected during home interviews conducted by trained interviewers equipped with Computer-Assisted Personal Interviewing (CAPI) systems. Information of gender, age, race/ethnicity, socioeconomic status including reference person's highest level of education in the household and family income was self-reported. Family income was assessed using the family's income to poverty ratio, categorized on the basis of Supplemental Nutrition Assistance Program (SNAP) eligibility as $\leq$1.30, 1.31–3.50 and $>$ 3.50 (Johnson et al., 2013). Missing data of household reference person education level and ratio of family's income to poverty were imputed by weighted hot-deck imputation using PROC SURVEYIMPUTE procedure in SAS (SAS Institute Inc, 2015).

2.4. Statistical approach

All statistical analyses were conducted using appropriate survey weights in SAS version 9.4 (SAS Institute Inc., Cary, NC). Frequencies and medians (where applicable) with corresponding 95% confidence intervals (95% CI) of sociodemographic, anthropometric, and biochemical characteristics of study participants were estimated based on survey weights. Descriptive characteristics were compared between the final analytical sample and age-eligible participants who were excluded from the analysis because of missing data.

Linear regression models with survey weights were used to estimate the association of creatinine-adjusted total urinary arsenic with TC and HDL concentrations. Covariates added to the model were identified based on existing literature that suggested their association with arsenic exposure and serum lipids (Porkka et al., 1994; Postma et al., 2011; Kant and Graubard 2012; Zhang et al., 2018). Covariates included age, gender, race/ethnicity, weight status, education level of household reference person, ratio of family income to poverty, and data cycle. Three covariate-adjusted models were built for both TC and HDL. Model 1 included adjustments for all of the aforementioned covariates; model 2 included adjustments for covariates and arsenobetaine to explore the associations between inorganic arsenic and the outcomes; and model 3 included adjustments for covariates, and arsenobetaine and DMA together, to explore the associations between unmethylated inorganic arsenic and the serum lipid outcomes.

To examine whether weight status modified the association of creatinine-adjusted total urinary arsenic concentrations with TC and HDL levels, models were stratified by weight status categories as

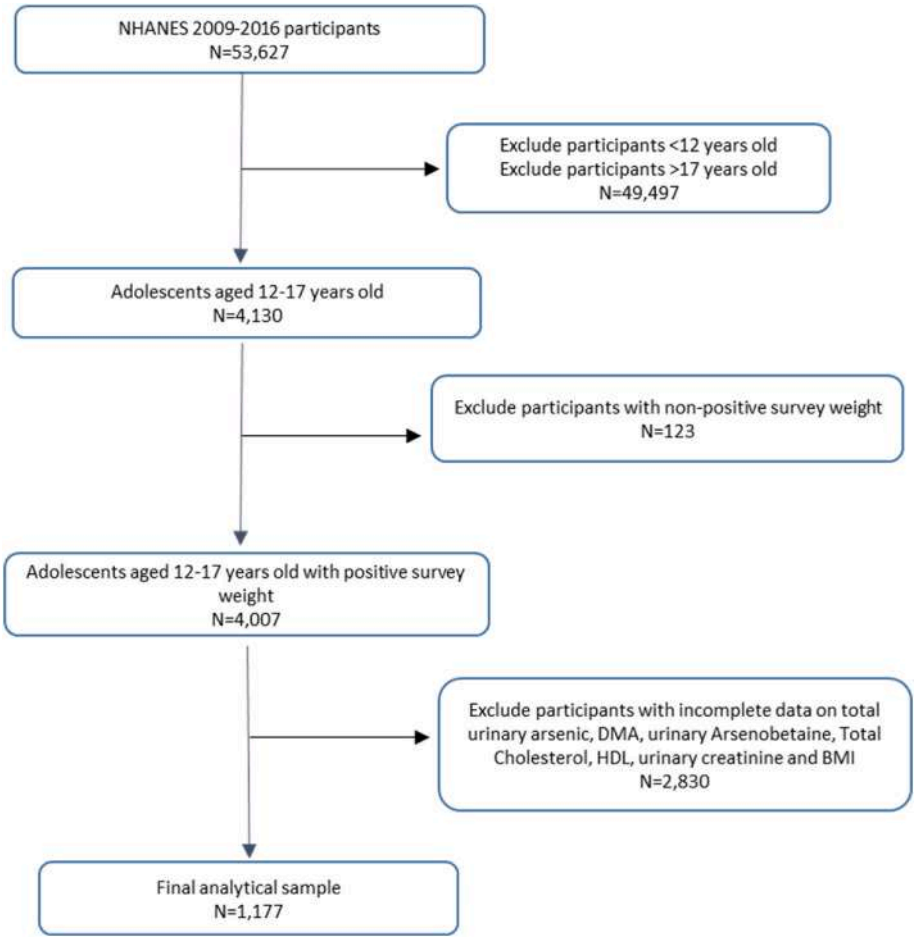

Fig. 1. Sample selection of 12-17-year-old National Health and Nutrition Examination Survey participants (2009–2016) for the study on the relationship between arsenic exposure and serum cholesterol.

underweight/healthy, overweight, and obese. In addition, the interaction term between creatinine-adjusted urinary arsenic and weight status was included in corresponding linear regression models, and the interaction was deemed statistically significant at p-value <0.05.

3. Results

The final analytical sample consisted of 1177 12–17-year-old participants from the 2009–2016 NHANES cycles with complete information on total urinary arsenic, DMA, TC, HDL, urinary arsenobetaine, urinary creatinine, and BMI (Fig. 1).

Table 1 describes the distribution of sociodemographic and biochemical characteristics of study participants. Forty-seven percent and 53% of the study participants were aged 12–14 and 15–17 years old, respectively. Forty-nine percent of the study sample were males, and 56% were Non-Hispanic White. Supplemental Table 1 presents the comparison of characteristics between participants who were included in the analysis to those who were excluded because of missing data; these groups were similar in their characteristics. Medians and corresponding 95% CI of arsenic, creatinine-adjusted arsenic, HDL and TC in different categories of participant characteristics are presented in Supplemental Tables 2 and 3.

Table 2 presents the beta coefficients and 95% CI that estimated the crude and covariate-adjusted associations of creatinine-adjusted total urinary arsenic with TC and HDL. No evidence for an association was found for either outcome in model 1, which included adjustments for sociodemographic and anthropometric covariates. However, after additionally adjusting for arsenobetaine to approximate the association between inorganic arsenic exposure and the outcomes, a modest positive association was observed with HDL (β: 0.063; 95% CI: 0.007, 0.119). Upon further adjusting the models for DMA to approximate unmethylated inorganic arsenic exposure, the associations existed for HDL with a β of 0.079 (95% CI: 0.015, 0.143) and TC with a β of 0.258 (95% CI: 0.002, 0.515).

The potential effect modification of the associations of creatinine-adjusted total urinary arsenic with TC and HDL by weight status is shown in Table 3. Statistically significant positive associations were found among underweight/healthy weight participants for HDL and among obese participants for TC, respectively. However, the 95% CI in the weight status categories were largely overlapping for both outcomes, indicating that there was no effect modification by weight status. Consistent with stratified analyses, the p-values associated with the interaction terms were not statistically significant.

Table 1
Descriptive characteristics of the final analytical sample (N = 1177).

Characteristics	N	% (95% CI)[a]
Age		
12-14	572	47.07 (43.33, 50.81)
15-17	605	52.93 (49.19, 56.67)
Gender		
Male	587	49.38 (45.63, 53.13)
Female	590	50.62 (46.87, 54.37)
Race/Ethnicity		
Mexican American	266	14.29 (12.42, 16.16)
Other Hispanic	124	6.54 (5.33, 7.75)
Non-Hispanic White	322	55.61 (52.1, 59.11)
Non-Hispanic Black	291	14.39 (12.6, 16.19)
Other	174	9.17 (7.41, 10.93)
Weight Status		
Underweight	26	1.81 (0.83, 2.79)
Normal	690	60.9 (57.29, 64.51)
Overweight	202	17.17 (14.35, 20)
Obese	259	20.11 (17.27, 22.96)
Education level of Household Ref. Person		
<9th grade	126	7.32 (5.76, 8.88)
9-11th Grade	196	13.49 (11.21, 15.77)
High School or Equivalent	250	21.17 (18.08, 24.27)
College or AA degree	334	31.87 (28.28, 35.46)
College Graduate or above	238	26.14 (22.61, 29.68)
Missing	33	2.43 (1.29, 3.57)
Ratio of family income to poverty		
≤1.30	435	27.76 (24.8, 30.72)
1.31–3.50	422	40.94 (37.06, 44.82)
>3.50	221	31.3 (27.34, 35.26)
Missing	99	6.1 (4.62, 7.57)
Data release number		
2009–2010	324	27.7 (24.42, 30.97)
2011–2012	271	23.85 (20.41, 27.28)
2013–2014	295	22.68 (19.61, 25.75)
2015–2016	287	25.77 (22.56, 28.99)
	Median	95%CI
Urinary Total Arsenic (μg/L)	5.18	4.81, 5.56
Urinary Dimethylarsonic acid (μg/L)	2.96	2.72, 3.2
Urinary Arsenobetaine (μg/L)	0.82	0.56, 1.07
Urinary Creatinine (μg/L)	122.71	115.69, 129.72
Direct HDL-Cholesterol (mg/dL)	49.18	48.36, 49.99
Total Cholesterol (mg/dL)	151.12	148.52, 153.72

Note.
[a] Percentages (%) and 95% confidence intervals (95% CI) were weighted to represent the national level.

Table 2
Associations between creatinine-adjusted total urinary arsenic and serum lipids in a nationally-representative sample of 12-17-year-old children participating of the 2009–2016 National Health and Nutrition Examination Survey (N = 1177).

Serum Lipids	β-Coefficients (95% CI[a])			
	Crude	Model 1[b]	Model 2[c]	Model 3[d]
Total Cholesterol (mg/dL)	0.121 (−0.094, 0.337)	0.121 (−0.07, 0.312)	0.241 (−0.012, 0.495)	**0.258 (0.002, 0.515)**
High Density Lipoprotein Cholesterol (mg/dL)	0.037 (−0.011, 0.086)	0.03 (−0.009, 0.07)	**0.063 (0.007, 0.119)**	**0.079 (0.015, 0.143)**

Note.
Bolded values indicate statistical significance at an alpha level of 0.05.
[a] 95%CI: 95% confidence interval.
[b] Adjusted for age, gender, race/ethnicity, weight status, data release cycle, ratio of family income to poverty and household reference person education level.
[c] Adjusted for age, gender, race/ethnicity, weight status, data release cycle, ratio of family income to poverty, household reference person education level, and arsenobetaine.
[d] Adjusted for age, gender, race/ethnicity, weight status, data release cycle, ratio of family income to poverty, household reference person education level, DMA and arsenobetaine.

4. Discussion

Arsenic is a carcinogenic metalloid found in many foods including rice. This study sought to examine the association of creatinine-adjusted total urinary arsenic exposure with TC and HDL and to evaluate the potential effect modification by weight status among 12-17-year-olds in the United States. We found that higher unmethylated inorganic arsenic was associated with higher TC. Further, contrary to our expectation, we found a modest, positive association of inorganic arsenic concentrations with HDL levels. We found no evidence of effect modification by weight status for either outcome.

Considering cholesterol tracking and the modifiable nature of both arsenic exposure and lipid concentrations to varying extents, evidence on the associations between arsenic exposure and lipid concentrations among adolescents is critical. Akhtar, Roy et al. (2021) examined the longitudinal associations between cadmium and arsenic exposure and multiple biomarkers of cardiometabolic disease, including lipid levels, among children at 4.5 and 9 years of age. Their results indicated that exposure to arsenic in utero was associated with reduced TC and HDL and are in direct contrast to our study findings. A birth cohort study in

Table 3
Associations between creatinine-adjusted total urinary arsenic and serum lipids in a nationally representative sample of 12–17-year-old participants of the 2009–2016 national health and nutrition examination survey stratified by weight status (N = 1177).

	Underweight/ Healthy (n = 716)	Overweight (n = 202)	Obese (n = 259)	P for interaction
Total Cholesterol (mg/dL)				
Crude	0.096 (−0.159, 0.352)	0.281 (−0.037, 0.599)	0.244 (-0.302, 0.789)	0.136
Model 1[a]	0.08 (−0.124, 0.283)	0.216 (−0.025, 0.457)	0.299 (-0.296, 0.895)	0.127
Model 2[b]	0.209 (−0.06, 0.479)	0.216 (−0.025, 0.457)	0.637 (−0.138, 1.411)	0.099
Model 3[c]	0.201 (−0.057, 0.458)	−0.37 (−1.015, 0.275)	**0.87 (0.033, 1.706)**	0.13
High Density Lipoprotein-Cholesterol (mg/dL)				
Crude	0.021 (−0.032, 0.075)	0.053 (−0.067, 0.174)	−0.093 (-0.205, 0.018)	0.654
Model 1[a]	0.036 (−0.01, 0.082)	0.037 (−0.06, 0.134)	−0.106 (-0.233, 0.021)	0.644
Model 2[b]	**0.071 (0.005, 0.137)**	0.037 (−0.06, 0.134)	−0.012 (−0.188, 0.164)	0.776
Model 3[c]	**0.082 (0.007, 0.156)**	−0.088 (−0.319, 0.144)	0.026 (−0.166, 0.219)	0.784

Note.
Bolded values indicate statistical significance at an alpha level of 0.05.
[a] Adjusted for age, gender, race/ethnicity, weight status, data release cycle, ratio of family income to poverty and household reference person education level.
[b] Adjusted for age, gender, race/ethnicity, weight status, data release cycle, ratio of family income to poverty, household reference person education level, and arsenobetaine.
[c] Adjusted for age, gender, race/ethnicity, weight status, data release cycle, ratio of family income to poverty, household reference person education level, DMA and arsenobetaine.

Taiwan found that 14-year-old children in a high and rising total arsenic exposure trajectory were at increased risk of both high TC and LDL levels and increased levels of non-HDL cholesterol compared to those in a stable low exposure trajectory (Kuo et al., 2018).

While our study yielded mixed findings, animal studies have consistently revealed that arsenic exposure has the potential to increase HDL levels (Afolabi et al., 2015; Ahangarpour et al., 2018). In general, however, evidence from human studies has been inconclusive. It should be noted that most existing evidence on the relationship between arsenic exposure and cardiometabolic markers comes from studies of adults, typically with high level of exposure. Karim, Rahman et al. (2013) found that arsenic concentrations in drinking water sources [mean (SD) concentration = 2.3 (2.77) μg/L in nonendemic regions and 173.46 (156.59) μg/L in endemic regions], hair, and nails of 324 adult residents (mean age: ~36 years) of Bangladesh were inversely associated with TC, HDL, and LDL measured in plasma. One study among Korean adults (20 years or older) assessing urinary arsenic and another among Chinese seniors (mean age: 64.26 years) assessing plasma arsenic both found inverse associations between internal arsenic exposure and HDL levels (Bae et al., 2013; Jiang et al., 2021), while another study, also among adults (aged 50–75 years), found no associations of blood arsenic with TC or HDL (Rotter et al., 2015). On the other hand, a cross-sectional study in Chihuahua, Mexico among 1038 adults (mean age = 45.6 years) showed that higher urinary speciated arsenic concentrations were associated with higher odds of diabetes and higher levels of triglycerides, TC, as well as HDL (Mendez et al., 2016). Furthermore, a cross-sectional study in Bihar, India conducted among 150 adults suggested that total arsenic intake from drinking water and food was associated with lower risks of general hypertension and higher levels of HDL (Xu et al., 2021). Findings from a study among Taiwanese adults chronically exposed to arsenic suggest that although urinary arsenic can increase the risks of hypertension and ischemic heart disease, such associations are independent of serum lipid profile (Hsueh et al., 1998). Direct comparison between these studies and our own is not appropriate given the differences in ages and exposure levels. Several reasons could contribute to our observed association between arsenic exposure and HDL levels, including the lack of adjustments for dietary elements as well as other toxicants such as mercury exposure, and low exposure levels. More severe cardiometabolic effects of arsenic exposure are suggested at higher exposure levels (Ledda et al., 2018), thereby highlighting the need to conduct more studies at low exposure levels and to understand if there are exposure thresholds above which such effects are observed.

Several mechanisms may underlie the positive association that we observed between arsenic exposure and TC. Exposure to arsenic in animal models is associated with increased expression of pro-inflammatory cytokines (Calatayud et al., 2014). Additionally, arsenic has contributed to the generation of oxidative stress in humans through the production of reactive oxidative species and free radicals (Tseng 2004). This increased oxidative activity may result in DNA damage in human vascular smooth muscle cells, such as those comprising the cardiovascular system (Lynn et al., 2000). It is also likely that the cardiometabolic effects of arsenic exposure may manifest via alterations to gene structure and expression (Chien-Jen 1994; Chen et al., 1995).

We observed no evidence of effect modification of the arsenic-lipid associations by participants' weight status. One reason for this may be that the variability in urinary arsenic concentrations and both HDL and TC levels were low [median (95% CI) = 5.18 (4.81, 5.56) μg/L for urinary arsenic, 49.18 (48.36, 49.99) mg/dL for HDL and 151.12 (148.52, 153.72) md/dL for TC]. We did not have data on other factors influencing lipid profiles such as physical activity and other lifestyle variables, diets, family history of dyslipidemia, genetic susceptibility etc. Although it is difficult to pinpoint the reasons behind the null results of effect modification in our study, the absence of the aforementioned information, low variability in the exposure and outcome variables, and the low levels of exposure might explain our findings to an extent.

Our study aim was to assess the association of total urinary arsenic concentrations with TC and serum HDL levels. However, total urinary arsenic concentrations include inorganic as well as organic arsenic species. Organic arsenic and its compounds such as arsenolipids, arsenosugars, and arsenobetaine are generally considered less toxic (Navas-Acien et al., 2011). Further, the methylated form of inorganic arsenic, DMA, is the least toxic biproduct of the methylation cycles; it is also formed when organic arsenic compounds are metabolized (Navas-Acien et al., 2011). Assessing the associations of inorganic arsenic exposure with TC and serum HDL levels is critical, given the highly toxic nature of inorganic arsenic (ATSDR 1991). To arrive at the approximate inorganic arsenic exposure in our study, we adjusted our analytical models for arsenobetaine. We further adjusted our models for DMA to approximate the exposure to unmethylated inorganic arsenic and MMA, which have high toxicity. We observed that urinary arsenic concentrations in models adjusted for arsenobetaine (indicating inorganic arsenic) were positively associated with HDL, and the models adjusted for both arsenobetaine and DMA (indicating unmethylated inorganic arsenic exposure) were positively associated with TC and HDL. Our findings need to be confirmed by conducting longitudinal studies among adolescents exposed to low-level arsenic and focusing specifically on measured urinary inorganic arsenic concentrations.

Low-level arsenic exposure has consistently been associated with numerous adverse health outcomes including diabetes (Brauner et al., 2014), cardiovascular disease (Monrad et al., 2017; Xu et al., 2020),

cancers (Zhang et al., 2020), and neurodevelopmental deficits (Tsuji et al., 2015). Hence, despite observing a positive association between arsenic exposure and HDL levels, we do not support any change in arsenic exposure-related recommendations or policies. Rather, our results highlight the need to conduct prospective studies among adolescents from varying geographic areas exposed to low-to-moderate levels of arsenic in relation to their lipid profiles. Understanding the variations in multiple factors such as their arsenic methylation capacities, nutritional status, body compositions, and genetic susceptibility to cardiovascular disease will help in explaining how these variables influence the arsenic-lipid associations.

Our study is not without limitations. The cross-sectional design precludes any statements of causality on the relationship between urinary arsenic exposures and cholesterol markers. Additionally, the conclusions drawn from this study may only be generalizable to adolescents living in the United States or in settings with low-level arsenic exposure. Further, measures of other less toxic urinary arsenic species such as arsenocholine were largely below the limit of detection (91.6%) and did not lend themselves to be included in the analyses. Similarly, we adjusted our analytical models for urinary arsenobetaine concentrations, which gave an approximate idea of the association between inorganic arsenic exposure and the outcomes. The intake of seafood (main source of arsenobetaine) in our population was low. However, we acknowledge that this approach is not ideal particularly in populations with moderate-to-high seafood intake. Further, high level of missing data for triglyceride levels and LDL precluded their inclusion in these analyses. Finally, other toxic metals such as mercury can also influence the level of cardiovascular biomarkers (Eom et al., 2014). We did not include mercury concentrations in the analytical models because of the high proportion of missing data on for that variable.

Nevertheless, this study has numerous strengths. It was conducted using a large nationally representative sample of adolescents from the NHANES. Appropriate survey weights were used in all our analytical models to make the findings representative of the general US population of 12-17-year-olds. Further, our analytical models included adjustment for DMA and arsenobetaine, which helped approximate the association between inorganic arsenic exposure and our outcomes. We examined two different markers of cholesterol levels, providing useful and specific measures of outcomes.

5. Conclusions

We found modest and positive associations of urinary arsenic concentrations that approximated participants' unmethylated inorganic arsenic exposure with HDL levels and TC levels. Our findings need to be confirmed by conducting longitudinal studies among adolescents exposed to low-level arsenic and focusing specifically on measured urinary inorganic arsenic concentrations.

Declaration of competing interest

The authors declared no conflict of interest.

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Appendix A. Supplementary data

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Biomonitoring of per- and polyfluoroalkyl substances (PFAS) exposure in firefighters: Study design and lessons learned from stakeholder and participant engagement

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ABSTRACT

Firefighters may be occupationally exposed to per- and polyfluoroalkyl substances (PFASs) through Aqueous Film-Forming Foam (AFFF), smoke, dust and turnout gear, in addition to other background exposure sources. Epidemiological assessment of PFAS exposure in an occupational cohort of firefighting staff commenced in 2013–2014, following cessation of PFAS-based AFFF in Australian aviation. Here we present the study design and methodology of a follow-up study conducted in 2018–2019. We focus on our experiences engaging with stakeholders and participants with the establishment of an inclusive study group and highlight the key lessons learned from implementing a co-design process in the study. The study included a cross-sectional assessment of blood serum concentrations of 40 PFASs, including perfluorooctanoic acid (PFOA), perfluorooctane sulfonate (PFOS) and perfluorohexane sulfonate (PFHxS), and 14 health-related biomarkers in 799 current and former Aviation Rescue Firefighting Services employees. A large proportion (87%) of the participants from the preliminary exposure study in 2013–2014 were re-recruited in the follow-up study. This enabled further longitudinal analyses in this subset of 130 participants. Participants included employees from different work roles and timeframes, reflecting the periods when three different firefighting foams were utilised in Australia. Establishment of a collaborative and inclusive study group (including stakeholders and participants) contributed to several components of the study design, including the expansion of robust analytical quality assurance and control measurements, and tailoring of communication and dissemination strategies. These outcomes were key factors that improved transparency of the research design, methods and results. Additionally, implementing elements of co-design helped build trust between researchers and participants, which is an important consideration for studies funded by stakeholders related to the exposure source.

1. Introduction

Monitoring human exposure to emerging contaminants that threaten environmental and human health is a vital aspect of public health surveillance and exposure assessment, with increasing global interest in the determinants of exposure and potential health effects (Joas et al., 2012; Latshaw et al., 2017). Per- and polyfluoroalkyl substances (PFASs) are synthetic chemicals classified as contaminants of emerging concern

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that remain stable under varying conditions due to their unique physicochemical properties. These properties make PFASs suitable for a wide range of industrial applications and consumer products, including Aqueous Film-Forming Foam (AFFF) for extinguishing hydrocarbon-fuel fires (Barzen-Hanson et al., 2017; Buck et al., 2011; Wang et al., 2017). Health agencies are investigating blood serum concentrations of PFASs in populations worldwide, particularly in communities affected by environmental contamination and in occupationally exposed cohorts (Frisbee Stephanie et al., 2009; Ingelido et al., 2018; Olsen et al., 2003; Winquist et al., 2013).

Occupational exposure related to repeated use of AFFF in fire emergencies and training is associated with significantly higher PFAS blood serum concentrations in firefighters compared to the general population (Leary et al., 2020; Rotander et al., 2015; Trowbridge et al., 2020). Exposure to PFASs occurs predominately through ingestion and inhalation pathways, including incidental ingestion of AFFF and inhalation of aerosolised AFFF during firefighting activities (De Silva et al., 2021; Rotander et al., 2015). In addition, dermal exposure to AFFF may contribute to exposure in firefighters through direct skin contact. Exposure can also occur without direct AFFF use, such as through smoke, dust and turnout gear, in addition to background exposure sources (De Silva et al., 2021; Muensterman et al., 2022; Peaslee et al., 2020; Tao et al., 2008; Young et al., 2021).

The long elimination half-lives of long-chained perfluoroalkyl acids (PFAAs) contribute to concerns of the potential for these chemicals to adversely impact human health. The half-lives of the main constituents of historical formulations of AFFF range from 1 to 3 years for perfluorooctanoic acid (PFOA) through to 2 to 7 years for perfluorooctane sulfonate (PFOS) and perfluorohexane sulfonate (PFHxS) (Li et al., 2018; Xu et al., 2020). Increased blood serum concentrations of these PFASs are associated with potential health effects, including a reduction in kidney and liver function, increased cholesterol levels and other cardiometabolic health markers, and increased incidence of some cancers. Studies also report adverse effects on reproduction, development and immune function associated with increased PFAS exposure (Gaston et al., 2020; Steenland and Winquist, 2020; Sunderland et al., 2019). However, there have been few longitudinal studies reporting health risks in populations with higher PFAS exposure than the general population.

Airservices Australia (Airservices) manages Aviation Rescue and Firefighting (ARFF) Services at 27 fire stations at major Australian airports. Historically, firefighting activities used PFAA based Lightwater AFFF (3M) for fire training and emergencies from the 1980s until 2001 when the product was phased out of use and replaced with a fluorotelomere-based firefighting foam, Ansulite AFFF (ANSUL). The phase out of Lightwater AFFF was a gradual process over the course of approximately three years. Further, acquirement of additional stations where Lightwater AFFF remained in use delayed the replacement at those stations. In 2010, Ansulite was replaced with a fluorine-free foam formulation (Solberg RF6), which remains in current use.

In 2013–2014, Rotander et al. conducted a biomonitoring study (2013–2014 Airservices Exposure Study) to investigate exposure to and the health effects of PFASs in ARFF staff in Australia (Rotander et al., 2015). Of 731 eligible ARFF employees at that time, 149 employees (20%, 149/731) from 18 stations participated in the study. The study reported elevated serum PFOS and PFHxS concentrations compared to the Australian population (Rotander et al., 2015). PFAS concentrations reflected the timeline of AFFF use. Participants who commenced employment after the phase-out of historical Lightwater AFFF product containing PFOS and PFHxS had serum levels of these compounds that were consistent with those in the general population (Barzen-Hanson et al., 2017; Rotander et al., 2015). Study participants did not have elevated PFOA concentrations compared to the general population, indicating that PFOA concentrations were not associated with AFFF exposure. Further, PFAA concentrations were not associated with any significant change in the assessed biomarkers. While the 2013–2014 Study successfully established that elevated PFAS exposure occurred in the ARFF staff, the low participation rate and sample size and the cross-sectional design limited the assessment of temporal trends and health-related effects in these study participants.

In this paper, we aim to discuss the key lessons learned from engagement with stakeholders and participants throughout the 2018–2019 Airservices Exposure Study (2018–2019 Study), developed as a follow-up study to the 2013–2014 Airservices Exposure Study (2013–2014 Study). We present the study design and methodology, as well as the final profile of the study participants and discuss the outcomes of engagement with stakeholders and participants. We introduce the strategies implemented to increase participant recruitment and considerations for communication of results as elements of co-design. Lessons learned from conducting this study support recommendations for future biomonitoring studies in populations at risk of increased exposure to PFASs, or other emerging contaminants.

2. Methods

2.1. Study establishment and recruitment

2.1.1. Planning and development

The study design for the 2018–2019 Study was developed collaboratively with the establishment of a 'study group' which consisted of members of the 'research team' and members of the 'working team'. The research team included project managers, analytical chemists, toxicologists, epidemiologists, a medical doctor and other research professionals. The working team included employer representatives, employee representatives who worked in different employment positions, and the United Firefighters Union representatives. A series of study group meetings and workshops were held to discuss and agree on the overall aims, study design and methods. During this process the research team presented alternative study designs and discussed strengths and limitations with each design. The working team had the opportunity to discuss the options with their peers between the meetings, prior to decision-making. The study group also identified limitations of the 2013–2014 Study that helped to transform 2018–2019 Study's design. Table 1 presents the major aspects of the 2013–2014 Study that were improved for the current study through this co-design process. An overview of the co-design process is provided in Fig. 1 demonstrating how the study group communication intersected with the different research phases throughout the timeline of the 2018–2019 study.

The study aims, outcomes and expectations were discussed and agreed on by all members of the study group. The rationale for the follow-up 2018–2019 Study was to determine if the occupational health and safety interventions for ARFFS staff, such as the replacement of AFFF foams, effectively reduced PFAS exposure. In addition, the study was designed to contribute to the broader understanding of PFAS exposure and potential relationships with health outcomes. After the initial meetings and workshops, the following aims for the 2018–2019 Study were established:

1. Assess PFAS blood concentrations in Airservices current and former staff and evaluate links to work history
2. Evaluate PFAS exposure trends
3. Assess PFAS relevant biochemical markers and/or confounders associated with PFAS serum concentrations

Table 1
Major aspects of the 2018–2019 Airservices Exposure Study, how these aspects compared to the previous 2013–2014 Airservices Exposure Study and how these aspects were empowered by the study group[a] co-design process.

Aspect	Study Design	Participant eligibility and recruitment	Sample Collection	Sample Analysis and QA/QC	Reporting of results
2013–2014 Airservices Exposure Study	Cross-sectional	Currently employed firefighters	Questionnaires: Paper format Blood sample: Collection at a nearby clinic	Measurements: 10 biomarkers, 15 PFASs. Laboratory method: 200 μL serum for analysis. Analytical replicates, reference material	Communication of results included: - Individual letters - Study report
2018–2019 Airservices Exposure Study	Cross-sectional and longitudinal	Current staff and ex-staff. All positions that may have had contact with AFFF. Focus on recruiting firefighters who participated in 2013–2014	Questionnaires: Option of online or paper format Blood sample: Option of onsite collection or at a nearby clinic	Measurements: 14 biomarkers, 40 PFASs 1 mL serum for analysis. Laboratory method: Analytical duplicates replicated both within and between batches, reference materials and an inter-laboratory comparison—re-analysis of stored serum samples from the 2013–2014 study	Communication of results included: - Individual letters (separate letters for PFAS and biochemical results) - Study report - Study summary - Frequently asked questions document
Strengths of the 2018–2019 Airservices Exposure Study as a result of the improved aspects and co-design process	• The longitudinal design overcame limitations associated with a cross-sectional design (confounding) and enabled assessments of temporal trends, PFAS elimination and half-lives • Co-designing the study allowed an understanding of the expectations from potential participants • Co-designed aims increased participants willingness to participate in the study • The working team suggested to include several working roles in as well as firefighters. Broader eligibility increased the number of participants and enabled possibilities to assess different exposure routes related to differing employment roles.	• By including both current and ex staff, 'healthy worker' bias may be reduced. • The working team acted as a point of contact for queries and concerns for participants throughout the study. This improved communication between participants and the study group	• Co-creation of the questionnaire refined relevant questions • Co-designing of sample collection allowed for improved flexibility with participant work-schedules. • Questionnaire and sample collection options to suit all participants. • The opportunity for onsite blood sample collection limited impact to work time • >The opportunity for offsite blood sample collection enabled separation from the funding agency	• Addition of biomarkers and PFASs in response to recent scientific reporting. • Larger sample volume improved detection limits. • Study group discussions lead to an expansion of the QC/QA. This improved confidence in the results and helped building trust.	• Workshops with the full study group to discuss results helped to provide answers for any new questions. Expectations of dissemination of results were discussed and co-designed to support the appropriateness of the communication of an individual's exposure and health results. • Separate individual letters for biochemical and PFAS results allowed quick reporting of results to participants. • Assistance from the working team when tailoring the communication provided clear language appropriate for the study audience. This was aimed to avoid potential confusion related to technical language or scientific concepts.

[a] The study design for the 2018–2019 Study was developed collaboratively with the establishment of a study group which consisted of members of the research team and members of the working team. The research team included project managers, analytical chemists, toxicologists, epidemiologists, a medical doctor and other research professionals. The working team included employer representatives, employee representatives who worked in different employment positions, and the United Firefighters Union representatives.

4. Provide ongoing advice to Airservices to assess and minimize exposure risks to PFASs.

2.1.2. Recruitment

The target populations for this 2018–2019 Study were current and former ARFF staff with potential exposure to firefighting foams. This included firefighters, instructors, station officers, operation managers and Emergency Vehicle Technicians (EVTs). Including participants from several occupational positions would allow the assessment of position-specific exposures. This decision to broaden the participation from the 2013–2014 criteria to other positions was suggested by the working team to ensure inclusion of all staff with potential exposure to firefighting foams. For example, EVTs would have come into direct contact with AFFF concentrate frequently while working on maintenance and repair of the fire engines. Whereas the main AFFF exposure route for firefighters was diluted AFFF in the firefighting foam used during fire emergencies and training. To enable exposure assessments in relation to the time periods of all firefighting foams, it was decided to recruit both current and former staff members. This allowed coverage of a broad employment history to represent the periods of the three different firefighting foams: Lightwater AFFF (1980–2002), Ansulite AFFF (2002–2010) and Solberg RF6 (2010-current) at the majority of stations. There was no requirement of length of time of employment to participate in the study.

After ethics approval, Airservices invited current and former employees to submit an expression of interest to participate in the exposure

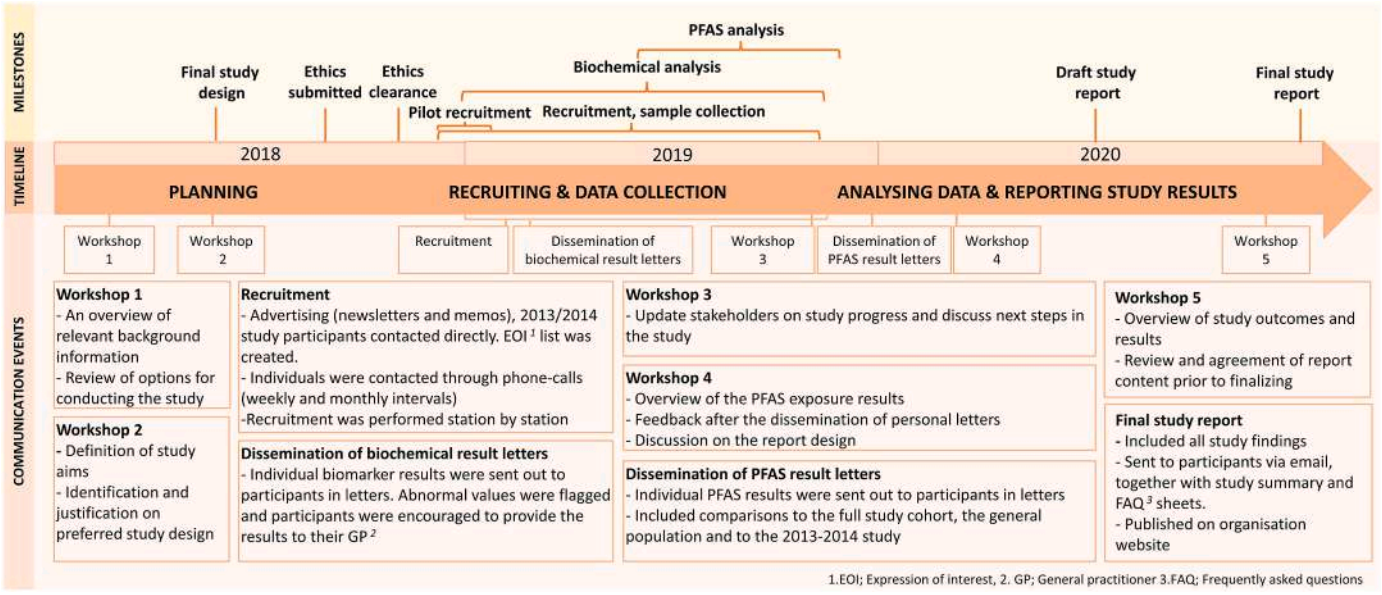

Fig. 1. Study timeline, milestones and main communication events of the 2018–2019 Airservices Exposure Study.

study to the research team. Airservices had no further involvement in the study. The study was promoted both internally and externally on behalf of the study group. People who had previously participated in the 2013–2014 Study were individually contacted and invited to participate.

The working team supported engagement with the study providing a contact point for staff queries and concerns. The working team could address these concerns independently or refer to the research team for further information. A pilot study was conducted at one fire station to test recruitment and data collection prior to recruitment for the main study and feedback was provided by members of the working team. Participants were then progressively recruited across all Airservices fire station locations across Australia. Potential participants were provided with information about the study and the consent form. Consenting participants were asked to submit questionnaires and blood samples. They received a regular reminder until these were received.

2.2. Measurement and analysis

2.2.1. Serum collection

As suggested by the working team, participants were given the option to provide their blood samples at a pathology collection clinic or a mobile phlebotomist. Fasting before the blood collection was not required. This minimised any impact on the participant's work and helped facilitate the collection process. Three serum-separating tubes (SST) of blood (total of 25.5 mL) were collected from each participant. Samples were transported to a single accredited pathology laboratory in Brisbane by courier; one SST was used for biochemical analysis and two SST were transferred to the research laboratory on dry ice and stored at −20 °C prior to PFAS analysis.

2.2.2. Questionnaire

The questionnaire was developed by the research team in consultation with the working team. It captured demographic, lifestyle and detailed work history information. The questionnaire is available in the Supplementary Material, including an overview of the questionnaire content. Participants had the option to fill in the questionnaire in electronic or paper format.

2.2.3. Sample analyses

Blood samples were analysed for 40 PFASs and 14 health biomarkers (Table 2). These PFASs include PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), PFHxS, perfluoroheptanesulfonate (PFHpS) and PFOS, which are routinely analysed and frequently detected in blood samples (Toms

Table 2
Biomarkers included in the serum analysis, 2018–2019 Airservices Exposure Study.

GROUP	BIOMARKERS
Exposure markers	
PFAS[a]	PFCAs: PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA, PFTeDA[b], PFHxDA[b], PFODA PFSAs: PFBS, PFPeS[b], PFHxS, PFHps[b], PFOS, PFNS[b], PFDS, PFDoDS[b] FASAs[b]: FOSA, N–Me FOSA, N–Et FOSA FASAAs[b]: FOSAA, N–Et FOSAA, N–Me FOSAA FASEs[b]: N–Et FOSE, N–Me FOSE Cyclic PFAS[b]: PFECHS FTSs[b]; 10:2 FTS, 8:2 FTS, 6:2 FTS, 4:2 FTS PAPs[b]: 6:2 PAP, 8:2 PAP, SAmPAP diPAPs[b]: 6:2 DiPAP, 8:2 DiPAP, 6:2 8:2 DiPAP
Health related markers	
Lipid profile (marker for cardiovascular disease, metabolic effects)	Cholesterol Triglycerides[c] High density lipoprotein (HDL) Low density lipoprotein (LDL)
Thyroid function [b] (marker for thyroid disease)	Thyroid Stimulating Hormone (TSH) Free Thyroxine (T4) Free Triiodothyronine (T3)
Liver function[b] (marker for liver disease)	Alanine Aminotransferase (ALT)
Kidney function (Marker for kidney disease)	Urate (Uric acid) Creatinine[d] Estimated Glomerular Filtration Rate (eGFR)
Serum proteins[e] (Provide binding sites for PFASs in blood)	Globulin Albumin Total protein (Globulin + Albumin)

[a] PFAS abbreviation list available in the Supplementary Material.
[b] Not quantified in the 2013–2014 Airservices Exposure Study.
[c] Triglycerides were tested but not further assessed as fasted blood samples were not provided.
[d] Creatinine measured to assess eGFR.
[e] Biomarkers were assessed as confounders (not outcomes).

et al., 2019).

The biochemical profile included the health biomarkers that were previously assessed in the 2013–2014 Study. This allowed for longitudinal assessment of trends for biomarkers of cardiovascular disease and kidney function which may be associated with PFAS exposure. Blood serum proteins were also measured in both studies. PFASs have been found to associate with serum proteins such as Albumin (Han et al., 2003). Thus, the protein profile may act as a confounder that might influence PFAS serum concentration. Additionally, biomarkers of thyroid and liver function were included in the current 2018–2019 Study to reflect updated scientific literature of potential health effects associated with PFAS exposure (Darrow et al., 2016; Lewis et al., 2015; Shrestha et al., 2015).

Biochemical analyses were performed at a commercial laboratory. The analysis was performed within days of sample collection and the results of the biochemical markers were reported back to the research team. PFAS analysis was performed at the research laboratory. The extraction and analysis of the blood samples were consistent with the methods used in the 2013–2014 Study, with minor adjustments to improve detection, such as the use of a greater volume of sample (See Table 1). Comprehensive quality control and quality assurance (QA/QC) was conducted, including re-analysis of stored serum samples from the 2013–2014 Study and an inter-laboratory validation with a subset of samples. Further details on the QA/QC are presented in a separate publication (Nilsson et al., 2021).

2.3. Reporting of results and study outcomes

The study group developed an approach to ensure all participants received their individual results in a timely manner. Individual letters, with health biomarker results together with the reference range, were sent to each participant within three weeks of blood sample collection. Any abnormal values were highlighted (Supplementary Material). All participants were encouraged to provide the results to their General Practitioner (GP) for inclusion in their health records. Participants with abnormal biochemical results were advised to consult their own GP for a follow-up. Participants had already been informed that they would be advised to do this during the consent process.

After completion of blood testing for PFASs, all participants were sent a letter containing their results together with a summary of the study's findings. The letter included a comparison of their results to the whole study population and to PFAS levels in the general population. The pro forma for these letters is shown in the Supplementary material. For participants who were involved in the 2013–2014 Study, the letter included their previous results for PFASs.

After dissemination of individual results, the study group held additional workshops to review the findings and discuss how these related to specific research questions. The working team also reviewed and provided comments for the draft report which was prepared by the research group. Following completion of the project, the results of this study were presented in a final report to Airservices. The report was published on the Airservices website and distributed to study participants. The report primarily discussed exposure to PFOA, PFHxS, PFHpS and PFOS but also provided the overall results of all PFASs that were analysed. A summary of the study outcomes and a 'frequently asked questions' document were published on the website and distributed with the report.

2.4. Ethical considerations

The study was approved by the University of Queensland Human Research Ethics Committee (Application number 2018001790). Details of ethical considerations for the study are presented in the Supplementary material.

3. Results & discussion

3.1. Participant profile

There were 880 participants recruited for the 2018–2019 Airservices Exposure Study, including 71% (625/880) who were current ARFF employees and 29% (255/880) who were former employees. The 625 current employees represent 72% (625/867) of the Airservices' ARFF employees in 2018–2019. Blood samples were provided by 91% (799/880) of recruited participants and 798 completed the questionnaire. Of those who completed the questionnaire, 15 did not provide a blood sample, and were excluded from the study.

All 799 participants who provided a blood sample were included in the cross-sectional analyses. Of these, 69% (555/799) were current employees and 30% (244/799) former employees. There were 16 participants who only provided a blood sample and did not complete the questionnaire. Of the 799 participants who provided a blood sample, 130 (16%, 130/799) had also participated in the previous 2013–2014 Study and formed the longitudinal population of this study (Table 3).

The majority (97%; 779/799) of participants were male, reflecting the gender ratio of the ARFF Airservices employees (96.4% male in 2019; 836/867). The median age of participants was 52 (range 22–82) years old in the cross-sectional study population and 57 (range 34–72) years old in the longitudinal population. The participants included

Table 3
Final participant profile, Airservices Exposure study 2018–2019.

Description	Category	Study population n (%[a])	Longitudinal population n (%[a])
In total		799	130
Gender	**Male**	779 (97.5)	126 (96.9)
	Female	20 (2.5)	<5
Age	**16–30**	28 (3.5)	0
	31–45	263 (32.9)	21 (16.2)
	46–60	308 (38.5)	64 (49.2)
	>60	200 (25.0)	45 (34.6)
Employment status	**Currently employed**	555 (69.5)	79 (60.8)
	Former employees	244 (30.5)	46 (35.4)
Year commenced service according to foam usage	**Lightwater AFFF (<2005[b])**	494 (61.8)	108 (83.1)
	Ansulite AFFF (2005–2010[b])	140 (17.5)	19 (14.6)
	Solberg RF6[c] (>2010)	135 (16.9)	<5
Position[d]	**Officer**	332 (41.6)	73 (56.2)
	Senior Officer	143 (17.9)	30 (23.1)
	Firefighter	744 (93.1)	120 (92.3)
	Instructor	102 (12.8)	19 (14.6)
	Emergency Vehicle Technician (EVT)	43 (5.4)	10 (7.7)
	Other	21 (2.6)	<5

[a] Percentage of total participants.
[b] The cessation of Lightwater use was a gradual process at Airservices, that started in 2001 and finalised before 2003. However, in 2003/2004 Airservices acquiesced a station where Lightwater AFFF was still used. Therefore, 2005 is used as a cut-off year in this study, to ensure to capture everyone with potential Lightwater AFFF exposure.
[c] Solberg RF6 is fluorine free.
[d] During employment at Airservices, they have held a position as listed; participants may have held more than one position during their employment.

officers, senior officers, firefighters, instructors and EVTs. They worked across 27 Airservices rescue fire stations, with an employment history ranging from 1965 to current. A detailed assessment of PFAS exposure is intended for separate publications. PFAS results from the 2013–2014 Study are available in Rotander et al. (2015).

This study is a novel investigation of serum PFAS concentrations over time in firefighters historically exposed to AFFF, where repeated PFAS measurements were obtained from a period longer than three months. In addition to the detailed information of the time periods of AFFF usage and work history, this dataset enabled assessment of serum PFAS concentrations in relation to changes in AFFF use over time, including the replacement of AFFF. However, investigation of serum PFAS concentrations in a cohort with different AFFF exposure histories has limitations. PFAS measurements from samples collected in 2013–2014 and 2018–2019—10–15 years after Lightwater AFFF was replaced, and 3–8 years after Ansulite AFFF was replaced—are not appropriate for assessment of PFASs with shorter elimination half-lives, which firefighters may have been exposed to while using AFFF. Only a subset of previous studies monitoring PFAS exposure in firefighters have also assessed relations to health outcomes (Goodrich et al., 2021; Leary et al., 2020; Tao et al., 2008). Similar to the current study, Tao et al. (2008), assessed the association between PFAS exposure and serum lipids in firefighter responders to the World Trade Centre emergency. This study reported inverse association between PFAS serum concentration and serum lipid concentration. However, the study was limited to a small sample size and cross-sectional design (Tao et al., 2008). Other health outcomes assessed in firefighters exposed to PFASs include metabolic syndrome and DNA methylation (Goodrich et al., 2021; Leary et al., 2020). Overall, the demographic features of our study population, with the high proportion of male participants and with age ranges of 20–80 years old, are comparable to those of other studies of occupationally exposed firefighters, except for Trowbridge et al. (2020) in which only female firefighters were recruited. In several studies that discuss the use of AFFF as a source of PFAS exposure, aviation firefighters were specifically targeted (Laitinen et al., 2014; Leary et al., 2020; Trowbridge et al., 2020). Other studies assessed PFAS exposure in different categories of firefighters, including rural, veteran, emergency response and general firefighters Dobraca et al. (2015); Jin et al. (2011); Leary et al. (2020); Shaw et al. (2013); Tao et al. (2008). A summary of the studies examining PFAS exposure in firefighters is presented in Table S2, **Supplementary Material.**

3.2. Key lessons learnt

3.2.1. Enhancing the recruitment process through broad promotion and assuring confidentiality

This study had a high number of participants and a high response rate. This is a key strength of the study as it reduces the potential for selection bias which is a common problem when considering epidemiological assessments of exposure and health. The 2018–2020 Study resulted in a 5-fold increase in recruitment of participants, compared to the 2013–2014 Study. There are a number of factors that contributed to this.

Extending the eligibility criteria to include both current and former employees enhanced the potential numbers, especially for the longitudinal cohort. Deciding to recruit all employees at Airservices who have had potential exposure to AFFF also helped to increase participation numbers compared to the 2013–2014 Study which focused on currently employed firefighters.

It is likely that the co-design process enabled the stakeholders to confidently engage with the researchers. The formation of the study group enabled the aims of the study to be defined together so that they were meaningful to both the researchers and the potential participants. The recruitment process including clear information for participants about how their data would be handled and an assurance of personal communication of their results in a timely manner. This communication strategy was developed during the co-design process.

Promotion of the study to potential participants was developed with the study group so that strategies for promotion with a broad reach to the target population were identified. Messaging was distributed both internally through the employing company and externally through relevant social media groups, union communications and direct contact. The high re-recruitment rate (87%, 130/149) of participants from the 2013–2014 Study suggests that implementing several different recruitment strategies was beneficial.

Other factors likely to have enhanced the response rate could include a general increased awareness of PFAS exposure between the two study periods.

Simplifying the blood collection process for participants, as suggested by the working team, is likely to have supported the high retention of participants. The collection of non-fasting blood samples and a choice of timing for the collection gave participants flexibility (Tolonen et al., 2017). Introducing the option to either provide blood at a pathology clinic or by a mobile phlebotomist improved access to blood collectors and decreased the time-cost for participants; addressing two common reasons for non-participation in health studies (Galea and Tracy, 2007; Tolonen et al., 2017). Interestingly, only 15% of the participants utilised the mobile phlebotomist service that was offered at the workplace. Thus, this service approach may not have been a cost-effective method to collect blood from participants. However, this method may have been important for the participants that did choose to use the mobile phlebotomist. This information was not collected. Stakeholder and participant feedback to the research team did indicate a preference for blood collection to be done outside of the work environment. The working team addressed this feedback by ensuring that there were opportunities for participants to complete the blood collection and questionnaires outside of the workplace. This ensured confidentiality for ARFF employees who did not wish to disclose their participation to their work colleagues. Maintaining confidentiality related to research participation is a key consideration of when designing occupational exposure studies, particularly those funded by stakeholders related to the exposure source, such as employers.

3.2.2. Benefits of engaging stakeholders and participants throughout the study included expansion of the study scope and improved study design

The successful completion of the study can be attributed to the collaborative engagement between the researchers, stakeholders and participants. The development and conduct of the study required partnership between a broad range of professionals with a breadth of expertise that were included in the study group. Major study aspects that were enabled as a result of the co-design process are summarised in Table 1. The co-design process ensured a more efficient utilisation of the research opportunity with the implementation of key improvements compared to the previous study. Beyond the benefits for recruitment already mentioned, the study group enabled an expansion of the scope of this study. Through a series of workshops, the study group co-designed the aims for the study through discussion of the rationale and expected outcomes of the study. Between these workshops, the research team encouraged the working team to discuss the proposed study with their peers. This contributed to an understanding of the expectations among the potential participants prior to the decision-making process and provided feedback to the study group about perceptions of the study. The initial rationale of the follow-up study, when the study was first commissioned by Airservices, was to assess the impact of the replacement of AFFF foam on exposure to PFAS. However, the co-design process established several additional aims for the study including assessment of work history and exposure to PFAS, examination of elimination half-lives of PFAS and analysis of the health effects associated with exposure to PFAS. The working team provided additional perspectives on examining exposure to PFAS throughout the methodology design stages, such as advice on the potential determinants of occupational exposure and relevant exposure pathways for aviation firefighters. Further, within

the working team, employee representatives were from different employment roles and firefighter stations. Communication of the occupational experiences among these participants provided insight into the potential variation in exposure to firefighting foams across roles and locations, prompting the research team to expand recruitment to all ARFF operations and refine relevant questions in the questionnaire. These insights were essential elements of the study and ensured that the aims and design of the study were not limited by the vision of stakeholders or the research team.

Throughout the study the working team also provided contact points for staff queries and concerns. These provided the research team with deeper insights into the expectations of the study, which were considered during analysis. Reporting of the study results was designed to address the participants' questions and concerns. Involving the working team in the study design promoted research engagement and participation and enhanced the research literacy of the working team. This helped improve the understanding of what could be expected of the study's findings, including the implications and limitations of the conclusions (Jagosh et al., 2012).

Introducing these elements of co-design also contributed to new research opportunities, including sustained research engagement beyond the length of the current study—exemplified by the continuation of the study currently underway.

3.2.3. Transparent communication helped to build trust with participants

Establishing trust benefits both the participants and researchers. It supports the opportunity for further follow-up studies. The significant recruitment rate, especially the re-recruitment from the 2013–2014 study, highlights the value of the trust that was built and maintained with participants through the previous study. Establishing a working team for this study facilitated open communication pathways between researchers, the ARFF employees (i.e., intended participants) and the funding agency. At the onset of the study, participants raised concerns about the potential biases associated with the inclusion of the funding agency in the working team. The inclusion of union representatives and participants in the working team played a crucial role in developing transparency and open communication pathways for the study. The study group provided a safe space for these conversations. The research teams' respectful engagement with working team members fostered and built trust from participants and others in the working team, enabling them to trust that they could speak their mind without fear of negative consequences, which may otherwise have been a potential drawback with the presence of employers in the working team. The funding agency's involvement in the study development, communication and dissemination of information was explicit and transparency was maintained at all times. This addressed this important concern related to the potential perception of employer influence over the results. It was made clear that the working team had no participation in the actual performance of the study, no control or influence over the outcomes of the study. Transparent and inclusive communication during stakeholder engagement supported the development of trust in the research methods (maintaining confidentiality in recruiting, independence in analysis and reporting) and therefore trust in the outcomes. The maintenance of such trust is a current challenge when conducting research in populations affected by PFAS exposure (Banwell et al., 2021; Christopher et al., 2008).

Ethical considerations for the study design, along with ethical governance arrangements were highlighted from the onset. As part of the development of research literacy, it was important that the working team and participants had a clear understanding of the ethics process. The conduct of the research team (including analysis and reporting of the findings) was open to scrutiny by the working team. Open communication and engagement with the working team allowed the research team to address concerns immediately as they arose, rather than at the end of the study. For example, during the study, members of the working team expressed concerns regarding the PFAS analysis. This resulted in an expansion of the QA/QC, to include an external validation via a national inter-laboratory study, further discussed below.

3.2.4. Robust analytical QA/QC measurements were a key component of the study

To enhance confidence regarding the PFAS analysis results, a robust QA/QC process for the PFAS analysis was developed. Including both external validations (via a national inter-laboratory study) and internal validations contributed to the precision of the analytical results. Additionally, re-analysis of stored blood serum samples from the 2013–2014 Study for participants of the longitudinal component of the study addressed potential analytical uncertainties. These uncertainties have been previously discussed for other longitudinal studies which relied on analytical PFAS data obtained over time (Li et al., 2018; Olsen et al., 2017). Re-analysing the stored samples decreased the analytical uncertainty and consequently increased the accuracy of elimination and half-life estimates (Nilsson et al., 2021). Analytical variances are well understood in the scientific community. However, communication of these results to participants was a significant consideration for the study group before the dissemination of the results. The outcomes of the QA/QC were clearly communicated including how this enhanced the reliability of the results for the study participants.

3.2.5. The inclusion of health measures in an exposure study can be valuable but unrealistic expectations from participants may arise if not proactively addressed

The introduction of health measures including biomarkers into studies measuring PFAS levels increases the potential benefits of the research. Such research can provide a deeper understanding of the impact of PFAS exposure on human health. The inclusion of health biomarker testing and self-reported health measures in this study enabled these research findings to contribute to a broader range of literature related to PFAS exposure and human health while also providing occupation-specific exposure measurements. However, there is a potential for an increased participant burden in such studies that can outweigh the benefits (Boyle et al., 2020).

Adding health measures can introduce other biases (e.g., when expanding an existing study to include health measures) if there is reduced participation, except for higher risk sub-groups of the study population (e.g., those with poorer health) (Boyle et al., 2020; Galea and Tracy, 2007). In this study, both blood sample collection and survey questionnaires were included in the original study design, reducing the risk of increased participant burden.

Ensuring that the research is addressing key issues that the participants are interested in may contribute to higher participation rates (Galea and Tracy, 2007). Populations impacted by elevated PFAS exposure may face higher stress due to their concerns about potential health risks associated with their exposure (Banwell et al., 2021; Calloway et al., 2020). The inclusion of health biomarkers in this study helped to address such concerns for the ARFF community. However, the research team had to consider the risk that participants could have unrealistic expectations of the health measured. This highlights the need for transparent communication of the study's limitations from the outset. The research team included research officers with the specific role of engaging with potential participants who had questions about the research and responding to participants to address any concerns as they were raised. Contact details were available in the communications about the research study.

3.2.6. Ethical and practical factors were considered during the communication of results

There are currently gaps in our understanding of how to communicate research results to study participants. Determining which results to share, how to present the results and how to minimize confusion or discomfort related to receiving results are challenges for researchers (Long et al., 2017). Although participants generally express their

interest in obtaining the results of studies they have participated in, researchers are concerned when participants report distress on receiving their results (Purvis et al., 2020). However, studies have suggested that when the results are communicated to participants in a manner that is easily understood and contextualised in a way that is meaningful for the participants, they are less likely to become distressed. Receiving results can also motivate changes, such as actions to minimize exposure and seek further knowledge (Brody et al., 2014).

In this study, the dissemination strategies were co-designed with all members of the study group, including the medical doctors, epidemiologists, analytical chemists, participants and stakeholders to support the appropriateness of the communication of an individual's exposure and health results (Brody et al., 2014; Chen et al., 2010; Exley et al., 2015). The working team reviewed the results letters template and the study report and assisted in tailoring the communication in clear language appropriate for the study audience, aiming to avoid potential confusion related to technical language or scientific concepts. After review of the final report, the working team also summarised a list of questions which arose after discussion among their peers. These questions were addressed by the research team and resulted in the writing of a companion document with 'frequently asked questions' which was distributed to participants with the final report.

It was important to mitigate potential participant distress associated with receiving their individual results. To improve the understanding of each individuals' results, the results letters aimed to put both the biochemical and PFAS results into context using summary data. Further, study participants were provided with a summary on the potential PFAS health effects of exposure to PFAS and relevant references. The biochemical results were assessed by a medical expert to confirm the content, and the reporting included clear statements that the participants would need to contact their GP to ensure their results could be included in their health record, or any potential abnormal results could be followed up. The immediate reporting of each participant's biochemical results addressed the best interest of participants with abnormal health results, ensuring timeframes for reporting allowed clinically appropriate follow-up. The dissemination of PFAS results to participants following the conclusion of the testing for the study allowed reporting of individual results contextualised with exposure results in the broader study population. Inclusion of graphical and text representation of the results; including individual results, study averages and general population benchmarks, aimed to improve participant understanding of their exposure to PFASs (Brody et al., 2014). Reporting results of emerging contaminants, such as PFASs, associated with uncertain clinical health implications may contribute to participant distress related to their exposure. Further, GPs may be not familiar with PFAS exposure and the potential health effects. To mitigate this risk, the research team distributed a PFAS information sheet with the individual results letters. The information sheet consisted of the summary of the Australian Government Department of Health, Expert Health Panel for PFAS Report, which presents a comprehensive review of research literature on the potential health effects of PFAS according to Australian and international evidence (Australian Government, 2018). The research team aimed to encourage study participants to engage in learning about PFAS exposure by providing the health summary and relevant references to study participants. After the dissemination of the results letters, the working team provided the research team with positive feedback about the process, suggesting that the communication approach was well-received.

4. Conclusions

The 2018–2019 Airservices Exposure Study provides the results of a large cross-sectional study and smaller longitudinal cohort study. This is the first longitudinal PFAS exposure study to assess ARFF employees exposed to AFFF in Australia that documents the effects of changes to AFFF exposure control measures. The study established a cohort of current and former ARFF employees for future research opportunities to assess PFAS exposure and health outcomes, which are currently underway in Australia. The study design was developed using a co-design process, which enabled improvements to multiple aspects of the study. Key recommendations for future biomonitoring studies include establishing an inclusive study group, which includes researchers, key stakeholders and participants. This enables ongoing communication throughout the research process. Successful biomonitoring studies rely on the transparency of the research design, methods and results, and building trust between researchers and participants, particularly for studies funded by stakeholders related to the exposure source. The co-design process used in this study enabled these issues to be addressed effectively.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

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Cancer mortality and chemical exposure in a retrospective zinc and lead smelter cohort: A 48-year follow-up

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Lead
Cadmium
Asbestos

ABSTRACT

Introduction: Very few studies to date have investigated cancer mortality in non-ferrous metal smelters. Existing studies mainly focus on lead exposure and have reported inconsistent results. The aim of this study was to investigate the risk of excess cancer mortality in the employees of a lead and zinc smelter located in the north of France by 1) comparing mortality in this cohort of employees with that of the regional population; 2) studying the associations between occupational exposure and cancer deaths.

Methods: The study cohort was composed of male workers, born in France, who had been employed by the company in question for at least 365 consecutive days. A company-specific job-exposure matrix was produced in order to calculate a cumulative exposure index for 15 toxic agents. Deaths of smelter employees which occurred between January 1, 1968 and December 31, 2015 were compared to those in the regional population (standardized mortality ratio, SMR). The relationships between the cumulative exposure indexes and mortality by cancer site were studied using Cox regression models with age and the 20-year lagged cumulative exposure index as time-dependent variables.

Results: Vital status was found for 2177 of the employees in the cohort (98%). Median follow-up was 34.8 years (interquartile interval = 24.3–44.8), totaling 74,437 person-years. Compared to the regional population, no excess risk of all-cause mortality (n = 913, SMR = 0.96, 95%CI:0.90–1.02), nor of cancer mortality (n = 338, SMR = 0.97, 95%CI:0.87–1.08) was found. An overall significant excess risk of cancer mortality was found for employees who worked in this non-ferrous metal smelter for a period of between 15 and 29 years (n = 139, SMR = 1.23, 95%CI:1.04–1.45). Asbestos exposure was found to be associated with an increased risk of mortality for all cancer sites (p = 0.0012), lip-oral cavity-pharynx malignant neoplasms (MN) (p = 0.0141) and trachea-bronchus-lung MN (p = 0.0018); lead exposure was associated with the same risk for lip-oral cavity-pharynx (p = 0.0378) and liver MN (p = 0.0155); aromatic amine exposure with bladder MN (p = 0.0002); chromium exposure with colon-rectum-anus MN (p = 0.0057) and colon MN (p = 0.0315); bismuth exposure with rectal MN (0.0011) and sodium hydroxide vapor exposure with laryngeal MN (0.0150).

Conclusion: Including occupational exposure to numerous toxic agents other than lead in this study of smelter mortality has made it possible to identify associations between different toxic agents and cancers, opening up new avenues for future research.

1. Introduction

Cancer is the leading cause of mortality in men in France (Boulat et al., 2019). Currently, occupational exposures represent a substantial proportion of the new cancer cases in France, around 4% in men (Marant Micallef et al., 2019). In France, a program for monitoring mortality by

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sector of activity has found a significant excess risk of cancer mortality in male employees in the metal industry, with a relative risk of 1.16 (Santé Publique France, 2018). The lead and zinc smelter investigated in this study, located in the north of France, operated for over a century and was shut down in 2003. Given the very high levels of lead to which the smelter employees were exposed, questions were raised regarding mortality rates. There was a particular concern regarding cancer mortality, even though the precise carcinogenic effects of lead have not yet been clearly established in humans (Fu and Boffetta, 1995; IARC Monographs, 2006). In the literature, several cohorts of smelter workers exposed to lead have already been studied, with each study coming to different conclusions. One Italian cohort (Cocco et al., 1997) studied 1388 workers and laborers in the production and maintenance units of a primary lead-smelting plant. Mortality from all cancers, stomach cancer, and lung cancer was lower than expected, and mortality from kidney cancer was not significantly elevated. A Swedish study followed a cohort of 3979 workers at a copper and lead smelter, with follow-up last updated in 1997 (Lundström et al., 1997). This study found significant excess mortality for all cancers and for lung cancer, an association which was ultimately attributed to arsenic exposure (Lundström et al., 2006). Furthermore, in a cohort of 2300 U.S. workers at 6 lead smelters, no significant excess mortality was found overall, despite the non-significant elevated risk found for certain cancer sites, in particular digestive and respiratory cancer (Wong and Harris, 2000). Finally, mortality in another cohort of 1990 U.S. workers at a primary lead smelter in Idaho has recently been reassessed (Bertke et al., 2016). Significant excess all-cause, cancer and lung cancer mortality were found in the cohort compared to mortality rates for the State of Idaho. A non-significant elevated mortality rate for kidney and stomach cancer was also found but no relationship to the level of cumulative lead exposure established. It is difficult to compare results between these cohorts due to the disparities in terms of production processes and lead exposure levels, and due to the combined exposure to multiple toxic agents which is often ignored or partially taken into consideration. A specific study of the lead and zinc smelter in the north of France was therefore deemed necessary in order to investigate the impact of the exposures workers were subjected to in this particular firm.

The aim of this study was to assess the risk of excess cancer mortality in the employees of a lead and zinc smelter by comparing mortality in this cohort of employees with that in the regional population, and by studying the associations between occupational exposures and causes of cancer deaths.

2. Material and methods

2.1. The lead and zinc smelter

This study was conducted in a metallurgy firm involved in the primary processing of precious and non-ferrous metals, specialized in lead and zinc production, implanted in the north of France from 1894 to 2003. This company was one of the largest employers in the area. It operated two pyrometallurgical smelters, a smelter using the imperial smelting process for mixed zinc-lead ore, and a water jacket furnace for lead ore. In its final years of operation annual production was 160,000 tons of lead and 105,000 tons of zinc. A quarter of its supply of raw materials came from metal recycling. At certain points in its history the company also had a germanium production workshop (1976–2003) and an indium workshop (1982–2003). The company was liquidated in March 2003. At this time the company had 830 employees, not including temporary workers and subcontractors.

Producing lead and zinc using pyrometallurgical processes involves roasting ore to remove the sulfur, followed by reduction smelting in an adapted furnace (a water jacket shaft furnace or imperial smelting process blast furnace). After reduction the raw lead and zinc are refined ready for sale. These processes not only expose workers to metals (namely lead, cadmium, arsenic, bismuth, mercury, antimony, thallium, zinc), but also to heat, carbon monoxide, sodium hydroxide, sulfur dioxide and sulfuric acid. There are five different sectors within the firm:

1) *Production:* lead and zinc production (including roasting, smelting and refining), sulfur dioxide workshop (where liquid sulfur dioxide is produced, using aromatic amines - toluidine and xylidine), sulfuric acid workshop, hydrometallurgy workshop (production of rare metals - germanium, gallium)
2) *Technical and purchasing units*: purchasing, supply chain, warehouse, maintenance and servicing workshops, electrical and instrumentation unit, design unit, civil engineering, fluids-energy, and garage for maintaining the site vehicles.
3) *Control and development*: metallurgy studies (for the continuous improvement of metal production techniques), sampling (taking and preparing samples for analysis), laboratory (carrying out chemical analyses) and investigations (various measurements taken in the plant).
4) *Logistics*: transporting raw materials, intermediate and finished products around the plant.
5) *Administrative sector*: administrative roles, security, IT unit, training.

Biomonitoring of lead exposure in the company's employees was introduced at the start of the 1970s, by monitoring urinary delta-aminolevulinic acid levels. The monitoring of blood lead levels was introduced in 1985. In France, the regulatory monitoring of lead exposure with blood lead level testing officially came into force with the decree dated February 1, 1988. Cadmium exposure monitoring was also implemented in the 1990s by monitoring urinary cadmium levels in exposed workers.

The biomonitoring results were anonymised and we were unable to link them to each of the employees in the cohort. However, averages per period and per sector were available. The workshops where workers were the most exposed to lead were the lead and zinc smelters. Blood lead levels decreased gradually over time until a mean level of around 500 μg L^{-1} for workers at these stations was reached at the start of the 2000s. Employees with blood lead levels approaching 800 μg L^{-1} (the upper regulatory biological limit value since 1988) were temporarily moved to less exposed workstations. In some rare cases, employees with blood lead levels over 800 μg L^{-1} with consequences in terms of renal function were declared unfit for positions exposed to lead and were moved permanently to the less exposed sectors on the site.

Atmospheric measurements were also taken as of the 1990s onwards. Mean exposure levels could reach 3000 μg m^{-3} for Pb and more than 100 μg m^{-3} for Cd for some workstations. This collective, atmospheric and biological data was synthesised by workstation, and enabled exposure levels to be estimated when a company-specific job-exposure matrix was produced.

2.2. Population

The cohort studied was composed of all former employees of the company who met the following inclusion criteria: male subjects, born in France, employed by the company on the site for at least 365 consecutive days and who worked there during the period from January 1, 1968 to March 24, 2003 (the date of the company's liquidation). Temporary workers and subcontractors were not included in the study (as there were no records of these workers).

When the firm closed, the association of former workers on the site entrusted the hard copies of the former employees' administrative files to the French national labor archives (*ANMT*). The list of employees included in the cohort was established based on the information in these files.

2.3. Data collection

The following information was collected for each employee included:

the administrative data required to obtain vital status from the National Institute of Health and Medical Research (*INSERM*) (surname, first name, sex, date and place of birth), their work history with the firm (official dates of entry and departure, positions held and sectors occupied by the employee during his time working on the site).

The vital status and individual medical causes of death were investigated for all subjects in the cohort. Only the initial causes of death (causes which triggered the process resulting in death) were considered in the data analysis. The causes of death were coded based on the classifications in force at the time of death: International Classification of Diseases, 8th revision (ICD-8) from 1968 to 1978; 9th revision (ICD-9) from 1979 to 1999; and the 10th revision (ICD-10) since 2000.

2.4. Assessment of occupational exposure in the company

A company-specific job-exposure matrix was created by former employees of the firm in collaboration with the research team. Exposure to fifteen toxic substances was assessed for the cancer mortality analysis: lead (Pb), cadmium (Cd), arsenic (As), bismuth (Bi), chromium (Cr), antimony (Sb), thallium (Tl), asbestos, aromatic amines (ortho-toluidine and xylidine), diesel exhaust, sodium hydroxide vapor, solvents, sulfur dioxide (SO_2), dust, silica (SiO_2). The assessment of exposure was carried out for each of the 2210 positions in the company identified as being held by employees during their time of employment. Temporal changes in exposure were taken into account by way of job titles, which evolved over the course of time. This enabled us to distinguish different exposure levels successively, within the same profession. The exposure assessment was based on an understanding of the production process, atmospheric exposure data, bioavailability (of the different chemical forms of lead) and/or biological data from former employees working in different roles. Four categories of exposure were defined for Pb, based on atmospheric Pb levels: 0–50 μg m^{-3}, 51–100 μg m^{-3}, 101–150 μg m^{-3} and >150 μg m^{-3}. These were coded as 1, 3, 5 and 8 respectively to approximately reflect the increase in mean levels in each of these groups. Similarly, four categories of exposure were defined for Cd, based on atmospheric Cd levels: 0–10 μg m^{-3}, 11–30 μg m^{-3}, 31–50 μg m^{-3} and >50 μg m^{-3} which were also coded as 1, 4, 8 and 12 respectively. For the other toxic agents investigated, four categories of exposure were defined: no exposure, possible exposure, indirect exposure and direct exposure, coded as 0, 1, 2 and 4 respectively. The coherency of the matrix as a whole was validated by the former occupational health physician for the firm. For each toxic substance, a cumulative exposure index was assigned to each employee, corresponding to the sum of the numbers of years of exposure weighted by the levels of exposure.

2.5. Legal and ethical requirements

The ethics committee (Advisory Commission on Information Processing in Health Research, *CCTIRS*) approved the terms and conditions for implementing the project on June 10, 2015. The French National Commission for Information Technology and Civil Liberties (*CNIL*) approved the study on December 16, 2016 (deliberation no. 2016-389 dated December 8, 2016). The *ANMT* approved the consultation of the firm's archives on 26 January 2017.

2.6. Statistical analysis of data

Person-time began on whichever was the earliest date between January 1, 1968 or one year after the hire date and continued until whichever was the most recent date between the date of death or December 31, 2015.

For each of the causes of death studied, the corresponding list of disease codes established according to the relevant version of the international classification of diseases is set out in detail in the Supplementary Materials, Table S1.

Deaths of former employees of the firm which occurred between January 1, 1968 and December 31, 2015 (endpoint) were compared to those in the regional population (the North and Pas-de-Calais departments). Mortality rates for the region were applied to the study cohort, taking into account age (by 5-year age groups) and the year of death (calculation of the standardized mortality rate, SMR). The SMR are presented with the 95% confidence interval, calculated using the exact method for 5 or fewer observed deaths and Byar's approximation method for more than 5 deaths (Breslow and Day, 1987; Sahai and Khurshid, 1996). Comparisons were made for all employees and according to: 1) the date of hire in the company, with 3 periods defined based on the company's production history: 1921–1936 (very high exposure to lead), 1937–1969 (high exposure to lead), and 1970–2003 (more moderate exposure to lead); 2) the duration of employment in the company, in 3 categories: less than 15 years, from 15 to 29 years, and 30 years or more; 3) the following four job sectors: production, technical and purchasing units, control and development, logistics. The employees taken into account were those who worked exclusively in one of these four sectors (for at least one year) and possibly in the administrative sector (where employees were considered not to be exposed). This analysis by sector therefore did not take into account employees who worked exclusively in the administrative sector nor those who worked in different sectors (excluding the administrative sector).

The relationship between the cumulative exposure to the 15 toxic agents and mortality by cancer site was investigated using Cox regression modelling with age and cumulative exposure index as time-dependent variables. This model enables us to take account of changes both in the age and the cumulative exposure index of employees, for each year's monitoring. For each toxic agent, a cumulative exposure index was incremented for each year of follow-up, first with no lag and then with a lag of 10, 15 and 20 years. Different cumulative exposure categories were constituted: three categories for Pb, Cd, asbestos and dust; two categories (exposed/non exposed) for all other substances (See Supplementary Materials, Tables S2–S3). Age-adjusted analyses were conducted for the causes of death for which at least ten deaths were found (that is 12 locations). For Pb, Cd, asbestos and dust, the median cumulative exposure index for each of the three categories was used to compute the linear trend tests. The overall statistical significance and linear trend (expressed as a global P-value and trend P-value, respectively) were assessed with a likelihood ratio statistic. Subsequently, for each of the 12 causes of death, we created a first multivariable Cox model in which we fitted all of the covariates for which global p-value was <0.15 in the age-adjusted model. Then we used a backward step-by-step procedure based on Akaike's information criteria (AIC), so as to finally keep only significant exposures ($p < 0.05$) in the final models. In the presence of collinear variables, a reasoned choice was made to select the toxicant to be retained in the model. The absence of violation of proportional hazard assumption was tested for each model.

All analyses were conducted using the R 3.6 software program (epitools and survival packages).

3. Results

3.1. Population description

The cohort of employees was composed of 2226 men born in France. Vital status and cause of death were established for 2177 of them (97.8%). Median follow-up was 34.8 years (interquartile interval = 24.3–44.8), totaling 74,437 person-years. A total of 2210 job titles were identified by examining the work histories conserved in the firm's administrative archives.

These employees were born between 1902 and 1980 (median year of birth: 1944) (Table 1) and started working for the firm between 1921 and 2002. Almost 60% were hired before 1970. Just over a quarter of the employees worked for the firm for less than 10 years and over 60% for 30 years or more. More than 40% of them had worked exclusively in the production sector and 22% in the technical and purchasing units. On

Table 1
Description of the cohort of workers studied (n = 2226).

Population characteristics	Numbers (%)
Decade of birth	
≤ 1920	161 (7.2)
1921–1930	275 (12.4)
1931–1940	491 (22.1)
1941–1950	586 (26.3)
1951–1960	417 (18.7)
1961–1970	171 (7.7)
1971–1980	125 (5.6)
Date of hire	
≤ 1950	298 (13.4)
1951–1960	343 (15.4)
1961–1970	687 (30.9)
1971–1980	415 (18.6)
1981–1990	257 (11.5)
1990–2002	226 (10.2)
Employment duration	
< 10 years	592 (26.6)
10–19 years	252 (11.3)
20–29 years	657 (29.5)
30–39 years	612 (27.5)
≥ 40 years	113 (5.1)
Employment sector	
Exclusive employment in production[a]	940 (42.2)
Exclusive employment in technical and purchasing units[a]	489 (22.0)
Exclusive employment in control and development[a]	128 (5.8)
Exclusive employment in logistics[a]	77 (3.5)
Other situations (various sectors, administrative sector exclusively)	592 (26.5)

[a] At least 1 year in the sector considered and no other (except administrative sector).

December 31, 2015, 913 of them had died. Cancer was the most common cause of death (338 deaths, 37.0%) (Table 2). Almost one-third of these cancer deaths were caused by trachea-bronchus-lung cancers (98 deaths, 10.7%).

3.2. Comparison with mortality in the regional population

Compared to the regional population, no excess risk of all-cause mortality (913 deaths, SMR = 0.96, 95%CI:0.90–1.02), nor of cancer mortality (338 deaths, SMR = 0.97, 95%CI:0.87–1.08) was found (Table 3). An excess risk of death from malignant neoplasms (MN) was observed in employees who had worked for the firm for between 15 and 29 years, SMR = 1.23, 95%CI:1.04–1.45. An excess mortality from colon-rectum-anus MN was observed in employees hired between 1921 and 1936 (6 deaths, SMR = 2.84, 95%CI: 1.04–6.18). Two deaths from gallbladder MN were found. Both of these employees had worked exclusively in the production sector (SMR = 11.50, 95%CI: 1.39–41.52), were hired between 1937 and 1970 (SMR = 4.68, 95%CI: 0.57–16.89), and had worked for the firm for between 15 and 29 years (SMR = 11.77, 95%CI: 1.42–42.24). Eight deaths from pancreatic MN were found, with an excess risk for employees having worked exclusively in the logistics sector (3 deaths, SMR = 6.20, 95%CI: 1.28–18.10). Deaths from leukemia (n = 8) were over-represented in employees having worked exclusively in the technical and purchasing units (7 deaths, SMR = 3.34, 95% CI: 1.34–6.88).

3.3. Cancer mortality by exposure category

Of all the exposures identified within the firm, exposure to Pb, Cd, asbestos and dust were those which affected the largest number of employees. For all the other toxic substances studied, more than 75% of the cumulative exposure indexes with a 20-year lag were null (See Supplementary Materials, Tables S2–S3). High positive correlations (correlation coefficients >0.60) were found between Pb, Cd, asbestos and dust, between As, Sb and sodium hydroxide vapor, between solvents and silica, Cr and silica, and between aromatic amines and sulfur dioxide (See Supplementary Materials, Fig. S1).

Table 2
Number and distribution of cancer deaths.

Causes of death	N	%
All causes	913	100.0
Neoplasms	338	37.0
Malignant neoplasms (MN)	329	36.0
- MN lip, oral cavity & pharynx	22	2.4
– MN lip & oral cavity	7	0.8
– MN oropharynx	6	0.7
– MN nasopharynx	1	0.1
– MN hypopharynx	7	0.8
– MN other or unspecified sites	1	0.1
- MN esophagus	23	2.5
- MN stomach	9	1.0
- MN colon, rectum & anus	30	3.3
– MN colon	17	1.9
– MN rectosigmoid junction	1	0.1
– MN rectum	12	1.3
– MN anal canal	0	0.0
- MN liver & intrahepatic bile ducts	14	1.5
– MN liver	12	1.3
– MN intrahepatic bile ducts	2	0.2
- MN gallbladder	2	0.2
- MN other specified sites bile ducts & unspecified sites	2	0.2
- MN pancreas	8	0.9
- MN larynx	13	1.4
- MN trachea, bronchus & lung	98	10.7
- MN pleura	3	0.3
- Malignant melanoma of skin	2	0.2
- MN breast	1	0.1
- MN prostate	22	2.4
- MN kidney	5	0.5
- MN renal pelvis	0	0.0
- MN ureter	0	0.0
- MN bladder	14	1.5
- MN brain & other unspecified central nervous system sites	2	0.2
- MN thyroid	0	0.0
- MN lymphoma	4	0.4
– Hodgkin's lymphoma	1	0.1
– Non-Hodgkin's lymphoma	3	0.3
- Leukemia	8	0.9
– Lymphoid leukemia	2	0.2
— Acute lymphoblastic leukemia	0	0.0
— Chronic lymphocytic leukemia	2	0.2
— Other & unspecified lymphoid leukemia	0	0.0
– Myeloid leukemia	4	0.4
— Acute myelogenous leukemia	2	0.2
— Chronic myelogenous leukemia	2	0.2
— Other & unspecified myeloid leukemia	0	0.0
- Other MN of lymphoid and histiocytic tissue, multiple myeloma & immunoproliferative neoplasms	3	0.3
– Multiple myeloma	2	0.2
- Other MN	42	4.6
Benign neoplasms, carcinoma in situ & neoplasms of uncertain behavior	9	1.0

The study of the associations between the cumulative exposure indexes and the risk of death showed similar results regardless of the time lag applied (none, 10, 15 or 20 years). The 20-year lag analysis is presented in Table S4. When several different exposures were associated with a specific cause of death ($p < 0.15$) the final results of the multivariate analysis are summarized in Table 4.

Taking age into account (Table S4), a significant excess risk of death from MN was found in subjects exposed to Cd and asbestos. In the final model, only exposure to asbestos was significantly associated with an excess risk of death from MN (HR = 1.76, 95%CI: 1.29–2.41 and HR = 1.42, 95%CI: 1.03–1.96 for the intermediate and high exposure categories, respectively) (Table 4). Deaths from trachea-bronchus-lung MN were significantly associated with Pb, Cd, asbestos and dust exposure

Table 3
Comparison of mortality in the smelter cohort studied and mortality for the regional population: standardized mortality ratio (SMR) and 95% confidence interval (except for causes of death <2 events).

Causes of death	Total cohort		Exclusive sector of work[a]							
			Production		Technical & purchasing		Control & development		Logistic	
	O	SMR [95%CI]	O	SMR [95%CI]	O	SMR [95%CI]	O	SMR [95%CI]	O	SMR [95%CI]
All causes	913	0.96 [0.90–1.02]	341	1.06 [0.95–1.18]	197	0.78 [0.68–0.90]	46	0.85 [0.62–1.13]	46	1.13 [0.83–1.51]
Neoplasms	338	0.97 [0.87–1.08]	119	1.00 [0.84–1.20]	81	0.89 [0.71–1.10]	16	0.79 [0.45–1.28]	18	1.34 [0.79–2.12]
Malignant neoplasms (MN)	329	0.97 [0.87–1.08]	117	1.00 [0.84–1.20]	80	0.89 [0.72–1.11]	15	0.75 [0.42–1.24]	17	1.29 [0.75–2.07]
- MN lip, oral cavity & pharynx	22	0.78 [0.49–1.18]	8	0.78 [0.34–1.54]	6	0.85 [0.31–1.85]	1	_	1	_
– MN lip & oral cavity	7	0.64 [0.26–1.32]	4	1.01 [0.27–2.59]	2	0.73 [0.09–2.64]	0	_	0	_
– MN oropharynx	6	0.97 [0.35–2.11]	1	_	2	1.32 [0.16–4.77]	1	_	0	_
– MN hypopharynx	7	1.29 [0.52–2.66]	3	1.51 [0.31–4.41]	2	1.50 [0.18–5.42]	0	_	0	_
- MN esophagus	23	0.91 [0.58–1.37]	6	0.69 [0.25–1.50]	4	0.61 [0.17–1.56]	1	_	3	3.05 [0.63–8.91]
- MN stomach	9	0.87 [0.40–1.65]	3	0.89 [0.18–2.60]	2	0.74 [0.09–2.67]	1	_	0	_
- MN colon, rectum & anus	30	1.09 [0.74–1.56]	13	1.44 [0.77–2.46]	4	0.53 [0.14–1.36]	2	1.28 [0.15–4.62]	1	_
– MN colon	17	0.90 [0.52–1.44]	8	1.31 [0.56–2.58]	3	0.58 [0.12–1.69]	0	_	0	_
– MN rectum	12	1.78 [0.92–3.11]	5	2.26 [0.73–5.27]	1	_	2	5.24 [0.63–18.92]	1	_
- MN liver & intrahepatic bile ducts	14	0.79 [0.43–1.33]	8	1.30 [0.56–2.56]	2	0.42 [0.05–1.52]	0	_	0	_
– MN liver	12	0.68 [0.35–1.19]	7	1.15 [0.46–2.37]	1	_	0	_	0	_
– MN intrahepatic bile ducts	2	1.21 [0.15–4.37]	1	_	1	_	0	_	0	_
- MN gallbladder	2	3.66 [0.44–13.21]	2	**11.50 [1.39–41.52]**	0	_	0	_	0	_
- MN other specified sites bile ducts & unspecified sites	2	1.81 [0.22–6.53]	0	_	0	_	0	_	1	_
- MN pancreas	8	0.61 [0.26–1.20]	2	0.45 [0.05–1.62]	0	_	0	_	3	**6.20 [1.28–18.10]**
- MN larynx	13	0.92 [0.49–1.57]	5	1.04 [0.34–2.42]	3	0.82 [0.17–2.39]	0	_	0	_
- MN trachea, bronchus & lung	98	1.08 [0.88–1.31]	40	1.27 [0.91–1.73]	28	1.19 [0.79–1.72]	3	0.56 [0.12–1.64]	4	1.17 [0.32–3.00]
- MN pleura	3	1.03 [0.21–3.01]	0	_	2	2.53 [0.31–9.13]	0	_	0	_
- Malignant melanoma of skin	2	1.07 [0.13–3.86]	1	_	0	_	0	_	0	_
- MN prostate	22	1.16 [0.73–1.76]	6	1.06 [0.39–2.31]	7	1.27 [0.51–2.62]	1	_	2	2.19 [0.26–7.91]
- MN kidney	5	0.93 [0.30–2.17]	2	1.10 [0.13–3.97]	2	1.39 [0.17–5.02]	0	_	0	_
- MN bladder	14	1.25 [0.68–2.10]	5	1.38 [0.45–3.22]	3	0.96 [0.20–2.80]	1	_	1	_
- MN brain & other unspecified central nervous system sites	2	0.38 [0.05–1.37]	1	_	1	_	0	_	0	_
- MN lymphoma	4	0.88 [0.24–2.25]	2	1.24 [0.15–4.48]	0	_	0	_	0	_
– Non-Hodgkin's lymphoma	3	0.85 [0.18–2.48]	1	_	0	_	0	_	0	_
- Leukemia	8	1.03 [0.44–2.03]	1	_	7	**3.34 [1.34–6.88]**	0	_	0	_
– Lymphoid leukemia	2	0.99 [0.12–3.57]	0	_	2	3.62 [0.44–13.07]	0	_	0	_
— Chronic lymphocytic leukemia	2	1.41 [0.17–5.09]	0	_	2	4.96 [0.60–17.91]	0	_	0	_
– Myeloid leukemia	4	1.27 [0.35–3.25]	0	_	3	3.55 [0.73–10.37]	1	_	0	_
— Acute myelogenous leukemia	2	1.14 [0.14–4.12]	0	_	1	_	1	_	0	_
— Chronic myelogenous leukemia	2	2.12 [0.26–7.65]	0	_	2	7.92 [0.96–28.59]	0	_	0	_
- Other MN of lymphoid and histiocytic tissue, multiple myeloma & immunoproliferative neoplasms	3	0.60 [0.12–1.75]	0	_	0	_	1	_	0	_
– Multiple myeloma	2	0.67 [0.08–2.42]	1	_	0	_	0	_	0	_

Table 3. (continued)

Causes of death	Period of hire						Employment duration					
	1921–1936		1937–1970		1971–2002		<15 years		15–29 years		≥30 years	
	O	SMR [95%CI]	O	SMR [95%CI]	O	SMR [95%CI]	O	SMR [95%CI]	O	SMR [95%CI]	O	SMR [95%CI]
All causes	67	0.93 [0.73–1.18]	710	0.99 [0.92–1.06]	136	0.83 [0.70–0.99]	229	1.05 [0.92–1.19]	347	1.10 [0.99–1.22]	337	0.80 [0.72–0.89]
Neoplasms	17	0.88 [0.51–1.41]	271	1.01 [0.90–1.14]	50	0.82 [0.62–1.09]	76	0.95 [0.76–1.19]	139	**1.22 [1.03–1.44]**	123	0.80 [0.67–0.96]
Malignant neoplasms (MN)	17	0.90 [0.52–1.44]	263	1.00 [0.89–1.13]	49	0.83 [0.62–1.09]	74	0.94 [0.75–1.19]	137	**1.23 [1.04–1.45]**	118	0.78 [0.65–0.94]
- MN lip, oral cavity & pharynx	2	2.19 [0.26–7.91]	17	0.79 [0.46–1.26]	3	0.51 [0.11–1.49]	6	0.89 [0.32–1.94]	10	1.04 [0.50–1.91]	6	0.50 [0.18–1.09]
– MN lip & oral cavity	1	_	5	0.60 [0.19–1.40]	1	_	1	_	3	0.80 [0.16–2.34]	3	0.64 [0.13–1.87]
– MN oropharynx	0	_	6	1.30 [0.47–2.83]	0	_	3	2.01 [0.41–5.87]	1	_	2	0.78 [0.09–2.82]
– MN hypopharynx	1	_	4	0.98 [0.27–2.51]	2	1.68 [0.20–6.06]	2	1.52 [0.18–5.49]	4	2.19 [0.60–5.61]	1	_
- MN esophagus	1	_	20	1.01 [0.62–1.56]	2	0.47 [0.06–1.70]	5	0.88 [0.29–2.05]	11	1.31 [0.65–2.34]	7	0.63 [0.25–1.30]
- MN stomach	0	_	6	0.78 [0.28–1.70]	3	2.03 [0.42–5.93]	3	1.33 [0.27–3.88]	3	0.90 [0.19–2.63]	3	0.64 [0.13–1.87]
- MN colon, rectum & anus	6	**2.84 [1.04–6.18]**	18	0.83 [0.49–1.31]	6	1.52 [0.56–3.31]	6	0.97 [0.35–2.11]	11	1.25 [0.62–2.24]	13	1.02 [0.54–1.74]
– MN colon	3	2.17 [0.45–6.34]	9	0.60 [0.27–1.14]	5	1.93 [0.63–4.50]	6	1.44 [0.53–3.13]	6	1.00 [0.37–2.18]	5	0.57 [0.18–1.33]
– MN rectum	2	3.38 [0.41–12.20]	9	1.75 [0.80–3.32]	1	_	0	_	5	2.31 [0.75–5.38]	7	2.28 [0.91–4.70]
- MN liver & intrahepatic bile ducts	0	_	11	0.78 [0.39–1.40]	3	0.97 [0.20–2.83]	1	_	7	1.25 [0.50–2.58]	6	0.76 [0.28–1.65]
– MN liver	0	_	10	0.72 [0.34–1.32]	2	0.65 [0.08–2.35]	1	_	6	1.08 [0.39–2.35]	5	0.64 [0.21–1.49]
– MN intrahepatic bile ducts	0	_	1	_	1	_	0	_	1	_	1	_
- MN gallbladder	0	_	2	4.68 [0.57–16.89]	0	_	0	_	2	**11.77 [1.42–42.24]**	0	_
- MN other specified sites bile ducts & unspecified sites	1	_	1	_	0	_	1	_	0	_	1	_
- MN pancreas	0	_	6	0.59 [0.22–1.28]	2	0.87 [0.11–3.14]	1	_	5	1.19 [0.39–2.77]	2	0.35 [0.04–1.26]
- MN larynx	0	_	10	0.88 [0.42–1.62]	3	1.53 [0.32–4.47]	4	1.35 [0.37–3.46]	2	0.42 [0.05–1.52]	7	1.10 [0.44–2.27]
- MN trachea, bronchus & lung	1	_	82	1.19 [0.96–1.47]	15	0.87 [0.49–1.44]	19	0.90 [0.54–1.41]	38	1.26 [0.89–1.73]	41	1.04 [0.75–1.41]
- MN pleura	1	_	2	0.84 [0.10–3.03]	0	_	0	_	2	2.11 [0.26–7.62]	1	_
- Malignant melanoma of skin	0	_	2	1.49 [0.18–5.38]	0	_	0	_	1	_	1	_
- MN prostate	3	1.34 [0.28–3.91]	16	1.07 [0.61–1.74]	3	1.70 [0.35–4.96]	5	1.25 [0.41–2.91]	11	1.91 [0.95–3.42]	6	0.65 [0.24–1.41]
- MN kidney	0	_	4	0.95 [0.26–2.43]	1	_	2	1.63 [0.20–5.88]	3	1.74 [0.36–5.08]	0	_
- MN bladder	0	_	13	1.46 [0.78–2.50]	1	_	3	1.22 [0.25–3.56]	7	1.96 [0.79–4.04]	4	0.77 [0.21–1.97]
- MN brain & other unspecified parts of central nervous system	0	_	2	0.53 [0.06–1.91]	0	_	1	_	0	_	1	_
- MN lymphatohematopoietic system	0	_	3	0.87 [0.18–2.54]	1	_	2	1.77 [0.21–6.39]	2	1.35 [0.16–4.87]	0	_

(*continued on next page*)

Table 3 (*continued*)

Table 3. (continued)

Causes of death	Period of hire						Employment duration					
	1921–1936		1937–1970		1971–2002		<15 years		15–29 years		≥30 years	
	O	SMR [95%CI]	O	SMR [95%CI]	O	SMR [95%CI]	O	SMR [95%CI]	O	SMR [95%CI]	O	SMR [95%CI]
Non-Hodgkin's lymphoma	0	-	3	1.12 [0.23–3.27]	0	-	1	-	2	1.77 [0.21–6.39]	0	-
- Leukemia	0	-	6	1.00 [0.37–2.18]	2	1.68 [0.20–6.06]	2	1.15 [0.14–4.15]	3	1.20 [0.25–3.50]	3	0.86 [0.18–2.51]
– Lymphoid leukemia	0	-	1	-	1	-	1	-	1	-	0	-
— Chronic lymphocytic leukemia	0	-	1	-	1	-	1	-	1	-	0	-
– Myeloid leukemia	0	-	4	1.64 [0.45–4.20]	0	-	0	-	3	2.92 [0.60–8.53]	1	-
— Acute myelogenous leukemia	0	-	2	1.48 [0.18–5.34]	0	-	0	-	1	-	1	-
— Chronic myelogenous leukemia	0	-	2	2.72 [0.33–9.82]	0	-	0	-	2	6.53 [0.79–23.57]	0	-
- Other MN of lymphoid and histiocytic tissue, multiple myeloma & immunoproliferative neoplasms	0	-	3	0.75 [0.15–2.19]	0	-	2	1.84 [0.22–6.64]	0	-	1	-
– Multiple myeloma	0	-	2	0.84 [0.10–3.03]	0	-	0	-	1	-	1	-

- SMR and 95%CI are not presented when specific causes of death <2.
[a] At least 1 year in the sector considered and in no other (except administrative sector).

(age-adjusted analysis); in the final model, only exposure to asbestos showed a significant excess risk of death from trachea-bronchus-lung MN (HR = 2.01, 95%CI: 1.06–3.83 and HR = 2.96, 95%CI: 1.56–5.64, for the intermediate and high cumulative exposure categories, respectively). Deaths from lip-oral cavity-pharynx MN were associated with Pb and asbestos exposure (age-adjusted analysis), which both remained significant in the final model (HR = 12.75, 95%CI: 1.42–114.7 and HR = 14.87, 95%CI: 1.30–169.6 for the intermediate and high cumulative exposure categories of asbestos, respectively; HR = 2.86, 95%CI: 0.69–11.96 and HR = 0.79, 95%CI: 0.13–4.80 for the intermediate and high cumulative exposure categories of lead, respectively). Taking age into account, numerous toxic agents were associated with deaths from colon-rectum-anus MN and rectum MN. In the final model, only the relationship to Cr exposure remained for deaths from colon-rectum-anus MN, and bismuth exposure for deaths from rectum MN. Colon cancer deaths were significantly associated with exposure to Cr and silica. The four subjects who died from colon cancer were exposed to both Cr and silica. To be consistent with the results observed with deaths from colon-rectum-anus MN, we chose to keep Cr in the final regression model. Only Pb exposure was significantly associated with death from liver-intrahepatic bile duct MN and more specifically to deaths from liver cancer (HR = 3.26, 95%CI: 0.25–41.72 and HR = 13.36, 95%CI: 1.30–137.2 for intermediate and highly exposed workers, respectively). Sodium hydroxide vapor was the only toxic agent significantly associated with death from laryngeal cancer (HR = 6.82, 95%CI: 1.45–32.06 for exposed versus unexposed workers). Deaths from bladder MN were significantly linked to aromatic amine and sulfur dioxide exposure; in the final model, only exposure to aromatic amines (HR = 4.63, 95%CI: 1.01–21.22) remained significant. Lastly, none of the exposures studied were significantly linked to death from prostate MN nor to death from esophageal neoplasm.

4. Discussion

Compared to the regional population, an overall excess risk of cancer mortality was found for the employees who worked in this non-ferrous metal smelter for a period of between 15 and 29 years (n = 139, SMR = 1.23, 95%CI:1.04–1.45). Deaths from leukemia were significantly more frequent in employees having worked in the technical and purchasing units and deaths from pancreatic cancer more common in employees from the logistics sector. Asbestos exposure was found to be associated with an increased mortality risk for all cancer sites (p = 0.0012), lip-oral cavity-pharynx malignant neoplasms (MN) (p = 0.0141) and trachea-bronchus-lung MN (p = 0.0018); lead exposure was associated with the same risk for lip-oral cavity-pharynx (p = 0.0378) and liver MN (p = 0.0155); aromatic amine exposure with bladder MN (p = 0.0002); chromium exposure with colon-rectum-anus MN (p = 0.0057) and colon MN (p = 0.0315); bismuth exposure with rectal MN (p = 0.0011) and sodium hydroxide vapor exposure with laryngeal MN (p = 0.0150).

This study has a number of weaknesses, in particular the small number of deaths for some cancer sites which limits the statistical power of the analyses, notably when investigating the relationship to occupational exposure. Furthermore, the absence of data on individual risk factors means factors such as smoking could not be taken into account. However, the study also has a number of strengths: the very low percentage of subjects lost to follow-up (2.2%); the detail and accuracy of the employees' work histories (2210 jobs identified); and the company-specific job-exposure matrix which makes it possible to take into account a broad range of occupational exposures. Moreover, the levels of exposure to Pb and Cd in the job-exposure matrix were determined based on atmospheric levels.

As in the present study, an overall deficit in all-cause mortality has been found in most studies of non-ferrous metal smelters (Lundström et al., 1997; Marsh et al., 2009; Wong and Harris, 2000) which is probably due to a healthy worker effect bias. Nonetheless, elevated mortality was reported in a cohort of 1990 lead smelter workers in Idaho

Table 4
Final cancer mortality models according to categories of cumulative exposure index (Cox regression models, with age and 20-year lag cumulative exposure index as time-dependent variables): hazard ratio and 95% confidence intervals for each MN location with more than 10 deaths[a].

Toxic agents & cumulative exposure[b]	Malignant neoplasms				MN lip, oral cavity, pharynx				MN colon, rectum, anus				MN colon				MN rectum			
	Eff	HR	[95%CI]	Global p value (*p trend*)	Eff	HR	[95%CI]	Global p value (*p trend*)	Eff	HR	[95%CI]	Global p value (*p trend*)	Eff	HR	[95%CI]	Global p value (*p trend*)	Eff	HR	[95%CI]	Global p value (*p trend*)
Pb								**0.0378**												
≤500 µg/m^{33} x years				_[c]	3	1.00		*(0.25)*				NS[d]				_				NS
>500–2000 µg/m^3 x years					14	2.86	[0.69–11.96]													
>2000 µg/m^3 x years					5	0.79	[0.13–4.80]													
Bismuth																				
Non exposed				_				_				NS				_	8	1.00		
Exposed																	4	7.70	[2.27–26.14]	**0.0011**
Cr																				
Non exposed				_				_	23	1.00			13	1.00						NS
Exposed									7	3.43	[1.43–8.22]	**0.0057**	4	3.56	[1.12–11.31]	**0.0315**				
Asbestos																				
Non exposed	62	1.00		**0.0012**	1	1.00		**0.0141**				NS				_				NS
>0 to <20	140	1.76	[1.29–2.41]	*(0.56)*	14	12.75	[1.42–114.7]	*(0.15)*												
20+	127	1.42	[1.03–1.96]		7	14.87	[1.30–169.6]													
Aromatic amines																				
Non exposed				_				_				_				_				NS
Exposed																				
Sodium hydroxide vapor																				
Non exposed				_				_				NS				_				NS
Exposed																				
Other exposures taken into account in the models but non-significant in the final model	Cadmium, sulfur dioxide								Cadmium, arsenic, antimony, thallium, diesel exhaust, solvents, sulfur dioxide, silica				Thallium, silica				Arsenic, antimony, thallium, dust, diesel exhaust, solvents, sulfur dioxide, silica			
Pb																				
≤500 µg/m^3 x years	1	1.00		**0.0121**	1	1.00		**0.0155**				_				NS				_
>500–2000 µg/m^3 x years	3	4.89	[0.45–53.62]	***(0.009)***	2	3.26	[0.25–41.72]	***(0.009)***												
>2000 µg/m^3 x years	10	14.51	[1.49–141.7]		9	13.36	[1.30–137.2]													
Bismuth																				
Non exposed				NS				NS				_				_				_
Exposed																				
Cr																				
Non exposed				_				_				_				_				_
Exposed																				
Asbestos																				
Non exposed				_				_				_	16	1.00		**0.0018**				_
>0 to <20													33	2.01	[1.06–3.83]	***(0.002)***				
20+													49	2.96	[1.56–5.64]					
Aromatic amines																				
Non exposed				_				_				_				_	10	1.00		
Exposed																	4	9.06	[2.78–29.49]	**0.0002**

(*continued on next page*)

Table 4 (*continued*)

Toxic agents & cumulative exposure[b]	Malignant neoplasms				MN lip, oral cavity, pharynx				MN colon, rectum, anus				MN colon				MN rectum			
	Eff	HR	[95%CI]	Global p value (*p trend*)	Eff	HR	[95%CI]	Global p value (*p trend*)	Eff	HR	[95%CI]	Global p value (*p trend*)	Eff	HR	[95%CI]	Global p value (*p trend*)	Eff	HR	[95%CI]	Global p value (*p trend*)
Sodium hydroxide vapor																				
Non exposed				-				-	11	1.00						-				NS
Exposed									2	6.82	[1.45–32.06]	**0.0150**								
Other exposures taken into account in the models but non-significant in the final model																				
					Arsenic, antimony								Cadmium, dust, sulfur dioxide				Cadmium, dust, sulfur dioxide			

[a] Final models for esophagus and prostate MN mortality are not presented due to the absence of any significant exposure linked with.
[b] Cumulative index of exposure with a 20-year lag, expressed as $\mu g/m^3$ x years for Pb and as cumulated scores for asbestos.
[c] Exposure not taken into account in the multivariate analysi0073.
[d] NS: exposures taken into account in the models but non-significant for the specific cause of death.

(SMR = 1.23, 95%CI: 1.17–1.23) with an exposure-response relationship found for cumulative exposure to lead (Bertke et al., 2016; Steenland et al., 1992). In an Italian cohort of 1388 workers, non-significant excess all-cause mortality was found (SMR = 1.05, 95%CI: 0.97–1.14) (Cocco et al., 1997). However, it should be noted that in these two cohorts, unlike the other studies in the literature, smelter employees mainly working in administrative roles were excluded from the analysis. As a point of comparison, our study also found a non-significant excess risk in the production and logistics sectors which were considered to be the most exposed.

Similarly to other mortality studies in smelters, no increase in mortality from all cancers was found (Cocco et al., 1997; Marsh et al., 2009). Nor was an increased incidence of cancer found for lead-exposed workers in a primary lead and copper smelter in Sweden (Englyst et al., 2001). However, excess cancer mortality was reported in other smelter studies. Excess cancer mortality was reported by Bertke et al. (2016) (SMR = 1.29, 95%CI: 1.16–1.43) but the study did not establish any relationship with lead exposure levels. Lundström et al. reported excess cancer mortality (SMR = 1.2, 95%CI: 1.0–1.5) in a Swedish cohort of 3979 workers exposed to lead, but this excess was not found to be significant for the workers with the highest levels of lead exposure (SMR = 1.2, 95%CI: 0.9–1.5). However, the incidence of cancer in this same cohort was not significantly elevated (SIR = 1.1, 95%CI: 0.9–1.2).

Studies of employees in non-ferrous metal smelters usually use lead exposure as their point of reference for occupational exposure. However, smelting operations expose workers to many other toxic substances which should also be taken into consideration. In our cohort, although there was a significant increased risk of overall cancer mortality in workers with moderate and high exposure to cadmium (age-adjusted analysis), only asbestos exposure was significantly associated with excess all-cancer mortality (multivariate analysis). In 2004, the IARC upgraded its classification of inorganic lead compounds to Group 2A carcinogens, based on evidence from studies in experimental animals (IARC Monographs, 2006). Indeed, it is difficult to obtain firm evidence of a causal link between lead exposure and cancer from epidemiological studies in the general and working population due to combined exposures to other suspected or confirmed carcinogens.

Regarding lung cancer mortality, the results from smelter studies are inconsistent. An increased risk of lung cancer mortality was reported for workers in a primary copper and lead smelter in northern Sweden (SMR = 2.8, 95%CI: 2.0–3.8) along with increased risk of incidence of lung cancer (SIR = 2.9, 95%CI: 2.1–4.0) (Lundström et al., 1997). The latter was even higher for the subgroup with the highest exposure to lead (cumulative blood-lead index). In an update of the same cohort, the increased incidence of lung cancer in the 481 workers exposed in the smelter's lead workshop was confirmed (SIR = 2.4, 95%CI: 1.2–4.5); but as the workers were also exposed to arsenic, the effects of these two carcinogens could not be untangled (Englyst et al., 2001). In the Idaho smelter cohort, a significant excess mortality of lung cancer was reported (SMR = 1.94, 95%CI: 1.64–2.27), but there was no exposure-response relationship for lead (Bertke et al., 2016). The authors suggested that potential confounders such as arsenic, cadmium or smoking, which were not controlled in the study, might explain this finding. In our cohort, as in other smelter studies, there was no evidence of excess lung cancer mortality (Cocco et al., 1997; Marsh et al., 2009). Our study did however reveal an exposure-response relationship between exposure to lead, cadmium, asbestos, dust and lung cancer mortality. In the multivariate analysis, only asbestos remained significantly associated with lung cancer mortality. The presence of asbestos in the smelter is attested to in the furnaces, buildings and the personal protective equipment used. Asbestos is known to be carcinogenic for the lungs and this is a convincing explanatory factor which probably suffices to explain the increased incidence of lung cancer (IARC Monographs, 2012a). However, asbestos exposure is only rarely taken into consideration in other smelter mortality studies. Naturally, we cannot exclude a synergistic effect from other pulmonary carcinogens such as tobacco,

arsenic, chromium and cadmium. Several studies investigating smelters or other sectors of activity have reported links between lead exposure and lung cancer incidence or mortality (Anttila et al., 1995; Barry and Steenland, 2019; Fu and Boffetta, 1995; McElvenny et al., 2015; Steenland et al., 2017). However, to date there still appears be a lack of conclusive evidence to support such a link, in particular given the exposure to other pulmonary carcinogens.

In our study, lip-oral cavity-pharynx cancer mortality was associated with asbestos exposure. The results from the literature regarding this association are still conflicting whereas the association between laryngeal cancer and asbestos has been proven. As for Marsh et al. (2009) et Wong and Harris (2000), no increase in laryngeal cancer mortality was found for the cohort as a whole. No links were found between asbestos exposure and laryngeal cancer mortality, but there was a significant excess risk related to exposure to sodium hydroxide vapor. Caustic soda was used in the lead refining process, thus exposing employees to sodium hydroxide vapor. Sodium hydroxide is corrosive and can cause severe burns in any tissues it comes into contact with. Irritation of the nose, throat, and respiratory airways is induced by inhalation of low levels of sodium hydroxide as dusts, mists or aerosols. There are no data available on the direct carcinogenicity of sodium hydroxide. However, cancer of the esophagus has been reported to develop on damaged tissue many years after exposure to sodium hydroxide by ingestion. Although the association with laryngeal cancer found here has not been previously reported in the literature, it is plausible from a pathophysiological point of view due to the causticity of these vapors, as for strong inorganic acid mists which are recognized carcinogens due to their corrosivity (IARC Monographs, 2012b). In our study, lip-oral cavity-pharynx cancer mortality was also associated with lead exposure, but without excess risk in the highest exposure category. To date, the literature does not support this association.

Regarding digestive cancers, no significant excess mortality was found in this study. Significant associations were highlighted between lead exposure and liver cancer mortality, chromium exposure and colon-rectum-anus MN and colon MN mortality and between bismuth exposure and rectum cancer deaths.

The finding from our cohort of no excess mortality from esophageal cancer is consistent with the results from other smelter studies (Marsh et al., 2009; Wong and Harris, 2000). Furthermore, we did not find any exposure related to esophageal cancer deaths. In contrast, Steenland et al. (2019) reported a link between lead exposure and esophageal cancer mortality in a large multi-center cohort studying cancer incidence in lead-exposed workers in Finland and Great Britain.

To the best of our knowledge, no smelter studies have reported any significant excess mortality relating to stomach cancer. In our study, the results showed non-significant decreased mortality due to stomach cancer (SMR = 0.87, 95%CI: 0.40–1.65) in line with the findings of Cocco et al. (1997) (SMR = 0.97, 95%CI: 0.53–1.62) for Italian lead smelter workers, and those of Marsh et al. (2009) in workers at a copper smelter in Tennessee (SMR = 0.78, 95%CI: 0.31–1.60). Other studies found a non-significant excess risk (Bertke et al., 2016; Gerhardsson et al., 1986; Wong and Harris, 2000). Similarly, no increased incidence of gastro-intestinal cancers was found in lead-exposed workers in the Swedish cohort (Lundström et al., 1997). Very few smelter studies have specifically investigated the relationship between lead exposure and stomach cancer (Bertke et al., 2016). Nonetheless, increased rates of stomach cancer have been reported for lead-exposed workers in a range of sectors of activity (Fu and Boffetta, 1995; Steenland et al., 2017, 2019; Steenland and Boffetta, 2000; Wong and Harris, 2000). The very small number of cases in our study (n = 9) meant it was impossible to study the association between different exposures (including lead) and deaths due to stomach cancer.

Like Marsh et al. (2009) and Wong and Harris (2000) no excess colon cancer mortality was found in our cohort. However, mortality for this cancer site was significantly higher for workers exposed to chromium and silica. To our knowledge, no significant association with chromium exposure has yet been reported in the literature. A meta-analysis of digestive cancers among workers occupationally exposed to Cr(VI) was published in 2010, including 13 studies with non-significant relative risk estimates for colon cancer ranging from 0.33 to 3.08 (Gatto et al., 2010). The colon cancer meta-SMR was 0.89, 95%CI: 0.70–1.12 for any Cr(VI) exposure. Another meta-analysis also failed to report links between Cr (VI) exposure and colon MN mortality (Deng et al., 2019). To the best of our knowledge, the association with silica is not currently supported by the literature. In our study, chromium exposure was also significantly linked to colon-rectum-anus MN mortality. The recent meta-analysis of Deng et al. (2019) pointed out a meta-SIR of 1.03, 95%CI: 0.96–1.12 for bowel cancer (intestine, colon, and rectum) exposed to hexavalent chromium.

A non-significant excess mortality for rectal cancer was found (SMR = 1.78 95%CI: 0.92–3.11) as reported in two other cohorts of smelter workers, with four cases in the cohort investigated by Marsh et al. (2009) (SMR = 1.60, 95%CI: 0.44–4.10), and eight cases in the study by Wong and Harris (2000) (SMR = 1.23 95%CI: 0.53–2.42). This association has also been found in other sectors of activity involving lead exposure (McElvenny et al., 2015; Steenland et al., 2019). In our study there was no link to lead exposure but a strong association between bismuth exposure and death by rectum MN was found. This association has not yet been reported. Bismuth is not recognized as a carcinogen. This metal is widely used in therapeutics for leishmaniasis and helicobacter pylori treatment, and more recently in cancer therapy. In foundries exposure to bismuth occurs during the refining phase, which also involves exposure to other metallic elements such as lead, arsenic, antimony, as well as carcinogens such as asbestos, for which doubts exist regarding its carcinogenicity to the rectum (Paris et al., 2017).

No excess liver cancer mortality was found in our study, as in other smelter studies (Cocco et al., 1997; Marsh et al., 2009; Wong and Harris, 2000). However, an exposure-response relationship was found in our study between lead exposure and cancer at this site. To date, there is little data to support this association. In one Australian cohort of 4114 workers exposed to lead in different sectors of activity, not including the primary lead industry, Gwini et al. (2012) found both significant excess liver cancer mortality and incidence. As the link with blood lead levels could not be studied, the authors attributed this increased risk to possible excessive alcohol consumption.

There was no evidence of excess mortality from bladder cancer. This was also the case in other cohorts of smelter workers (Cocco et al., 1997; Englyst et al., 2001; Marsh et al., 2009; Wong and Harris, 2000). The meta-analysis by Fu and Boffetta (1995) reported an association between lead exposure and bladder cancer mortality (meta-SMR = 1.41, 95%CI: 1.16–1.71), but this was not supported by the findings of Steenland et al. (2019) in their study of almost 30,000 lead-exposed workers, even at levels of over 400 μg L^{-1}. In our cohort, bladder cancer mortality was not found to be associated with lead exposure but was associated with exposure to aromatic amines. Indeed, ortho-toluidine was used in the smelter to absorb sulfur dioxide from lead and zinc ore roasting workshops. Ortho-toluidine is carcinogenic and there is sufficient evidence that exposure leads to an elevated risk of urinary bladder cancer in humans (IARC Monographs, 2012b).

No evidence was found of excess kidney cancer mortality in our study, as in the majority of smelter worker cohorts (Marsh et al., 2009; Wong and Harris, 2000). Steenland et al. (1992) found a significant excess risk (SMR = 2.39, 95%CI: 1.03–4.71) in the smelter workers most severely exposed to lead, but this finding was no longer present in the update by Bertke et al. (2016). Cocco et al. (1997) identified a non-significant elevated risk of kidney cancer (SMR = 1.75, 95%CI: 0.46–4.49) with a significant increase in risk in line with duration of employment. Finally, Englyst et al. (2001) found no increase in the incidence of kidney cancer in lead-exposed workers (SMR = 0.9, 95%CI: 0.1–3.2). The number of kidney cancer deaths in our cohort was too low to study any possible associations with specific exposures. The kidney is, however, a target organ for lead and other metals in smelters, such as

cadmium. Several authors have investigated lead's role in kidney cancer but none have found any significant association (Callahan et al., 2019; Fu and Boffetta, 1995; Gwini et al., 2012; McElvenny et al., 2015; Steenland and Boffetta, 2000).

Most lead smelter cohorts reported no significant excess mortality for cancer of the central nervous system, notably Marsh et al. (2009) (SMR = 1.39, 95%CI: 0.69–2.48), Cocco et al. (1997) (SMR = 2.17, 95%CI: 0.57–5.57) et Wong and Harris (2000) (SMR = 0.74, 95%CI: 0.24–1.74). Similarly, Lundström et al. (1997) did not find any significant excess risk of central nervous system cancer in the workers most exposed to lead (SIR = 1.6, 95%CI: 0.4–4.2). Our study only identified two deaths for this cause and therefore could not investigate any associations with exposure. A recent meta-analysis found suggestive evidence of an association between lead exposure and malignant brain tumors (pooled OR = 1.13, 95%CI: 1.04–1.24) (Ahn et al., 2020), whilst the meta-analysis by Meng et al. found a non-significant elevated risk of meningioma or brain cancer (Meng et al., 2020).

In our study, significant excess leukemia deaths were found for employees who worked in the technical and purchasing sector. Exposure to hematotoxic solvents in this sector cannot be ruled out. However, the titles of the jobs held by these employees, the period of professional activity and the time lag between the end of their working life and death, were not suggestive of work-related cancers.

5. Conclusion

One of the strengths of this study is that it investigated a broad range of chemical substances present in non-ferrous metal smelters which have not often been considered in other studies conducted to date. Studying mortality in relation to exposure to these substances has confirmed previously-known associations, such as an excess risk of bladder cancer mortality in employees exposed to aromatic amines and lung cancer mortality in those exposed to asbestos. However, these associations have not been reported in previous smelter studies. Furthermore, new associations were found, namely between lead exposure and liver cancer mortality; lead exposure and lip-oral cavity-pharynx cancer mortality; bismuth exposure and rectum cancer mortality; chromium exposure and colon cancer/colon-rectum-anus cancer mortality; and finally sodium hydroxide vapors exposure and laryngeal cancer mortality. These findings should be further consolidated by future studies.

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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.2022.113955.

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Declining semen quality and polybrominated diphenyl ethers (PBDEs): Review of the literature to support the derivation of a reference dose for a mixture risk assessment

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ABSTRACT

To support a mixture risk assessment for chemicals that interfere with male reproductive health, we reviewed the literature to identify studies of polybrominated diphenyl ethers (PBDEs) and poor semen quality. Several epidemiological studies have shown associations of PBDE exposures with declining semen quality, non-descending testes and penile malformations. In rodent studies, poor semen quality, changes in testosterone levels and reproductive tissues have been observed. *In vitro* studies with reporter gene constructs show PBDE congeners as androgen receptor antagonists, and mixture studies in these systems have demonstrated that PBDE congeners act together with other androgen receptor antagonists. These observations led us to attempt the estimation of reference doses for specific PBDE congeners that can be used in a future mixture risk assessment for deteriorations of semen quality. While epidemiological studies provide support for such associations, they were uninformative for derivations of reference doses, due to the incompatibility of dose metrics used in exposure assessments. We therefore based our estimates on animal studies. Using a rigorous confidence rating approach, we found robust evidence that BDE-47 produced reductions in semen quality. We identified only one high confidence study of BDE-99 and accordingly evaluated the strength of evidence as moderate. One high confidence, and several medium confidence experimental studies observed declines in semen quality after BDE-209 exposure. Using established risk assessment procedures, we estimated that BDE-47 exposures below 0.15 μg/kg/d are unlikely to lead to reductions in semen quality. The corresponding exposures for BDE-99 and BDE-209 are 0.003 μg/kg/d and 1000 μg/kg/d. It is planned to use these estimates as reference doses in a mixture risk assessment of deteriorations in semen quality, involving multiple other chemicals also contributing to poor semen quality.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are a group of organobromine chemicals used as flame-retardants in a wide range of products such as plastics, textiles and electronic equipment. There are 209 congeners which all share a common structural motif of two phenyl rings linked by an oxygen atom. PBDEs have been sold as commercial mixtures, named pentaBDE, octaBDE and decaBDE in reference to their average bromine content.

PBDE congeners differ in their chemical stability but are generally persistent and bioaccumulative. Due to their widespread use in the past, they are ubiquitous environmental contaminants. They accumulate in human and animal tissues. The production and use of hexaBDE, heptaBDE, tetraBDE, pentaBDE and decaBDE has been restricted under the Stockholm Convention on Persistent Organic Pollutants (POPs) (Sharkey et al., 2020).

Human exposure to PBDEs occurs through the diet, through inhalation and ingestion of dust, and by dermal contact. The European Food Safety Authority (EFSA) found the main route of exposure to be food of animal origin with a high lipid content such as meat, fish and dairy products (EFSA 2011).

PBDE congeners and their commercial mixtures have endocrine disrupting properties. *In vitro* assays with reporter gene constructs have revealed androgen receptor (AR) antagonist properties of several congeners (BDEs-19, -28, -38, -39, -47, -49, -79, -99, -100, -127, -153, -155, -181, -190) (Ermler et al., 2010; Harju et al., 2007; Stoker et al., 2005). They also interfere with male reproductive development, as

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Abbreviations

AF	Assessment factor
AR	Androgen receptor
BB	Body burden
BMDL	Benchmark dose level
BPA	Bisphenol A
EFSA	European Food Safety Authority
EHDI	Estimated human daily intake
GD	Gestational day
HBGV	Health-based guidance value
HI	Hazard Index
LOAEL	Lowest observed effect level
NOAEL	No observed effect level
NTP	National Toxicology Program
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
PECO	Populations Exposures Comparators Outcomes
PND	Postnatal day
PoD	Point of departure
POP	Persistent organic pollutant
RfD	Reference dose
RoB	Risk of Bias

demonstrated in rodent studies where they produce declines in semen quality, changes in reproductive tissue and hormone levels (Zhang et al., 2020). Several epidemiological studies show associations of PBDE exposures with declining semen quality (Akutsu et al., 2008; Albert et al., 2018; Yu et al., 2019), non-descending testes (Goodyer et al., 2017; Main et al., 2007) and penile malformations (hypospadias) (Poon et al., 2018).

Experimental mixture studies have shown that PBDEs can act in concert with other AR antagonists *in vitro* (Orton et al., 2014). Although supporting *in vivo* studies are missing, it is conceivable that PBDEs will contribute to anti-androgenic mixture effects also *in vivo*. Multiple other chemicals are known to interfere with normal male reproductive development and health. These include phthalates, bisphenol A (BPA), parabens, some azole pesticides, polychlorinated biphenyls and dioxins, as well as analgesics (Kortenkamp 2020). Mixture effects of combinations of some of these anti-androgens have been shown *in vivo*, with effects ranging from retained nipples in male offspring (Axelstad et al., 2014) to declines in semen quality (Axelstad et al., 2018). Furthermore, human exposure to anti-androgens is widespread (Apel et al., 2020; Bauer et al., 2021; EFSA 2018; Koch et al., 2012; Moos et al., 2017). As co-exposures to some or all of these chemicals are a reality (Frederiksen et al., 2020), the impacts of possible mixture effects on male reproductive health warrant systematic examination. PBDE congeners must be included in such an assessment.

The risks from exposures to multiple compounds in chemical risk assessment can be assessed by using the Hazard Index (HI) approach (Teuschler and Hertzberg 1995). The HI is the sum of so-called Hazard Quotients, the ratio of exposure and a reference dose or health-based guidance value (HBGV) for specific toxicities of all chemicals considered together in the assessment. By evaluating this sum against a reference value of 1, the HI expresses fold-exceedances of combined "acceptable" chemical exposures. To achieve consistency in the assessment and to reduce uncertainties, it is important that the reference doses selected for the Hazard Quotient are for similar, ideally identical, toxicity endpoints. A mixing of reference doses related to different toxicities is not advisable as this would introduce bias in the mixture risk assessment.

PBDEs not only interfere with the male reproductive system but also produce a wide range of other toxicities. In their assessment of four PBDE congeners (BDE-47, -99, -153, and -209), EFSA identified neurodevelopmental toxicity as the critical toxicity and derived corresponding points of departure (PoDs) (EFSA 2011). However, these values are not suitable as reference doses to build HIs in a mixture risk assessment for disruption of male reproductive health. To derive reference doses for such an assessment, it is necessary to search for appropriate studies of PBDE effects on the male reproductive system.

In this review we examined the literature with the aim of locating studies of the adverse effects of PBDE exposures on male reproductive development. We were interested in deriving corresponding reference doses for specific PBDE-congeners. To be able to utilise existing PBDE exposure data which is available for individual congeners, it was necessary to search for congener-specific toxicity data (EFSA 2011). We were particularly interested in aligning the mixture risk assessment with currently observed deteriorations in semen quality in Western countries (Levine et al., 2017). We therefore selected semen quality as the basis for deriving the PBDE reference doses (exposures no longer associated with declines in semen quality) and reviewed the literature for relevant experimental studies. We were able to build on the systematic review by (Zhang et al., 2020). We also considered the epidemiological literature but found this to be of limited use for deriving a reference dose, for several reasons. First, the dose metric in epidemiological studies is often PBDE tissue levels, especially hair, which complicates conversion to daily intakes, the metric used in most exposure assessments. Second, epidemiological studies do not normally allow attribution of effects to specific PBDE congeners. We therefore focused on experimental studies with animals and assessed the strength of evidence for links between PBDE exposure and declines in semen quality.

2. Materials and methods

2.1. Literature search

Through a scoping search of the literature we identified a recent systematic review and meta-analysis on the toxicity of PBDEs on the rodent male reproductive system (Zhang et al., 2020). Instead of conducting another full systematic review of the literature on the adverse effects of PBDEs on male reproduction, we opted for using this review as the basis for identifying relevant studies and as a starting point for an update. We complemented the records in Zhang et al. (2020) with additional literature searches for the period after 2020, by conducting citation searches of papers describing PBDE effects on semen quality. Briefly, we generally conducted our study according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) Statement (Shamseer et al., 2015). For inclusion of studies we used the PECO principle (Populations: laboratory mammalian species; Exposures: PBDEs by oral gavage, drinking water or diet; Comparators: animals not exposed to PBDEs; Outcomes: semen quality parameters, Supplementary Table 1). Additional literature searches for studies post 2020 were performed in PubMed and Web of Science using the keywords "PBDE", "Polybrominated diphenyl ether", "semen", "sperm", "semin", "reproduction", using MeSH terms and wildcards as appropriate. We also used search alerts in Web of Science, as well as references cited in the EFSA Scientific Opinion on Polybrominated Diphenyl Ethers (PBDEs) in Food (EFSA 2011). A flow diagram with details of the selection process is shown in Fig. 1.

The focus of our analysis was on mammalian animal studies of the effects of PBDEs on semen parameters. Studies that analysed sperm parameters such as count, concentration, motility, morphology or vitality, but not sperm DNA damage or aneuploidy were included in our analysis. Studies with non-mammalian test species were excluded. Data

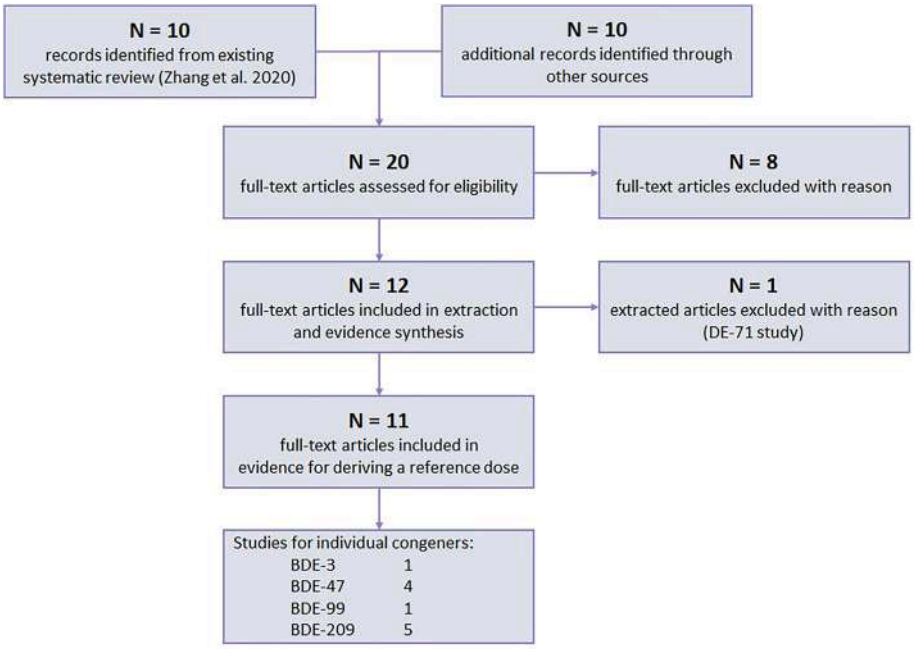

Fig. 1. Literature flow diagram for animal studies of PBDE exposures and semen quality.

from exposures during the sensitive window of exposure for male reproductive toxicity was used, but in the absence of foetal exposure studies, data from postnatal, juvenile, or adult animals were also considered. The eligibility criteria for experimental studies with laboratory animals are listed in Table 1.

2.2. Data extraction

The studies identified in the systematic literature review by Zhang et al. (2020) and additional searches were used to compile self-reported data on the respective PBDE doses related to no observed adverse effect levels (NOAELs) or lowest observed adverse effect levels (LOAELs), or effect doses at predetermined effect magnitudes (benchmark dose levels, BMDL).

The data was extracted into a template based on the one described in the National Toxicology Program (NTP) OHAT 2019 handbook (NTP OHAT 2019) and adapted for animal studies on declining semen quality and exposure to BPA (Kortenkamp et al., 2022; Martin et al., 2021). The data items included elements to summarise the study design, experimental model methodology and results. Some additional minor changes were made regarding the chemical identity and purity and the final data items are listed in the Supplement (Supplementary Table 2).

2.3. Study evaluation

The internal validity of the animal studies was appraised through a risk of bias (RoB) assessment based on a protocol defined for BPA studies by EFSA (EFSA 2017a, 2019) and further developed in the protocol for a systematic review on and declining semen quality (Kortenkamp et al., 2022; Martin et al., 2021). We utilised the NTP OHAT RoB Tool (NTP OHAT 2019). We adapted the assessment further to evaluate the studies we identified for PBDE exposure and male reproductive toxicity endpoints. The key elements of assessment included exposure characterisation (including purity and stability of test compounds, and absence of contaminations), outcome assessment (blinding of the outcome assessors) and power of detecting effects (sufficient number of animals per

Table 1
Eligibility criteria for experimental studies.

		Inclusion criteria	Exclusion criteria
Populations	Laboratory mammalian species including rats, mice, rabbits, guinea pigs, dogs and monkeys	Mammalian species	Non-mammalian test species such as fish or amphibians
Exposures	Polybrominated diphenyl ethers (PBDEs)	Administered by gavage, via drinking water or through the diet	Administered subcutaneously or intraperitoneally
Comparators	Animals not exposed to PBDE	Control group (same species as exposure group (s))	No control group
Outcomes	Semen quality	• Total sperm count • Sperm concentration • Sperm motility • Sperm morphology • Sperm vitality	• Sperm DNA damage • Aneuploidies • Fertility and fertilization outcomes

Criteria for the eligibility of experimental studies on the effects of PBDE exposure of laboratory animals on semen quality.

dose group). Due to the nature of the effects we additionally included a key element for laboratory proficiency (use of a reliable and sensitive animal model and inclusion of a positive control). We considered the characterisation of PBDE exposures as critical, particularly the purity of test compounds and the measurement of contaminants. The presence of dioxins and furans as contaminants is often observed and these compounds can exert similar toxic effects as PBDEs or might even mask effects exhibited by the PBDEs. We considered the use of phytoestrogen-free chow (i.e. soy-free feed) to be relevant for examinations of semen quality. Accordingly, we included this aspect in the RoB assessment, but did not consider it a key element.

A detailed list of all the elements of the RoB assessment can be found in Supplementary Table 3.

Each RoB element was evaluated using the NTP OHAT scores: ++ *definitely low risk of bias*; + *probably low risk of bias*; ~ *probably high risk of bias*; ~~ *definitely high risk of bias*. We used a tiered system to rate the studies, adopted from the system described by EFSA (EFSA 2019). This comprises three tiers, and each study was allocated to one tier as follows: *TIER 1 – high confidence,* where all key elements were scored + or ++ AND no more than one additional question was scored ~ or ~~; *TIER 2 – medium confidence* was assigned to all combinations not covered by *TIER 1* or *3*; the lowest tier, *TIER 3 – low confidence* was used when any one of the key elements was scored ~ or ~~ OR more than 50% of the additional questions were scored ~ or ~~.

2.4. Data synthesis

The findings and characteristics of eligible studies were summarised in a narrative synthesis. The data synthesis included summaries of PBDE exposure ranges (not) associated with declines in semen quality in animal studies as concluded from the derived NOAELs or LOAELs. Only studies we rated as high or medium confidence (*Tier 1* and *Tier 2*) were included in the summary. Studies that were allocated to *Tier 3* were not further analysed in detail.

2.5. Evidence synthesis

We synthesised the evidence from animal studies, using frameworks previously devised for BPA and phthalates (EFSA 2019; Radke et al., 2018). The evidence was categorised as *Robust* if multiple studies with a *Tier 1* confidence rating showed similar adverse effects. Any evidence not explained by study design or difference in animal model was considered of lower confidence, *Tier 2* or *Tier 3*. We rated the evidence as *Moderate* when it was insufficiently strong for *Robust*, but contained at least one *Tier 1* study and additional information supporting the findings. The rating of *Slight* was given in situations where studies suggested a possible decline in semen quality, but with weak or conflicting findings. *Indeterminate* was used for inconsistent, weak or conflicting findings. *Compelling evidence of no effect* was assigned when studies with high confidence ratings consistently demonstrated a lack of biological effects across species, sexes and exposure levels.

2.6. Derivation of a reference dose for individual PBDE congeners for declines in semen quality

In deriving a reference dose for individual PBDE congeners we followed the procedure used by EFSA for other toxicity endpoints (EFSA 2011). Each study where a PoD was discernible, was considered for the derivation of a reference dose. The PoDs under consideration were NOAELs or BMDLs.

Where a NOAEL was reported, but in addition sufficient data was available for BMDL modelling, we employed both, by using the PROAST tool via the EFSA web application (https://efsa.openanalytics.eu/) (EFSA 2017b) and the US-EPA tool BMDS3.x (https://www.epa.gov/bmds) (US EPA 2012).

In cases where no BMDL could be derived, either due to insufficient data or because the models did not deliver a BMDL, we used the reported NOAEL. If only a LOAEL could be estimated from the available data, the NOAEL was extrapolated using a standard assessment factor (AF = 3).

PBDEs are persistent compounds which bioaccumulate in tissues. They can exhibit different kinetic properties in different species which is relevant for extrapolations from rodent studies to humans. To scale the doses across different species we used the body burden approach. The body burden approach has previously been applied to derive health based guidance values for dioxins and dioxin-like polychlorinated biphenyls (PCBs) (EFSA 2015, 2018) and was the basis for margin-of-exposure considerations for PBDEs (EFSA 2011). We employed this approach to estimate rodent body burdens of PBDE congeners associated with PoDs ("critical" body burden). These were then used to derive human intake estimates which would lead to a human body burdens equivalent to the critical body burden in rodents.

We first estimated the body burden at the experimental PoD in the animal study. For studies which used a single oral PBDE dose, the body burden was derived by multiplying the PoD with the fraction of the compound absorbed into the animal body (Equation (1)). The absorbed fraction was derived from the oral absorption of the chemicals. For repeat administration studies, the body burden at the end of treatment was estimated by taking account of the absorption as well as the half-life of the chemical in the animal body. All kinetic parameters were collected from (EFSA 2011).

$$BB_a = F_{abs,a} \cdot PoD \quad (1)$$

with BB_a = body burden in the animal (amount/kg bw); $F_{abs,a}$ = fraction of chemical which is absorbed into the animal body; and PoD = point of departure, such as BMDL or NOAEL.

In a second step, we estimated the equivalent human daily intake (EHDI) by using the assumptions outlined in the EFSA opinion on PBDEs. Accordingly, we used a one compartment model to calculate the EHDI by multiplying the animal body burden derived in step one (Equation (1)) with the rate constant for the elimination from humans, divided by the fraction of compound absorbed into the human body (Equation (2)).

$$EHDI = \frac{BB_a \cdot k_{el,h}}{F_{abs,h}} \quad (2)$$

with $k_{el,h}$ = rate constant for removal from human body (1/day) and $F_{abs,\,h}$ = Fraction of chemical absorbed into the human body. In the one compartment model $k_{el,h}$ can be calculated according to Equation (3).

$$k_{el,h} = \frac{ln2}{t_{½\ ,\ h}} \quad (3)$$

with $t_{1/2,h}$ = halflife of excretion in humans. After substituting $k_{el,h}$ in Equation (2) with Equation (3) the EHDI was calculated according to Equation (4).

$$EHDI = \frac{BB_a \cdot \ln 2}{t_{½\ ,h} \cdot F_{abs,\,h}} \quad (4)$$

An additional assessment factor (AF = 2.5) was then applied to the EHDI to derive the reference dose for the individual PBDE (EFSA 2011). The AF of 2.5 was used to account for inter-species differences (EFSA 2011; WHO 1999). No further AFs were considered to be required because i) the reference dose was derived from developmental toxicity and the body burden applied to the entire human lifespan; and ii) the longest possible half-lives for the congeners were used, resulting in conservative estimates (EFSA 2011).

For PBDE congeners (such as BDE-209) with similar toxico-kinetics in rodents and humans, the body burden approach is not required, and the external PoD derived from the rodent study can be directly used as EHDI. The reference dose can then be calculated directly by application of an additional assessment factor. This is for instance the case for BDE-209 (AF = 100) (EFSA 2011).

3. Results

The selection process for animal studies to be included for the estimation of reference doses for PBDEs relevant for declines in semen quality is shown in Fig. 1.

3.1. Study selection and evaluation

Overall, 12 studies of PBDE congeners and their effect on semen quality *in vivo* were identified. Four of those studies were included in the systematic review by Zhang et al. (2020). Eight additional studies were identified through further searches, by citation searches and search alerts. One of the retrieved studies was conducted with the commercial PBDE mixture DE-71, whilst 11 studies investigated the effects of individual PBDE congeners. One study examined the effects of BDE-3 (Wei et al., 2018), four studies those of BDE-47 (Khalil et al., 2017; Li et al., 2021; Wang et al., 2013; Zhang et al., 2013), one study examined BDE-99 (Kuriyama et al., 2005) and five studies looked at BDE-209 (Miyaso et al., 2012; Sarkar et al., 2016, 2019; Tseng et al., 2006, 2013). All these records were included in the data extraction process.

One eligible study investigated the effects of the commercial PBDE mixture DE-71 on various sperm parameters (Van der Ven et al., 2008). This study identified a BMDL of 9.6 mg/kg/d for DE-71 on sperm morphology. Due to the lack of information on specific PBDE congeners, we could not include this study in our efforts of deriving a reference dose but considered it as supporting evidence for the adverse effects of PBDEs on male reproduction.

To evaluate the internal validity of studies on individual congeners we conducted a RoB analysis (Table 2).

The only eligible study on BDE-3 in mice (Wei et al., 2018) did not provide information about the purity of the test compound and lacked characterisations in terms of contaminations. We therefore rated this study as of low confidence (*Tier 3*).

We identified four studies that investigated semen parameters after exposure to BDE-47 (Khalil et al., 2017; Li et al., 2021; Wang et al., 2013; Zhang et al., 2013). The only mouse study on BDE-47 and semen quality raised concerns as the purity of the compound was not reported. Accordingly, we assigned *Tier 3* (Wang et al., 2013). The remaining three studies were conducted in rats. Two of these ranked "definitely low" or "probably low risk" on all points and where thus evaluated as high confidence studies and assigned to *Tier 1* (Li et al., 2021; Zhang et al., 2013). The study by Khalil et al. (2017) used soy containing diet and lacked a conflict of interest statement. We considered it to be of medium confidence and assigned it to *Tier 2*.

One study investigated the effect of BDE-99 on semen quality in rats (Kuriyama et al., 2005). This study scored "definitely low" or "probably low risk" on all key assessment elements and most of the other assessment aspects, except for the use of soy feed, and was thus rated as high confidence (*Tier 1*). We utilised this study to estimate a reference dose.

Our search returned five studies which investigated BDE-209, all of them examining semen quality in mouse models (Miyaso et al., 2012; Sarkar et al., 2016, 2019; Tseng et al., 2006, 2013). Two of these studies did not provide any information on the purity of their test compound or whether potential contaminants were assessed and thus were rated low confidence studies in *Tier 3* (Kim et al., 2009; Zhai et al., 2019). These studies also used soy containing diet and lacked conflict of interest statements. Two of the studies scored as "probably high" and "definitely high risk" on one or two non-key elements (see Table 2) and were thus of medium confidence and assigned to *Tier 2* (Sarkar et al., 2016; Tseng et al., 2006). Both employed soy containing diet for their experimental procedures and lacked a conflict of interest statement, and one had additional "probable high risk" due to a lack of information on randomisation and blinding (Tseng et al., 2006). The remaining study ranked "definitely low" or "probably low risk" on all assessment points and was therefore considered to be of high confidence (*Tier 1*) (Sarkar et al., 2019).

3.2. Overall study confidence ratings

Overall, four of the 11 studies included in the analysis were assigned to *Tier 1* (high confidence). These included two of the BDE-47 studies, the only BDE-99 study and one BDE-209 study. Three of the 11 studies were assigned to *Tier 2* (medium confidence), including one BDE-47 study and two BDE-209 studies. We allocated a "definitely high risk" in all these studies because they lacked a conflict-of-interest statement. The remaining four studies all obtained a rating of low confidence (*Tier*

Table 2
Outcome of RoB analysis for BDEs -3, -47, -99 and -209.
Shown is the scoring for each Risk of Bias (RoB) element for the selected animal studies. Questions in red represent key element, questions in green are the remaining elements.

The studies were rated as follows: definitely low risk of bias, DLR, in dark green; probably low risk of bias, PLR, in light green; probably high risk of bias, PHR, in yellow; definitely high risk of bias, DHR, in red. The RoB Tier assigned to each study is shown at the bottom. More information on the elements of the RoB assessment is shown in the detailed list in Supplementary Table 3.

RoB analysis for PBDEs		PBDE:	BDE 3	BDE 47				BDE 99	BDE 209				
		Author:	Wei et al.	Zhang et al.	Khalil et al.	Wang et al.	Li et al.	Kuriyama et al.	Miyaso et al.	Tseng et al.	Sarkar et al.	Tseng et al.	Sarkar et al.
		Year:	2018	2013	2017	2013	2021	2005	2009	2011	2016	2006	2019
Detection bias	1. Was exposure sufficiently characterised, including purity and stability of test substance?		PHR	PLR	PLR	PHR	PLR	PLR	PHR	PHR	DLR	DLR	DLR
Detection / Performance bias	2. Where the outcome assessors blinded to study groups?		PLR	PLR	DLR	PLR	PLR	PLR	PLR	PLR	PLR	DLR	PLR
Detection bias	3. Was the number of animals per dose group sufficient?		DLR	DLR	DLR	DLR	DLR	DLR	DLR	DLR	DLR	DLR	DLR
Detection bias	4. Was a reliable and sensitive animal model used and a positive control included the showed an effect?		PLR	PLR	PLR	PLR	PLR	DLR	PLR	PLR	PLR	PLR	PLR
Selection bias	5. Was administered dose adequately randomised?		PLR	DLR	PLR	DLR	PLR	PLR	PLR	PLR	PLR	PHR	PLR
Selection bias	6. Was allocation to study groups adequately concealed?		PLR	PLR	PLR	PLR	PLR	PLR	PLR	PLR	PLR	PLR	PLR
Performance bias	7. Were experimental conditions identical across study groups?		DLR	DLR	DLR	DLR	DLR	DLR	DLR	DLR	DLR	DLR	DLR
Performance bias	8. Was exposure consistently administered across treatment groups (method, time frame etc)?		DLR	DLR	DLR	DLR	DLR	DLR	DLR	DLR	DLR	DLR	DLR
Performance bias	9. Was the diet soy-free or soy-poor?		PLR	PLR	PHR	PLR	PLR	PHR	PHR	PHR	PHR	PHR	PLR
Attrition bias	10. Were outcome data complete without attrition or exclusion?		PLR	PLR	PLR	PLR	PLR	PLR	PLR	PLR	PLR	PLR	DLR
Detection bias	11. Were reliable and sensitive methods used for investigating the selected endpoint?		DLR	PLR	DLR	DLR	DLR	PLR	PLR	DLR	DLR	DLR	DLR
Detection bias	12. Were measurements collected at suitable timepoints?		PLR	PLR	DLR	PLR	PLR	DLR	PLR	DLR	PLR	DLR	DLR
Detection bias	13. Were statistical methods appropriate & can we be confident about the estimation of doses associated with low effects (NOAEL, LOAEL etc)?		DLR	DLR	PLR	PLR	DLR	DLR	PLR	DLR	PLR	PLR	PLR
Selective reporting bias	14. Have all study outcomes been reported?		PLR	PLR	PLR	PLR	PLR	PLR	PLR	PLR	PLR	PLR	PLR
Selective reporting bias	15. Have funding sources and conflicts of interest been reported?		DLR	DLR	DHR	DHR	DLR	DLR	DHR	DHR	DHR	DHR	DLR
		RoB TIER:	3	1	2	3	1	1	3	3	2	2	1

Table 3
Evaluation of experimental animal studies on semen quality and additional male reproductive endpoints after treatment with PBDE.
Colours: Key appraisal elements – Dark green: definitely low risk; light green: probably low risk; light red: probably high; (dark red: definitely low risk – not applicable). Study outcomes – Yellow: highlight of problematic finding despite medium rating.

Abbreviations: Key appraisal elements – PTU: Propylthiouracil.

	Study description			Key appraisal elements				Study outcomes		Study evaluation	
Reference	Species, strain	Outcome measures	PBDE congener	Purity of chemical, check for contamination	Randomisation, concealment, blinding	Number of animals per group	Model sensitivity, positive control	Outcomes	Comments	Tier	Overall confidence
Wei at al. 2018	Mouse, C57BL/6J gpt delta mice	Sperm count, vitality, morphology	**BDE-3**	not reported	yes	6	no positive control	Decrease in sperm count		**3**	Low
Zhang et al. 2013	Rat, Sprague Dawley	Daily sperm production	**BDE-47**	>98.7%	yes	10	no positive control	Decrease in daily sperm production	Serum testosterone also decreased	**1**	High
Khalil et al. 2017	Rat, Wistar	Sperm motility and morphology, daily sperm production	**BDE-47**	100%	yes	21 (3 per litter, 7 litters per group)	no positive control	Disruption of sperm morphology	Only one dose tested!	**2**	Medium
Wang et al. 2013	Mouse, B6 mice	Sperm morphology, motility, capacitation	**BDE-47**	not reported	yes	10	no positive control	Disruption of sperm capacitation and some motility parameters		**3**	Low
Li et al. 2021	Rat, Sprague Dawley	Sperm quantity and motility parameters	**BDE-47**	99.90%	yes	9	no positive control	Disruption of sperm motility		**1**	High
Kuriyama et al.2005	Rat, Wistar, HsdCpb:WU strain	Spermatid number, sperm count and morphology	**BDE-99**	98%	yes	16-20	PTU (for thyroid endpoint)	Decrease in sperm number and production	Sperm quality was not affected	**1**	High
Tseng et al. 2011	Mouse, CD-1	Sperm count, motility, motion analysis, morphology	**BDE-209**	not reported	yes	15	no positive control	Disruption of sperm morphology	No changes in count or motility	**3**	Low
Miyaso et al.2012	Mouse, ICR	Sperm count	**BDE-209**	not reported	yes	5	no positive control	Decreased sperm count	Sperm count only decreased at lowest concentration; Testes weight also decreased	**3**	Low
Sarkar et al. 2016	Mouse, Parkes (P) strain	Sperm number and viability	**BDE-209**	>98% analytical grade	yes	9	no positive control	Decreased sperm count and viability	Testis weight also decreased	**2**	Medium
Tseng et al. 2006	Mouse, CD-1	Sperm motility, motion, morphology	**BDE-209**	98%	not reported	10	no positive control	Disruption of sperm morphology	No changes in count or motility	**2**	Medium
Sarkar et al. 2019	Mouse, Parkes	Sperm count, motility, viability, morphology	**BDE-209**	> 98%	yes	7	no positive control	Decrease in sperm number, motility, and morphology	All parameters at all concentrations affected	**1**	High

3). In all cases this was due to a lack of information on the purity of the tested PBDE congener and the potential for contaminants. The *Tier 3* studies comprised the BDE-3 study, one BDE-47 and two BDE-209 studies.

3.3. Evidence synthesis

The evaluation of the studies is summarised in Table 3. The table shows that all of the studies observed some adverse effects on semen quality after administration of individual PBDE congeners. All studies showed declines in various semen parameters, irrespective of their confidence rating.

In the BDE-3 study, a decline in semen quality was observed, however, the study was ranked as low confidence and was therefore not included in the derivation of a reference dose. Due to the low confidence of the only available study, we rated the evidence for an effect of BDE-3 on semen quality as *Slight*.

All four studies that tested BDE-47 consistently reported disrupted sperm parameters and only one of these studies was rated as low confidence. Two studies were of high and one of medium confidence. Thus, the evidence that BDE-47 exposures lead to declines in semen quality is considered to be *Robust*.

We identified only one study investigating the effect of BDE-99 on sperm parameters. We rated this study as high confidence. Due to the lack of additional studies, we evaluated the evidence for semen quality declines from BDE-99 as *Moderate*.

The effects of BDE-209 on semen quality were studied the most and were consistently found to be adverse. The evaluation of the studies only found one to be of high confidence, with an additional two being of medium confidence. Two studies on sperm parameters scored low confidence. Due to the consistency of adverse findings for BDE-209 but the scarcity of high-quality studies, we considered the evidence for association between BDE-209 and semen quality declines to be *Moderate*.

3.4. Derivation of reference doses for declines in semen quality for BDE-47, -99 and -209

We derived reference dose values for three PBDE congeners, BDE-47, -99 and -209. Where data such as responses from three or more different dose groups were available, we attempted BMD modelling to estimate a $BMDL_5$. However, even for studies with sufficient numbers of dose groups, no adequate model could be fitted and the resulting $BMDL_5$

Table 4
Derivation of reference doses from rodent studies that full-filled all inclusion criteria and passed RoB assessment.

Congener/Study	Tier	Species	LOAEL (μg/kg/d)	NOAEL (μg/kg/d)	BB at NOAEL (μg/kg/d)	EHDI (μg/kg/d)	RfD (μg/kg/d)
BDE-47 Zhang et al. (2013)	1	Rat	1.00E+03	30[a)]	500	0.374	**0.15**
BDE-47 Li et al. (2021)	1	Rat	1.00E+03	100[a)]	2.00E+03	1.497	0.6
BDE-99 Kuriyama et al. (2005)	1	Rat	60	*20*[b)]	15	0.00721	**0.003**
BDE-209 Sarkar et al. (2016)	2	Mouse	9.50E+05	7.50E+05	n.a.	7.50E+05	7500
BDE-209 Tseng et al. (2006)	2	Mouse	5.00E+05	1.00E+05	n.a.	1.00E+05	**1000**

The reference doses chosen for mixture risk assessment are shown in bold.
LOAEL: Lowest observed adverse effect level; NOAEL: No observed adverse effect level; BB: Critical body burden; EHDI: Estimated human daily intake associated with rodent BB at NOAEL; RfD: Reference dose derived by dividing the EHDI by 2.5 for (BDE-47 and BDE-99) or 100 (BDE-209).
The NOAEL values shown in italics are extrapolations from studies where only a LOAEL, but no NOAEL was observed. A NOAEL was extrapolated by dividing the LOAEL by a factor of 3.
[a)] Repeat administration, BB estimated taking absorption and excretion into account.
[b)] Single administration.

values had too wide confidence intervals to be reliable. We therefore decided to use NOAEL values as PoDs for all congeners. If only a LOAEL was available, the NOAEL was extrapolated by using an AF of 3.

Table 4 shows the PoDs derived from the studies which we included in the calculation of reference dose values.

BDE-47: We based the derivation of a BDE-47 reference dose on two *Tier 1* studies which all used repeated dose administration in the rat (Li et al., 2021; Zhang et al., 2013). Li et al. (2021) exposed dams in 3 dose groups from 10 days pre-gestation to PND21, covering the critical period of male reproductive development (Gestational Day (GD) 9 to Postnatal Day (PND) 10). Zhang et al. (2013) exposed adult males in 3 dose groups for eight weeks, 6 days per week. The PoDs in these studies were 30 μg/kg/d (NOAEL) (Zhang et al., 2013) and 100 μg/kg/d (NOAEL) (Li et al., 2021). By using the toxicokinetic parameters for BDE-47 ($t_{1/2,a}$ = 23 days, $F_{abs,a}$ = 0.75 for the rat and $t_{1/2,h}$ = 926 days, $F_{abs,h}$ = 1 for the human, see (EFSA 2011)) we first calculated the cumulative critical body burdens at the NOAEL in the rat before estimating the EHDIs for BDE-47. The critical body burdens were 500 μg/kg/d (Zhang et al., 2013) and 2000 μg/kg/d (Li et al., 2021) and the estimated EHDIs were 0.374 μg/kg/d (Zhang et al., 2013) and 1.497 μg/kg/d (Li et al., 2021) respectively. Finally, the reference dose was derived by applying an AF of 2.5 to the EHDIs to account for variability between rodents and humans. Accordingly, the reference doses for BDE-47 (Table 4) were 0.15 μg/kg/d (Zhang et al., 2013) and 0.6 μg/kg/d (Li et al., 2021). Although the study by Zhang et al. (2013) was conducted in adult rats, it produced the lower PoD which we chose as our final estimate.

BDE-99: One (*Tier 1*) study qualified for derivation of a reference dose for BDE-99 (Kuriyama et al., 2005). It covered the critical period of male reproductive development. The study observed a LOAEL of 60 μg/kg/d based on administration of single oral doses in 2 dose groups at GD 6. Therefore, the NOAEL was estimated as 20 μg/kg/d, by application of a factor of 3. Considering an oral absorption in rodents of 75%, we calculated the critical body burden of BDE-99 at PoD by multiplication of the PoD of 20 μg/kg/d with the absorbed fraction as 15 μg/kg/d. With the toxicokinetic parameters for BDE-99 ($t_{1/2,a}$ = 20 days, $F_{abs,a}$ = 0.75 for the rat and $t_{1/2,h}$ = 1442 days, $F_{abs,h}$ = 1) we estimated 0.00721 μg/kg/d as EHDI in accordance with EFSA (2011). By application of an additional factor of 2.5 to account for inter-species variability in rodents and humans this gave a reference dose of 0.003 μg/kg/d (Table 4).

BDE-209: The two studies which we used to derive a reference dose for BDE-209 were conducted in juvenile or adult mice and were rated as medium confidence (*Tier 2*) (Sarkar et al., 2016; Tseng et al., 2006). The study in juvenile mice included 4 dose groups with a treatment duration of 50 days from PND21 (Tseng et al., 2006). In Sarkar et al. (2016), adult mice (12–14 weeks old) received BDE-209 in 2 dose groups for 35 days. The reported NOAELs in the studies were 7.5×10^5 μg/kg/d and 1×10^5 μg/kg/d. For BDE-209 the elimination half-life in animals and humans does not differ markedly and thus the corresponding external PoDs were used as EHDI to estimate the reference doses by application of an uncertainty factor of 100 following EFSA guidance (EFSA 2011). This produced possible reference doses of 7500 μg/kg/d (Sarkar et al., 2016) and 1000 μg/kg/d (Tseng et al., 2006) (Table 4). We had a higher confidence in the value produced by the (Tseng et al., 2006) study as juvenile mice with 4 dose groups were used. Accordingly, we chose the reference dose of 1000 μg/kg/d for BDE-209.

An additional study on BDE-209 which was rated as high confidence (*Tier 1*) (Sarkar et al., 2019) could not be included as BDE-209 administration was postnatally via gavage of the lactating dams which made it difficult to estimate the dosages received by the pups. However, at the maternal dose of 500 mg/kg/d (a LOAEL) reductions in sperm number and motility as well as changes in sperm morphology were seen in the offspring. This LOAEL is similar to those in the studies we used to derive the BDE-209 reference dose.

3.5. Extrapolation to untested PBDE congeners

Of the 209 possible PBDE congeners, relatively few are of environmental relevance, and even fewer have been tested toxicologically to a level required for risk assessments. However, limiting a mixture risk assessment only to toxicologically evaluated congeners while ignoring others that also contribute to human exposures will bias the assessment in the direction of underestimations of risk. To deal with this challenge, we adopted the read-across approach elaborated by us in an earlier PBDE mixture risk assessment for neurodevelopmental toxicity (Martin et al., 2017). Focusing on the congeners for which exposure data are available (EFSA 2011) – BDE-28, -47, -99, -100, -153, -154, -183 and -209 – we assumed that congeners with similar bromine content have similar toxicities. Congeners with similar bromine content also have similar half-lives in rodents and humans. Accordingly, we propose to assign the reference dose for BDE-47 (0.15 μg/kg/d) also to the untested BDE-28, the reference dose of BDE-99 (0.003 μg/kg/d) to BDE-153 and -154, and the reference dose for BDE-209 (1000 μg/kg/d) to BDE-183 and nonaBDE. Extrapolated reference doses that are close to exposure levels indicate a need to prioritise a congener for testing to refine the assessment.

3.6. Comparison with PBDE exposures

The average exposures to BDE-47 via food experienced by adults in Europe are around 0.7 ng/kg/d but can rise to 7.3 ng/kg/d through high consumption of PBDE-contaminated food items and additional high fish

Table 5
Calculation of Hazard Quotients for individual PBDE congeners.

BDE congener	RfD (μg/kg/d)	Average consumption		High consumption	
		Exposure (μg/kg/d)	HQ average	Exposure (μg/kg/d)	HQ high
BDE 28	0.15	0.00017	0.0011	0.00052	0.0035
BDE 47	**0.15**	**0.00072**	**0.0048**	**0.00733**	**0.049**
BDE 99	**0.003**	**0.00035**	**0.12**	**0.00142**	**0.47**
BDE 100	0.003	0.0003	0.1	0.00271	0.90
BDE 153	0.003	0.00026	0.087	0.00095	0.32
BDE 154	0.003	0.00028	0.093	0.00112	0.37
BDE 183	1000	0.00023	2.3E-07	0.001	0.000001
BDE 209	**1000**	**0.00169**	**1.69E-06**	**0.00479**	**4.79E-06**

Hazard Quotients have been calculated for average and high exposure to PBDEs via food, based on published consumption data (EFSA 2011; Martin et al., 2017). RfD: Reference dose; HQ: Hazard Quotient.
Data in bold shows congeners for which the RfD was derived, RfD values for the additional congeners was extrapolated.

consumption (Martin et al., 2017) based on data from EFSA (2011), well below the reference dose of 150 ng/kg/d. Similarly, average exposures to BDE-209 of adults via food are 1.7 ng/kg/d, with extremes of up to 4.8 ng/kg/d (Martin et al., 2017), far removed from the reference dose of 1000 μg/kg/d. The situation is different for BDE-99. Average intakes via food are around 0.35 ng/kg/d, which can reach 1.4 ng/kg/d for consumers of highly PBDE-contaminated food items and additional high fish consumption (Martin et al., 2017). This is relatively close to the reference dose of 2.9 ng/kg/d, yielding Hazard Quotients of 0.12 and 0.47, respectively.

We also used the extrapolated values for untested PBDE congeners to derive Hazard Quotients using the available exposure data for average and high consumption (EFSA 2011; Martin et al., 2017). The Hazard Quotients for the additional congers in the average exposure scenario ranged from 2.3×10^{-7} for BDE-183 to 0.12 for BDE-99 (Table 5). For high exposures, the range of Hazard Quotients was 1×10^{-6} for BDE-183 to 0.9 for BDE-100 (Table 5).

The sum of all Hazard Quotients, i.e. the HI for all congeners at average exposures had a value of 0.4, coming relatively close to a value of 1. The HI for the high consumption scenario was 2.1, exceeding the index value of 1.

4. Discussion

Mixture risk assessments for human health endpoints such as male reproductive health require the availability of relevant toxicity data. Here, we derived reference doses for three PBDE-congeners, BDE-47, -99 and -209, from animal studies on declines in semen quality. Although toxicity data for commercial PBDE mixtures and sums of congeners are also available, it was necessary to derive references doses for specific congeners in order to achieve a match with existing exposure data available e.g. from EFSA (EFSA 2011). Accordingly, we propose to utilise the reference doses estimated for BDE-47, -99 and -209 also for the evaluation of other congeners for which toxicity data are missing altogether.

We based our work on a recently published systematic review of the toxicity of PBDEs on the rodent male reproductive system (Zhang et al., 2020). Although the effects of PBDEs on male reproductive development have been studied in several animal studies, with respect to reproductive organ weights, anogenital distance or reproductive hormones, declines in semen quality were not always assessed. This meant that we could rely only on a limited number of studies.

We found that the quality of many eligible studies was compromised by a lack of information on the purity of the PBDE congeners. This is a significant shortcoming as other contaminants frequently found as impurities of PBDEs, such as dioxins, exert similar effects (EFSA 2018). Thus, we could not take account of studies which did not ascertain the absence of such contaminants.

We also did not consider studies that used the commercial mixture DE-71, due to their poorly defined composition. However, the majority of studies with DE-71 support the observations from studies with specific congeners, that PBDEs negatively affect semen quality and other markers of male reproductive development.

Ideally, exposure regimens would have covered the critical period when germline stem cell populations are established (mouse: gestational day 7 to postnatal day 8, rat: gestational day 9 to postnatal day 10). However, studies covering this period were not always available. In such cases (BDE-47: Zhang et al. (2013); BDE-209: Sarkar et al. (2016); Tseng et al. (2006)), we had to make recourse to exposure studies in adult male rodents. Possible concerns that this might have led to accordingly higher estimates of reference doses were not borne out in the case of BDE-47, where the study that covered the period of establishment of germ cell stem populations (Li et al., 2021) produced the higher reference dose.

In support of the observations from rodent studies, there are several reports of adverse effects of PBDEs on male reproductive health from human epidemiological studies. Declines in semen quality associated with elevated levels of PBDEs were observed in men attending fertility clinics (Abdelouahab et al., 2011; Den Hond et al., 2015) as well as in healthy men (Akutsu et al., 2008; Albert et al., 2018; Yu et al., 2019). However, others have found no association of selected congeners with semen parameters (Toft et al., 2014). The choice of congeners is often guided by their use as marker congeners and detection limits, and less by their toxicity and findings are reported linked to the sum of measured PBDEs. Overall, semen quality in men was found to be negatively associated with the individual congeners BDE-47, -100 and -153 and the sum of BDE-47, -99, -100 and -153. Furthermore, prenatal exposure to PBDEs has been linked with disrupted male reproductive development, namely an increase in cryptorchidism (Goodyer et al., 2017; Main et al., 2007) and hypospadias (Poon et al., 2018). However, many of these studies (Goodyer et al., 2017; Main et al., 2007; Poon et al., 2018) measured PBDEs in hair which makes it difficult to estimate daily exposures and to relate these observations to our reference doses.

The reference doses we estimated for declines in semen quality are higher than those which we used in a mixture risk assessment for developmental neurotoxicity of PBDEs (Martin et al., 2017) based on data from EFSA (2011) (BDE-47: 68.8 ng/kg/d versus 150 ng/kg/d for semen quality declines; BDE-99: 1.68 ng/kg/d versus 2.9 ng/kg/d; BDE-209: 17 μg/kg/d versus 1000 μg/kg/d). Thus, evaluated in a chemical-by-chemical approach, current exposures to single PBDE congeners are unlikely to be of concern in terms of declining semen quality. However, we find that the untested congeners BDEs-100, -153 and -154 were present at exposure levels close to the extrapolated reference dose of 2.9 ng/kg/d, indicating that toxicity data for those congeners is required to refine the assessment. Inclusion of all congeners with exposure data resulted in HIs of 0.4 for average and 2.1 for high consumption, indicating that combined exposures to these PBDEs warrants further investigation. It remains to be seen, how the contribution of PBDEs will play out in a risk assessment scenario that takes account of exposures to multiple chemicals implicated in disruptions of male reproductive development (Kortenkamp 2020).

Declaration of competing interest

The authors declare there are no conflicts 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.2022.113953.

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Estimating cobalt exposure in respirable dust from cobalt in inhalable dust

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ABSTRACT

Cobalt is a commonly used element in metal industry. Exposure to workers occurs mainly by inhalation of cobalt-containing dust. For the evaluation of cobalt exposure, risk assessment and investigations on occupational diseases, measurements of cobalt in respirable dust are needed. Up to now, often only data for cobalt in inhalable dust are available, which is due to the earlier classification of the limit value in this fraction. Therefore, a possibility to convert cobalt concentrations mathematically from inhalable into respirable concentrations is desirable. In this study, 639 parallel measurements of cobalt concentrations in inhalable ($c_{I(Co)}$) and respirable dust fractions ($c_{R(Co)}$) were extracted from the non-public exposure database MEGA (Measurement data relating to workplace exposure to hazardous substances, maintained at the Institute for Occupational Safety and Health of the German Social Accident Insurance) and investigated by regression analysis. For the whole dataset regression shows high quality measures (correlation coefficient $R = 0.888$, adjusted coefficient of determination *adj.* $R^2 = 0.788 - R^2$ is adjusted to sample size). Further description of the data is achieved by splitting the dataset according to the type of sampling (*'stationary'* and *'personal'*) and three working activity groups, *'high temperature processing'*, *'filling/transport/storage'*, and *'machining/abrasive techniques'* ($0.845 \leq R \leq 0.876$; $0.711 \leq$ *adj.* $R^2 \leq 0.762$). As subgroups of *'high temperature processing'* and *'machining/abrasive techniques'* two further groups could be determined. These groups are called heuristic groups, since they have to be formed non-systematically by trial and error. These heuristic groups are *'welding'* and *'grinding'*. They are more selective on the included working activities with *adj.* R^2 of 0.703 and 0.748 respectively. The resulting conversion functions of all groups are power functions with exponents between 0.704 and 0.794. For the estimation of cobalt in respirable dust in other studies, it is possible to use the conversion functions of the heuristic and working activity groups. Limitations of the possibility to use the conversion functions are discussed.

1. Introduction

Cobalt has a wide range of application in metallurgy. Main uses of cobalt in different application fields are formation of super alloys, cemented carbides and diamond grinding tools; colorizers (for glass, enamels, plastics, ceramics and fabrics); tire adhesives, soaps and driers, batteries and magnets (Barceloux, 1999; ASM, 2000). In terms of alloys, cobalt is added to improve wear heat, and corrosion resistance. Cobalt is used as matrix for the production of hard metals with tungsten carbide and small amounts of other metal elements like chromium, niobium, nickel, or molybdenum (Barceloux, 1999).

Occupational exposure of workers to cobalt occurs mainly by inhalation as a part of refining, alloy production and the processing of the metal and cobalt-containing alloys and salts especially in hard metal industries (Lison, 1996; Suh et al., 2016). Inhalation of hard metal and cobalt dust can cause severe health effects like asthma (Van Cutsem et al., 1987; Shirakawa et al., 1990) and the so-called hard metal disease, which seems to be directly associated with cobalt, causing health effects in a range from alveolitis to pulmonary fibrosis (Lison, 1996; Barceloux, 1999). Based on epidemiological data of exposure to workers in hard metal industry, in Germany the inhalable dust fraction of tungsten-carbide as well as cobalt-containing hard metal was classified by MAK (German Senate Commission for the Investigation of Health Hazards of Chemical Compounds) of DFG (German Research Foundation) as 'cancerogenic to humans' (category 1) if the total cobalt content is > 0.1% (AGS, 2017a,b). In contrast, the International Agency for Research on Cancer (IARC) classified in 2006 cobalt bound to tungsten-carbide in hard metal industry as 'probably carcinogenic to humans' (IARC: Group 2A) (CLP: Carc. 1A) and cobalt metal without tungsten carbide to 'possibly carcinogenic to humans' (IARC: Group 2B)

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(Wild et al., 2000; Wild et al., 2009; AGS, 2017a,b; ECHA, 2021). Besides that, the inhalable fraction of cobalt and its compounds are classified as 'possibly carcinogenic to humans' (MAK: 2; IARC: Class 2B; CLP: Carc. 1B) based on observations in animal testing (IARC, 2006; Suh et al., 2016; AGS, 2017a,b; ECHA, 2021). In Germany, Cobalt was mainly measured in the total dust fraction, based on a technical guide concentration [TRK], which was established 1984 in Germany. These legal values were adopted to the technical state of the art over the years and inhalable dust was measured. In 2004, a TRK of 0.1 mg m^{-3} was valid (in inhalable dust). In 2005, all TRKs for carcinogenic substances were suspended. Thereafter, in Germany for cobalt and its compounds, limit values derived from an exposure-risk-relationship (ERB) at 0.5 μg m^{-3} [AK] (acceptance concentration) in respirable dust and 5.0 μg m^{-3} [TK] (tolerance concentration) have been valid since 2017 in Germany (AGS, 2017a,b, 2021).

With the ERB values in Germany, subsequently the number of parallel measurements of cobalt in respirable and inhalable dust increased in recent years. For retrospective assessment, e. g. investigations on occupational diseases and monitoring of the development of exposure levels, it remains problematic if only data for cobalt in inhalable dust are available. In order to evaluate historical data, such as in epidemiological studies or retrospective view of a workplace, it would be valuable to determine a possibility to estimate cobalt in respirable dust from inhalable dust.

There are just a few studies concerning the conversion of cobalt concentrations in different dust fractions and they mainly focus on cobalt in total and inhalable dust in hard metal industries (Klasson et al., 2016; Ljunggren et al., 2019). Airborne cobalt concentrations in total, inhalable and respirable dust have also been measured in different working activities, such as finishing, molding, mixing, and weighing (Kim et al., 2015). Other studies mainly focus on biomonitoring and bioavailability after cobalt exposure of workers (Swennen et al., 1994; Broding et al., 2009; Klasson et al., 2017). Since this study focusses on air monitoring, with respect to the legal limit values, biomonitoring will not be discussed in further detail. For biomonitoring of cobalt in Germany, no threshold values in biological materials are established so far, besides a reference value of 1.5 μg/L [BAR] and a biological reference limit [BLW] of 35 μg/L cobalt in urine (DFG, 2020).

Following our recently published study, where we offered a mathematical solution for the estimation of nickel in respirable dust from nickel in inhalable dust (Wippich et al., 2021), we used the exposure data from the nonpublic database MEGA for this study to determine a possible relation between cobalt in inhalable and in respirable dust depending on working environments.

2. Materials and methods

2.1. Exposure database MEGA

The underlying data for this study bases on the surveillance activity of the German Social Accident Insurance and are stored in the exposure database MEGA (Gabriel et al., 2010). It holds over 3 million datasets with exposure data from over 870 hazardous substances, including information about the sampling procedure and analytical methods. The database was designed for the evaluation of cases of occupational diseases, hazard, and occupational exposure assessment in specific types of industry, as well as time-dependent analysis of exposure to hazardous substances at workplaces.

2.2. Sampling systems

The most commonly used system combination for collecting inhalable and respirable dust in parallel is GSP 3.5 with FSP-10 used for 356 measurement pairs, followed by GSP-10 with FSP-10 ($n = 240$). These systems can be used for both personal and stationary measurements (Mattenklott and Möhlmann, 2011). A complete list of sampling system combinations used, the sampling rate and the type of sampling, can be found in Table 1. The GSP sampler is used to collect inhalable dust through a cone directly on a filter (Riediger, 2001). With the FSP-sampler, coarse particles are pre-separated with a cyclone. Remaining respirable particles are subsequently sampled on a filter (Cossey and Vaughan, 1987; Siekmann, 1998). In some cases, in this study, the systems Gravikon VC-25 and Gravikon PM4 were also used for stationary measurements. Both systems can be used for measuring inhalable and respirable dust by applying different sampling heads. The VC-25 uses an additional impactor to collect respirable dust. For collecting inhalable dust, the sampling volume is drawn through an annular gap onto a filter (Coenen, 1981). Analogous to the FSP-sampler a cyclone is used as pre-separator for coarse particles in the sampling of respirable dust with the PM4 system. Inhalable dust is collected by drawing the volume into a filter cassette with an annular gap nozzle onto a filter (Siekmann, 1998).

All samplers used in this study are validated according to the international standard EN 13205 "Workplace exposure – assessment of sampler performance for measurement of airborne particle concentrations" (European Committee for Standardization, 2014) and comply with ISO 7708 (European Committee for Standardization, 1995).

2.3. Analytical methods

To collect dust samples, the sampling systems were equipped with cellulose nitrate filters (Sartorius, 0.8 μm pore size). After the application of dust, the filters were shipped to the Institute for Occupational Safety and Health of the German Social Accident Insurance for gravimetric and quantitative metal analysis. The filters were conditioned at a fixed temperature and humidity in laboratory atmosphere for at least one day for gravimetric analysis. For quantitative metal analysis, the filters were digested by a mixture of nitric and hydrochloric acid and cobalt was determined by inductively coupled plasma mass spectrometry (ICP-MS) (Pitzke, 2019).

2.4. Data selection

The formation of pairs is specified in our previous studies (Wippich et al., 2020, 2021). As a first step, the dataset for cobalt is extracted from the database MEGA. Between the years 1989 and 2020, a total of 17 445 cobalt measurements were collected. As a next step only concentrations higher or equal to the limit of quantification are considered, resulting in 10 238 cobalt measurements. The measured concentrations in this dataset are assigned to the associated dust fraction (considering the sampling system) and parallel pairs are formed if:

- the measurements have the same report number, industrial sector, working activity, type of sampling and the same day (remaining pairs: $n = 890$),
- the measurement procedure and the analytical process are standardized methods in the measurement system for exposure

Table 1
Measurement systems and sampling rates used for both dust fractions in parallel measurements.

sampler inhalable dust (sampling rate)	sampler respirable dust (sampling rate)	*n*	type of sampling
GSP (3.5 L/min)	FSP-10 (10 L/min)	356	Personal/ stationary
GSP-10 (10 L/min)	FSP-10 (10 L/min)	240	Personal/ stationary
VC-25 G (375 L/min)	VC-25 F (375 L/min)	33	Stationary
VC-25 G (375 L/min)	PM4-F (66.7 L/min)	4	Stationary
GSP (3.5 L/min)	PM4-F (66.7 L/min)	3	Stationary
PM4-G (66.7 L/min)	PM4-F (66.7 L/min)	2	Stationary
GSP (3.5 L/min)	FSP-2 (2 L/min)	1	Personal

assessment of the German Social Accident Insurance Institutions (MGU (Gabriel et al., 2010)) ($n = 862$)
- the measurements were executed at the same time (starting and ending times of both measurements do not differ by more than 5 min) and the sampling duration was ≥ 2 h ($n = 720$) and
- the type of sampling is limited to the categories 'personal' and 'stationary' ($n = 678$).

The parameter '*industrial sector*' describes the type of industry, where the measurement was performed, such as *plant engineering, chemical industry,* or *electroplating*. With the parameter '*working activity'*, the task and the process were combined. The restriction on measurements with a duration ≥ 2 h is based on the German Technical Guidance 402 (AGS, 2016), where the minimum number of samples, which have to be taken during a work shift with constant exposure, is dependent on the sampling duration. When the sampling duration is higher or equal 2 h, one measurement is sufficient (AGS, 2016). This is the reason why only measurements with a duration higher or equal 2 h have been included.

As an additional restriction, 'unphysical' results where the concentration of cobalt in respirable dust ($c_{R(Co)}$) exceeds the concentration of cobalt in inhalable dust ($c_{I(Co)}$), have been excluded from the dataset. Physically it is not possible that $c_{R(Co)}$ is higher than $c_{I(Co)}$ because respirable dust is a subset of inhalable dust; but because the parallel measurement values for $c_{R(Co)}$ and $c_{I(Co)}$ were measured independently, this can be observed occasionally. These concentrations can result from incorrect sampling, particle movement, thermal effects, or inhomogeneous materials. This criterion affects only 37 pairs of measurement. Further discussion on these values will be done later in this study.

Considering all restrictions and conditions for pair formation, a dataset of 641 pairs, gathered between the years 2011 and 2020, can be formed. The data have been collected during 149 different working activities and the majority of dust concentrations was recorded during 2 h-measurements. As described in following section 'Statistical and mathematical methods', two leverage points have been eliminated, so the whole dataset (group 0, see Table 2) consist of 639 pairs of parallel measured cobalt concentrations.

2.5. Statistical and mathematical methods

The statistical analyses are conducted with the statistical software IBM SPSS statistics, version 26 (IBM Corp.). For all tests, the significance level is fixed at $\alpha = 0.05$, equaling a confidence interval of 95 %.

In the first part of the statistical analysis, the data set was tested for log-normal or normal distribution. Both assumptions had to be rejected at the significance level of 0.05, using the Lilliefors-corrected Kolmogorov-Smirnov test (Sachs, 2004) for cobalt in both dust fractions. This is mainly caused by the heterogeneous working activities which are included in the total dataset. To separate the effect of the variables, *type of sampling* and *working activity*, and to identify possible interactions between these variables, a two-factor ANOVA was performed.

In our preliminary study (Wippich et al., 2020), we divided the data for parallel measured respirable and inhalable dust into groups with different *working activities* based on technical specifications for production processes or the attributed energy content of the process. Out of 818 working activity subgroup, six groups could be formed: *'surface treatment', 'high temperature processing', 'filling/transport/storage', 'machining/abrasive techniques', 'forming*' and *'others'*. Since the data for this study come from the same database, it was possible to form the same working activity groups. For some groups the amount of data was not sufficient ($n < 30$) and were therefore not considered in this study. Extracted working activity groups with sufficient data were: *'high temperature processing', 'filling/transport/storage',* and *'machining/abrasive techniques'*. The working activity subgroups and their assignment to the working activity groups can be found in the supplemental materials. For each group the ratio $c_{R(Co)}/c_{I(Co)}$ was calculated and homogeneity of variance was confirmed applying the Levene-Test (Janssen and Laatz, 2017). Differences (considering $c_{R(Co)}/c_{I(Co)}$) between the working activity groups were determined by ANOVA if variance homogeneity could be guaranteed and normal distribution was determined. Otherwise the non-parametric Kruskal-Wallis test was used. After those variance analyses a pair-by-pair comparison applying the Games-Howell post-hoc test (Sachs, 2004; Hilton and Armstrong, 2006) was performed, to show which specific working activity groups were different from each other. The group formation steps are illustrated by the flowchart, Fig. 1. This systematic approach leads to groups of parallel measured cobalt concentrations in inhalable and respirable dust.

In addition, to evaluate the impact of the *type of sampling,* the whole dataset was divided into the two subgroups *'stationary'* and *'personal'*. After calculating the ratio $c_{R(Co)}/c_{I(Co)}$, for each pair in both groups, they were compared using the non-parametric Mann-Whitney-U-Test for independent samples (Haviland, 1990; Sachs, 2004; MacFarland and Yates, 2016).

In order to perform a linear regression analysis in each group, several prerequisites had to be fulfilled. The residuals of working activity and type of sampling groups, as well as the residuals of the whole dataset have been checked graphically (histograms) for normality and absence of trends (scatterplot). There are no patterns discernible apart from the omission $c_{R(Co)}/c_{I(Co)}$ and all residuals are approximately normally distributed. Each group has been checked on the absence of autocorrelation by performing a Durbin-Watson test (Sachs, 2004). Before the regression functions, possible leverage points were identified and eliminated using the Cook's measure (Cook and Weisberg, 1982; Chatterjee and Hadi, 1986; Kleinbaum et al., 2014). Subsequently, the groups were subjected to a linear regression analysis. The quality of the regression parameters is evaluated using the correlation coefficient R and the adjusted coefficient of determination $adj.\ R^2$ (Janssen and Laatz, 2017):

$$adj.\ R^2 = R^2 - \frac{m}{n-m-1} * (1 - R^2) \quad (1)$$

The parameter is dependent on the number of variables m and the number of paired data n. Since in our case $n \gg m$, $adj.\ R^2$ is about R^2.

Table 2
Regression coefficients k, C_0 with standard errors for Equation (1), range of standard errors for regression function $s_{Fit}(\ln(c_{R(Co)}))$ within groups 1–3 for *working activity* and heuristic groups α and β, the concentrations must be inserted as mg m^{-3} in the conversion functions!

ID	Group	*n*	*R*	adj. R^2	C_0	k	$s_{Fit}(\ln(c_{R(Co)}))$	conversion function
0	*Entire dataset*	639	0.888	0.788	−3.331 ± 0.096	0.733 ± 0.015	0.088–0.252	$c_{R(Co)} = {c_{I(Co)}}^{0.733} * e^{-3.331}$
A	*Entire dataset only personal meas.*	515	0.864	0.747	−3.472 ± 0.109	0.704 ± 0.018	0.091–0.264	$c_{R(Co)} = {c_{I(Co)}}^{0.704} * e^{-3.472}$
Working activities								
1	*High temperature processing*	145	0.845	0.711	−3.083 ± 0.275	0.734 ± 0.039	0.131–0.474	$c_{R(Co)} = {c_{I(Co)}}^{0.734} * e^{-3.083}$
2	*Filling/transport/storage*	49	0.876	0.762	−3.407 ± 0.310	0.793 ± 0.064	0.333–0.753	$c_{R(Co)} = {c_{I(Co)}}^{0.793} * e^{-3.407}$
3	*Machining/abrasive techniques*	234	0.845	0.713	−3.418 ± 0.172	0.722 ± 0.030	0.122–0.426	$c_{R(Co)} = {c_{I(Co)}}^{0.789} * e^{-3.049}$
Heuristic groups								
α	*Welding*	96	0.840	0.703	−2.976 ± 0.365	0.747 ± 0.050	0.142–0.488	$c_{R(Co)} = {c_{I(Co)}}^{0.747} * e^{-2.976}$
β	*Grinding*	161	0.866	0.748	−3.195 ± 0.202	0.761 ± 0.035	0.150–0.550	$c_{R(Co)} = {c_{I(Co)}}^{0.761} * e^{-3.195}$

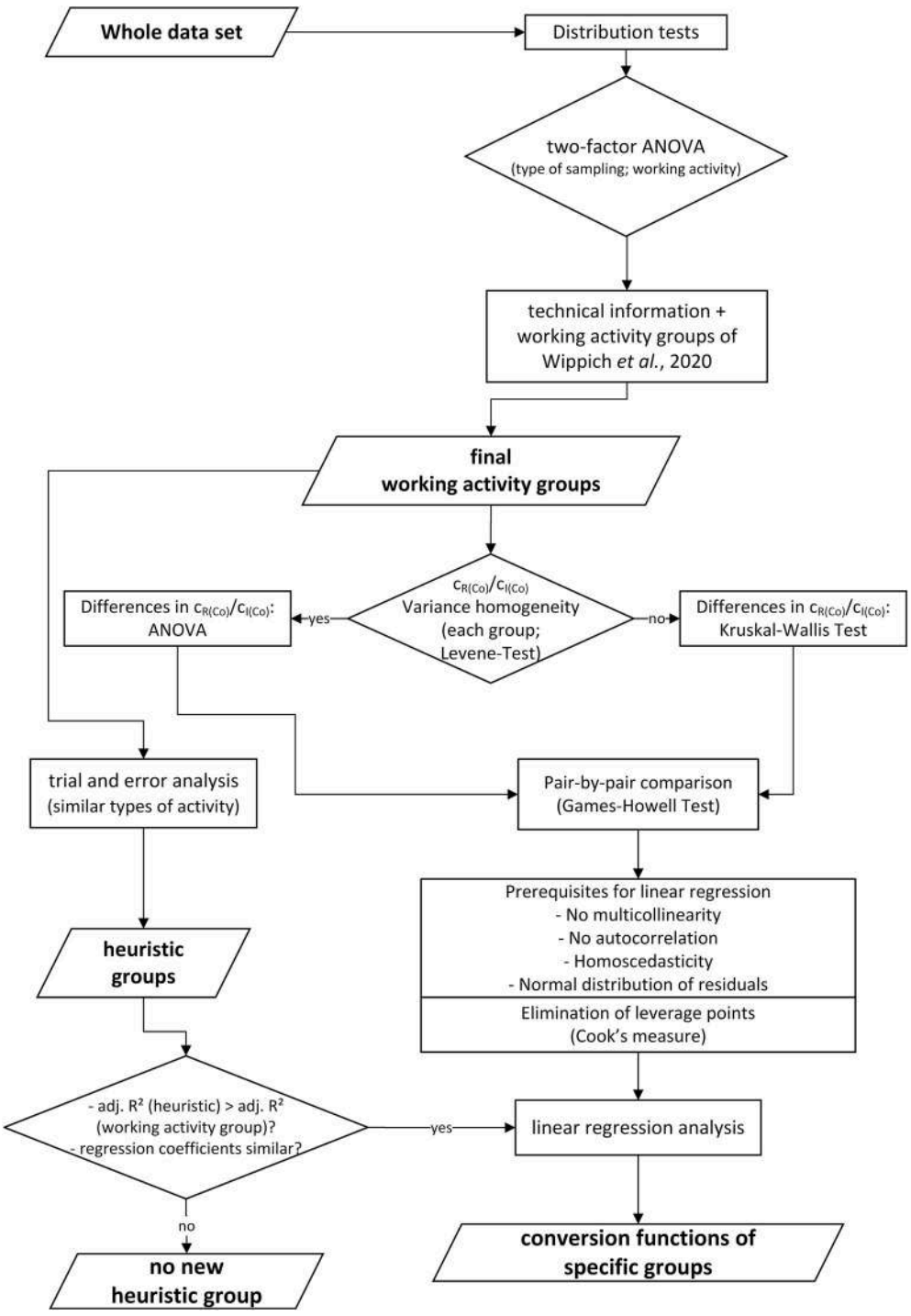

Fig. 1. Flowchart of the group formation steps and statistical tests.

This study describes a linear relationship between $\ln(c_{R(Co)})$ (natural logarithm of the cobalt concentration in respirable dust) and $\ln(c_{I(Co)})$ (natural logarithm of the cobalt concentration in inhalable dust):

$$ln(c_{R(Co)}) = k \cdot ln\left(c_{I(Co)}\right) + C_0 \quad (2)$$

The regression coefficients are k (slope) and C_0 (intercept), which can be determined by regression analysis and are shown with their standard errors (Table 2). To determinate the confidence intervals of the regression function at a given $ln(c_{I(Co)})$, the range of standard errors for the fitted regression function $s_{Fit}(ln(c_{R(Co)})$ (Draper and Smith, 1998), are also given. Equation (2) can be transformed back into a function with the original concentrations:

$$c_{R(Co)} = {c_{I(Co)}}^{k} \cdot e^{C_0} \quad (3)$$

From equation (3) two things can be derived: First, when $c_{I(Co)}$ tends to zero, $c_{R(Co)}$ also tends to zero. This is a necessary condition, since $c_{R(Co)} \leq c_{I(Co)}$. Second, the assumption of a linear relationship between $c_{R(Co)}$ and $c_{I(Co)}$ is included in equations (2) and (3), if the value 1 is a part of the 95 % confidence interval of k. The worst-case assumption $c_{R(Co)} = c_{I(Co)}$ is included, if $C_0 = 0$ and $k = 1$.

To evaluate the impact of other variables than inhalable dust on respirable dust, such as working activity, or sampling system, normally

equation (2) or (3) can be expanded with further (linear) variables, and a multilinear regression is performed. For this type of analysis, independent variables are required. This prerequisite is violated as all other predictor variables influence not only the predicted variable $c_{R(Co)}$ but also the predictor variable $c_{I(Co)}$. Since multilinear regression cannot be applied, we decided to form subgroups depending on *type of sampling* and *working activity* to evaluate each variable and their influence on the regression function individually.

Based on the identified 'working activity groups', more subgroups, so-called 'heuristic groups' (*'welding'* and *'grinding'*) were derived by trial and error. These groups cannot be formed systematically. They were formed based on two different criteria: describe similar working activities and show similar regression coefficients (k and C_0). For the first criterion as in on our preliminary study (Wippich et al., 2020), we determined similar heuristic groups by pooling similar working activities, such as various types of welding (e. g. metal active gas welding, gas tungsten arc welding or laser beam welding). As a second criterion to form these groups, the regression coefficients had to be similar and the *adj.* R^2 of the resulting heuristic group should be larger than for the relating working activity group. To meet these requirements, specific working activities were selected. The calculated regression functions are also just valid for measurements which can be assigned to those specific working activities. In this study the working activity groups, which were determined in the preliminary study, were used to form the heuristic groups. For many heuristic groups the requirements were not fulfilled and due to insufficient data, only two heuristic groups could be formed: *'welding'* and *'grinding'*.

3. Results

3.1. Cobalt in inhalable and respirable dust: description of the whole dataset

After the elimination of two leverage points, simple linear regression analysis was performed on the ln-transformed whole dataset of 639 pairs of parallel cobalt measurements. Considering only $c_{I(Co)}$ as predictor variable, one obtains $k = 0.733$ and $C_0 = -3.331$ in equation (1). The adjusted coefficient of determination and correlation coefficient are 0.788 and 0.888, respectively (Table 2). The arithmetic means (*AM*) for cobalt are 0.026 mg m^{-3} in inhalable dust, and 0.003 mg m^{-3} for cobalt in respirable dust respectively (Table 3).

Fig. 2 shows a scatterplot of the ln-transformed, parallel measured cobalt concentrations in inhalable versus respirable dust and the 95 % confidence interval. The cutoff values due to the restriction of $c_{R(Co)} > c_{I(Co)}$ are clearly visible. The data pairs of stationary and personal measurements can be distinguished by the different symbols.

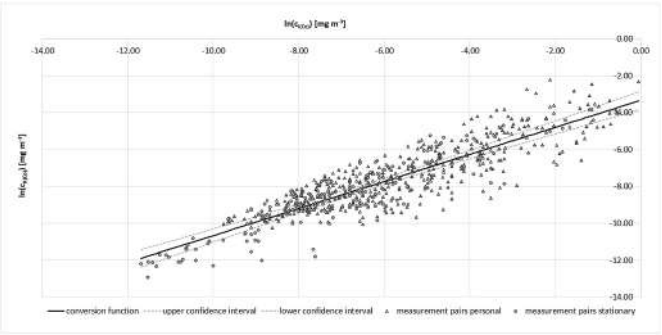

Fig. 2. Scatterplot $y = \ln(c_{R(Co)})$ versus $x = \ln(c_{I(Co)})$ with linear regression line and 95th confidence interval (equation (1)).

3.2. Type of sampling

From the total 639 datasets, 124 stationary and 515 personal measurements were recorded for cobalt in this study. The reason for the high amount of personal measurements results from the requirements of the Technical Guidance 402, which determines that stationary exposure measurements should be only conducted in exceptional situations (AGS, 2016). In the whole dataset (group 0) the median of the ratio $c_{R(Co)}/c_{I(Co)}$ in both groups *'stationary'* (median = 0.293) and *'personal'* (median = 0.186), as well as the distribution, are not identical. The proportion of cobalt in respirable dust is higher for stationary measurements. Test results of comparing the median ratios $c_{R(Co)}/c_{I(Co)}$ of both subgroups show significant differences (Median and Mann-Whitney-U-test: $p < 0.001$). A two-factor ANOVA was performed to check if the differences result from the type of sampling. The analysis revealed that both; *'working activity'* and *'type of sampling'* attribute to the differences in the subgroups (*'working activity'* $p < 0.001$; *'type of sampling:* $p = 0.036$). Interactions were not significant ($p = 0.470$). The concentrations for personal measurements (AM $c_{I(Co)} = 0.0298$ mg m^{-3}; AM $c_{R(Co)} = 0.0032$ mg m^{-3}) are higher than for stationary measurements (AM $c_{I(Co)} = 0.0099$ mg m^{-3}; AM $c_{R(Co)} = 0.0010$ mg m^{-3}). The majority of the stationary measured particles is < 0.020 mg m^{-3} (Co in inhalable dust) and < 1.0*10^{-3} mg m^{-3} (Co in respirable dust), respectively. For personal measurements the main part of $c_{I(Co)}$ is < 0.040 mg m^{-3} and $c_{R(Co)}$ is < 0.004 mg m^{-3} respectively. Fig. 3 shows the regression functions for both subgroups in direct comparison. As stationary measurements should only be performed in exceptional cases, larger variance in data can be determined (see also Fig. 3) and spurious differences in both subgroups occur, we decided to focus on personal measurements and calculated more specific conversion functions only for personal measurement data.

In case of personal measurements ($n = 515$) the quality measures for this regression model are $R = 0.864$, *adj.* $R^2 = 0.747$ (group A; Table 2).

Table 3
Descriptive statistics of respirable and inhalable cobalt samples used in the study, with the amount of paired cobalt concentrations (n) arithmetic mean (*AM*), standard deviation (*SD*), minimum measured concentration (*Min*), maximum measured concentration (*Max*).

ID	Group	Dust fraction	n	AM [mg m^{-3}]	SD [mg m^{-3}]	Median [mg m^{-3}]	Min [mg m^{-3}]	Max [mg m^{-3}]
0	*Entire dataset*	$c_{I(Co)}$	639	0.026	0.080	0.002	0.8*10^{-5}	0.950
		$c_{R(Co)}$	639	0.003	0.009	0.3*10^{-3}	0.2*10^{-5}	0.110
A	*Entire dataset only personal meas.*	$c_{I(Co)}$	515	0.030	0.087	2.5*10^{-3}	5.8*10^{-5}	0.950
		$c_{R(Co)}$	515	0.003	0.010	4.2*10^{-4}	2.6*10^{-5}	0.110
1	*High temperature processing*	$c_{I(Co)}$	145	0.007	0.027	7.0*10^{-4}	5.8*10^{-5}	0.240
		$c_{R(Co)}$	145	0.002	0.007	2.3*10^{-4}	2.6*10^{-5}	0.065
2	*Filling/transport/storage*	$c_{I(Co)}$	49	0.097	0.180	0.012	1.3*10^{-4}	0.950
		$c_{R(Co)}$	49	0.008	0.020	1.1*10^{-3}	3.6*10^{-5}	0.098
3	*Machining/abrasive techniques*	$c_{I(Co)}$	234	0.026	0.072	3.6*10^{-3}	6.6*10^{-5}	0.570
		$c_{R(Co)}$	234	0.003	0.009	5.2*10^{-4}	3.7*10^{-5}	0.110
α	*Welding*	$c_{I(Co)}$	96	2.7*10^{-3}	0.008	5.6*10^{-4}	5.8*10^{-5}	0.070
		$c_{R(Co)}$	96	1.2*10^{-3}	0.007	1.9*10^{-4}	2.6*10^{-5}	0.065
β	*Grinding*	$c_{I(Co)}$	161	0.027	0.078	2.9*10^{-3}	6.6*10^{-5}	0.570
		$c_{R(Co)}$	161	0.003	0.010	4.7*10^{-4}	3.7*10^{-5}	0.110

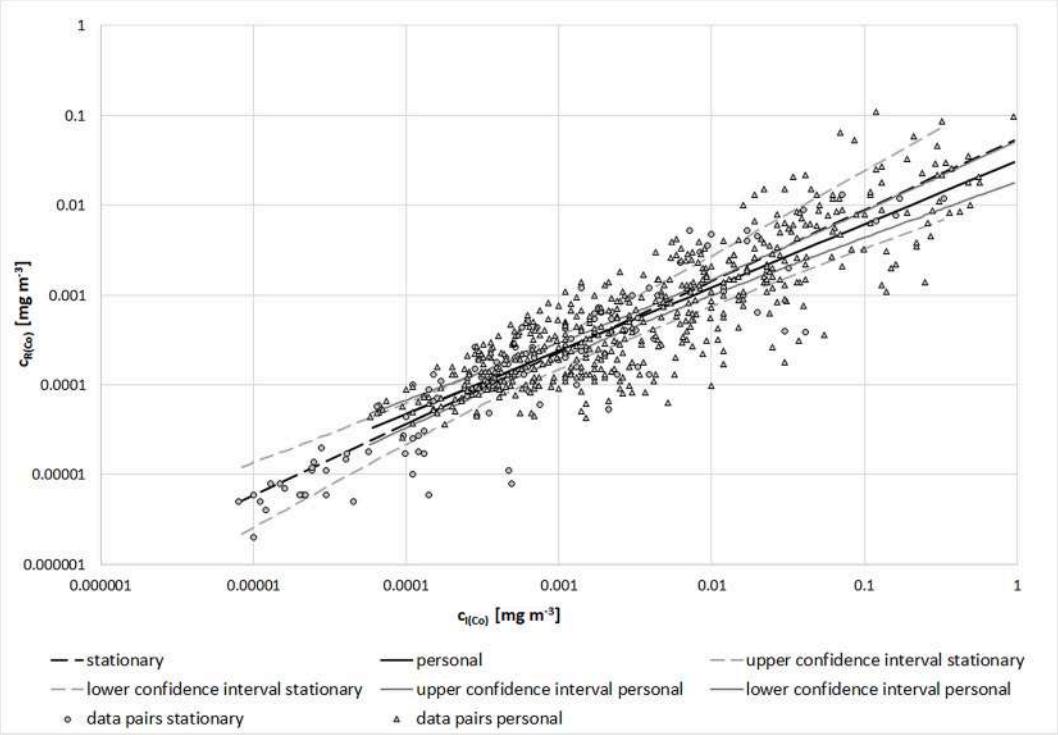

Fig. 3. Scatterplot of measured pairs from $c_{R(Co)}$ versus $c_{I(Co)}$ with direct comparison of personal and stationary sampling (n = 639) with 95th confidence intervals and logarithmic scale.

The regression coefficients for personal measurements are $k = 0.794 \pm 0.062$; $C_0 = -2.984 \pm 0.498$.

3.3. Working activity

In this study, three working activity groups are formed (Tables 2 and 3):

- *Group 1: High temperature processing* (such as welding, smelting, soldering)
- *Group 2: Filling/transport/storage*
- *Group 3: Machining/abrasive techniques* (such as grinding, milling, polishing)

Comparing the working activity groups, *'high temperature processing'* shows the lowest arithmetic means of the concentrations ($AM\ c_{I(Co)} = 0.007$ mg m^{-3}; $AM\ c_{R(Co)} = 0.002$ mg m^{-3}) . This is reflecting the low percentage of cobalt in steel, which is the material that is very often handled during high temperature processing tasks like welding. In contrast to that, highest concentrations are found in the working activity group '*filling/transport/storage*'. Here tasks can be found that are used in the hard metal industry, where materials with a higher content of cobalt are used. The arithmetic means, which were calculated for each group (Table 3), contain only concentrations equal or higher than the limit of quantification. About 1/3 of the measured cobalt concentrations in both dust fractions were neglected due to this criterion. But due to insufficient data, some of these groups could not be formed in this study (*'surface treatment'* n = 27; *'forming'* n = 24) (Wippich et al., 2020).

All conversion functions are power functions and no linear relation between $c_{R(Co)}$ and $c_{I(Co)}$ has been observed since the value 1 is not part of the confidence interval of k (Table 2). Comparing the three working activity groups, it can be seen that the standard errors of the regression function s_{Fit} increase with decreasing group size. Variance analysis shows that the three groups do not differ in the ratio $c_{R(Co)}/c_{I(Co)}$, except for group 1, which differs highly from all other groups ($p < 0.001$). Also, the regression coefficients of group 1 differ from the other groups (Table 2). With exception of group 1 the groups show similar intercept values (C_0) compared to group 0, while the slope (k) varies over the groups ($0.722 \leq k \leq 0.793$). The largest group, *'machining/abrasive techniques'* ($n = 234$), shows slightly decreased quality measures in comparison to the entire dataset ($\Delta R = 0.043$; $\Delta adj.\ R^2 = 0.075$).

In workplaces, there is often no spatial separation between welding and grinding processes. These processes can influence the particle distribution at a workplace. In the database MEGA the additional information about the grinding time fraction (GTF) for each measurement and whether the GTF is higher or lower than 5 % can be added but is not mandatory. It was not possible in this study to evaluate the impact of the GTF on the development of conversion functions, as there were just five datasets with information about GTF in the inhalable dust fraction.

The groups *'high temperature processing'* and *'machining/abrasive techniques'* are dominated by some single working activities. In group 2 about 66 % ($n = 96$) of the data were measured during welding processes and high temperature cutting accounts for further 12 % of the data. In group 3 the largest working activities are grinding 69 % ($n = 161$), polishing 9 % ($n = 21$) and milling 8 % ($n = 18$).

3.4. Heuristic groups

Apart from the systematic approach, two heuristic groups are formed (groups α and β) by trial and error (Tables 2 and 3). Group α (*welding*) is a subgroup of working activity group 1 *'high temperature processing'* and β (*grinding*) of group 3 *'machining/abrasive techniques'* respectively. As mentioned in section 'Statistical and mathematical methods', similar working activities were pooled. To form the *welding* group, the following activities were considered and pooled: laser welding, tungsten inert gas welding, metal active gas welding, metal inert gas welding, plasma welding and manual arc welding with coated rod electrode. For the *grinding* group the activities wet grinding, dry grinding and abrasive

grinding were pooled. Because of the small number of data pairs, it was not possible to form more heuristic groups. The regression model of the heuristic group *'grinding'* shows a better description of data than those with its relating working activity group (Table 2). The *adj.* R^2 is 0.866 in comparison to 0.845. Group *α 'welding'* shows similar results and quality measures compared to its working group *'high temperature processing'*.

In none of the heuristic groups a linear relation between $c_{R(Co)}$ and $c_{I(Co)}$ can be observed, as the value 1 does not lie within $k \pm$ standard error from these groups. Resulting conversion functions are power functions with k-values of 0.761 and 0.747 and C_0-values of −2.976 and −3.195 respectively (Table 2). The two regression functions are quite different, as it can be derived from the regression coefficients and Fig. 3. If one measures for example $c_{I(Co)} = 0.005\ mg\ m^{-3}$, the results for $c_{R(Co)}$ are $\approx 9.7*10^{-4}\ mg\ m^{-3}$ (*'welding'*) and $\approx 7.3*10^{-4}\ mg\ m^{-3}$ (*'grinding'*), respectively.

When estimating cobalt in respirable dust, e. g. for historical data of cobalt in inhalable dust during welding, one has to use the conversion function $c_{R(Co)} = {c_{I(Co)}}^{0.747} * e^{-2.976} = {c_{I(Co)}}^{0.747} * 0.051$. That means, if $c_{I(Co)}$ was measured with 1 mg m^{-3}, $c_{R(Co)}$ would be 0.051 mg m^{-3} or rather a proportion of 5.1%. Because k and therefore also the exponent of $c_{I(Co)}$ is < 1, the conversion function is not linear. As a consequence, the proportion of $c_{R(Co)}$ below 1 mg m^{-3} $c_{I(Co)}$ is higher than 5.1 % and the proportion of $c_{R(Co)}$ decreases for $c_{I(Co)}$ higher 1 mg m^{-3} continuously.

4. Discussion

4.1. Identification of groups

Inhalable dust is the most important variable. This variable already explains about 79 % of the variation in the dataset (Table 2, group 0). The resulting function is a power function with the coefficients $k = 0.733$ and $C_0 = -3.331$. Considering the additional variable *'working activity'* results in three systematic, more specific groups.

Considering only personal measurements, the group *'high temperature processing'* is described by $k = 0.734$ and $C_0 = -3.083$ and is different from all other groups. A subset of this group is *'welding'*. As already mentioned in the section *'Results'*, there is often no spatial separation of grinding and welding within workplaces. Thus, it cannot be ruled out that the grinding time fraction is influencing the regression functions, resulting in a lower R and *adj.* R^2. Notwithstanding this, in all groups the quality measures are quite high, supporting our model fit. In future measurements in welding workplaces additional information on the grinding time fraction (GTF), which also should be stored in the database MEGA, would be helpful to verify the conversion function of the groups 1 and α. In another study about nickel and its compounds, it was possible to evaluate the nickel concentrations in inhalable and respirable dust with respect to the amount of GTF at welding workplaces. The resulting functions for nickel in both fractions strongly differ between measurements with GTF < 5 % and GTF ≥ 5 % (Wippich et al., 2021). In case of nickel with GTF < 5 %, a better description of data was achieved with untransformed concentrations and the resulting regression function was a linear equation (*adj.* $R^2 = 0.724$ in comparison to *adj.* $R^2 = 0.377$). In contrast to that, nickel concentrations in inhalable and respirable dust showed a non-linear correlation regarding GTF > 5 %, resulting in a power function (*adj.* $R^2 = 0.455$ (non-transformed) vs. 0.830 (transformed)). It cannot be excluded, that for cobalt the same could be observed, since the heuristic group *'welding'* shows lower quality measures than the working activity group *'high temperature processing'* (compare Table 2, groups α and 1), as it was in case of nickel (Wippich et al., 2021). Due to insufficient data, an evaluation of GTF was not possible for cobalt.

The larger groups, *'high temperature processing'* and *'machining/abrasive techniques'*, are mainly characterized by some large, specific subgroups. Group 1 is mainly characterized by the heuristic group *'welding'* (group α), which accounts for 66 % of the data. In group 3, 69 % of the data contribute to the heuristic subgroup *'grinding'*. Therefore, these working activity groups might not be representative for the entire working activity group that they are supposed to describe. *'Filling/transport/storage'* is formed from heterogeneous subgroups but contains only a few measurement pairs. To validate the conversion function of this group further measurements should be done. Performing more measurements in general would also lead to the formation of more working activity groups, such as *'surface treatment'* or *'forming'*, similar to the six working groups which were formed for inhalable and respirable dust (Wippich et al., 2020) or nickel (Wippich et al., 2021). In case of cobalt for these potential working groups, a few datasets were available and therefore these groups were excluded from this study.

4.2. Application of equations (1) and (2)

There are two limiting cases of equation (1):

- The worst-case assumption $c_{R(Co)} = c_{I(Co)}$, equivalent to $C_0 = 0$ and $k = 1$.
- The linear assumption for $c_{R(Co)} > c_{I(Co)}$, equivalent to $C_0 < 0$ and $k = 1$.

The worst-case assumption has not been observed in our dataset. Additionally, all C_0 values for equation (1) throughout this study are negative ($-3.418 \leq C_0 \leq -2.976$), which is necessary to avoid unphysical values ($c_{R(Co)} > c_{I(Co)}$) in the data range if $k \neq 1$.

All k values of this study are smaller than 1 ($0.704 \leq k \leq 0.794$), although the regression analysis does not prohibit $k > 1$. For equation (1), this indicates that $k < 1$ is a systematic effect, which means that the resulting function is not linear. The ratio $c_{R(Co)}/c_{I(Co)}$ is declining with increasing values of $c_{I(Co)}$. A linear relation with $k = 1$ would imply that a single process is responsible for a constant ratio of emission for both dust fractions. Another possible explanation for the finding of $k < 1$ in this study are agglomeration effects, which become more important with increasing concentrations (Rumpf, 1976; Koch et al., 1999) and occur especially during welding processes (Zhang et al., 2013; Zimmer, 2002).

4.3. Exclusion of 'unphysical' cobalt concentrations

In this study pairs of cobalt concentrations are excluded if $c_{R(Co)}$ is higher than $c_{I(Co)}$. In fact, there are some cases where higher cobalt concentrations in respirable dust were observed. This is possible because independent sampling systems for both fractions are used. These data can result from inhomogeneous materials, particle movement, thermal effects, the distance of sampling systems to the source of emission, or air ventilation in workplaces. Another reason could be a carry-over due to wall deposits in FSP cyclones. With this restriction, 37 parallel cobalt measurements were excluded. If one includes these measurements for linear regression, the quality measures decrease slightly ($\Delta R = 0.021$; *Δadj.* $R^2 = 0.037$) in comparison to group 0. The regression coefficients vary by 0.029 *(ΔC_0) and* −0.012 *(Δk)*. Evaluating these differences, the exclusion of these data pairs does not have a large impact on the regression functions and the conversion of cobalt at this stage. However, to include these samples would introduce a bias of the analysis towards a physically uncommon situation. Therefore, these values remain excluded.

4.4. Type of sampling

In the entire dataset (group 0) the differences in median and distribution of $c_{R(Co)}/c_{I(Co)}$ are mainly caused by the working activity (Two-way ANOVA: $p < 0.001$) but also depend on the type of sampling (Two-way ANOVA: $p = 0.036$). In group 1 *'high temperature processing'*, the differences in the ratio are also significant. The p-value for the analysis of variance is borderline significant ($p = 0.049$) so the effect of the parameter *'type of sampling'* can be considered low. Personal measurements are collected close to the source of emission, while stationary sampling systems can only be directed to the source. This could be a

reason for the higher concentrations determined by personal measurements. This assumption is also supported by a study of Purdham et al. (1993) for example. They showed that the ratio of personal to static measurements ranges between 3.2 and 1.5 (in dependence from ventilation conditions). This leads also to the assumption that stationary measurements are less suitable for the evaluation of the particle exposure to workers at different workplaces. This is also supported by the fact that measurement data from stationary samplers show a larger variance than personal measurements.

Another reason for the differences between the two sampling types is that personal sampling systems might collect larger, heavier particles directly after the source of emission, while the amount of those particles decreases with increasing distance and thus are collected to a lesser extent using stationary sampling systems. This assumption is supported by the study of Cherrie (1999). They conducted near field and far field measurements, which can be compared to personal and stationary sampling. In their study, it was discussed that if the source was close to the worker (personal measurement), and the static measurement in some distance from the worker, then the personal exposure level would be higher than the static concentration in most cases for different workplaces, such as wood work or hard metal industry (Cherrie, 1999; Klasson et al., 2016). In many cases it is also reported that the magnitude of the ratio between the static and personal measurements are mainly influenced by the room size and the amount of room ventilation (Purdham et al., 1993; Cherrie, 1999). In our study, this cannot be proved using the technical information in the database MEGA, as it contains no information about the distance from the source of emission.

4.5. Application and limitations of results

All measurements in this study are representative for a whole 8-h-shift with regard to the German limit values. According to the German Technical Guidance 402, one measurement during a 2-h measurement is sufficient to report a representative exposure during the shift of a worker. The person which performs the measurement, must evaluate whether the measurement is representative for an 8-h work shift and the tasks of the worker or not. This information is also added to the measurement and stored in the database. At this point, there is no possibility to reproduce the situation in the workplace and one has to rely on the evaluation of the measurement technician (Kendzia et al., 2017). In our study only data, which is representative for the exposure during the shift of a worker, was used. The restriction $\geq$ 2-h measurement duration is also a limitation, because at workplaces with high particle load, the sampler could be loaded in a shorter time period. Defined groups contain heterogeneous working activities and subgroups which are described in the supplemental materials. One has to be careful to use the model parameters in toxicological or epidemiological analyses without a careful check of applicability. The data used in this study were derived from workplace cobalt measurements in the German industry between 2011 and 2020 and the working conditions described in the previous sections.

Especially in the *'welding'* group, it must be realized that the cobalt content of the welding material might influence the cobalt concentration in both dust fractions and therefore might influence the conversion function for *'welding'* (Kendzia et al., 2017). In our study different forms of welding are pooled, this can conceal the effect of different cobalt contents.

If the conversion functions from Table 2 are used to calculate $ln(c_{R(Co)})$ at a given $ln(c_{I(Co)})$, the result has a confidence interval of $\pm 1.96 \cdot s_{Fit} ln(c_{R(Co)})$. The smaller value for s_{Fit} in Table 2 is only valid around the AM of $ln(c_{I(Co)})$ (Table 3). This variance must be added to the measurement uncertainty, which should be calculated for the sampling process and the analytical procedure. The uncertainties of the analytical procedure regarding cobalt concentrations are equal in both dust fractions. The uncertainties caused by the sampling process are slightly different for each dust fraction and depend on the used sampling system (European Committee for Standardization, 2018). The measurement uncertainty (u) for the overall process (sampling and analytics) of cobalt in respirable dust is about 8.45 % (expanded measurement uncertainty (U): 16.9 %) for concentrations up to $1.0*10^{-4}$ mg m^{-3}, u = 10.7 % (U = 21.4 %) for concentrations up to $2.5*10^{-4}$ mg m^{-3}, and 7.58 % (expanded measurement uncertainty (U): 15.2 %) for concentrations up to 0.01 mg m^{-3} respectively (Pitzke et al., 2020). The calculations and estimations of measurement uncertainties comply with the demands and requirements in the international standards EN 482 (European Committee for Standardization, 2021), ISO 21832 (European Committee for Standardization, 2018) and ISO/IEC Guide 98-3 (GUM) (ISO/IEC Guide 98-3:2008, 2008). It must be emphasized, that the regression functions are only valid for measurement results in mg m^{-3}. Because the derived functions are power functions, measurement values in µg m^{-3} have to be converted into mg m^{-3} before using the regression functions.

The uncertainty of the measured concentrations is in this study limited to several percent of the measured value (European Committee for Standardization, 2010; Deutsches Institut für Normung, 2021). In the European Standard 482 it is stated that for long-term measurements (>2 h) the relative expanded measurement uncertainty must be lower or equal to 30 %, and for measurements with sampling duration $\leq$ 2 h it must be lower or equal 50 %. 15.2 %–21.4 % relative measurement uncertainty as we calculated in our study, is well below these limits. The concentrations themselves, on the other hand, cover up several orders of magnitude due to other influences such as the type of work and inter or intra worker effects. The difference of these two scales suggest that the estimates of the slope parameter are not severely biased (Draper and Smith, 1998). If such a bias existed, it would decrease the size of the slope parameter. However, a rigorous treatment of the effect of uncertainty in the concentrations is beyond the scope of this study, as it includes the transfer from the natural to a logarithmic scale in combination with non-constant uncertainties.

The conversion functions were calculated considering the sampling systems listed in Table 1. If these functions are applied on data associated with other sampling systems, other measurement uncertainties must be considered and u (and U) can differ. Nevertheless, the applicability of the functions of such data can be assumed, if the sampling systems were validated by the same international standards (ISO 13205-2; DIN EN 481) and collect the same dust fraction (inhalable or respirable dust, no total dust). For the estimation of cobalt in respirable dust, it is suggested to use the conversion functions of the heuristic groups if the concerning working activity matches these groups. If the working activity does not fall into one of these groups, groups 1 to 3 could be used. If they are also not applicable, the use of group 0 and therefore also group A is not recommended.

In case of the working activity *'welding'*, in further measurements and following studies, close attention should be paid to the grinding time fraction at work places, since it has been proved that it influences the dust concentration and therefore the applicability of the mathematical model and the conversion function (Wippich et al., 2021).

4.6. Comparison with literature

Klasson et al. (2016) determined the occupational exposure to cobalt in the hard metal industry. They measured cobalt in inhalable dust using also the GSP sampler, PM2.5 and PM10 by stationary and personal measurements. PM10 is considered to full-fill the same requirements as the thoracal dust fraction (Mattenklott and Höfert, 2009). Since PM4 is considered to equal the respirable dust fraction, PM2.5 is equivalent to the respirable dust fraction for risk groups (Mattenklott and Höfert, 2009). To evaluate the influence of different parameters, such as *'type of sampling'*, a linear regression was performed. Similar to our approach, Klasson et al. also transformed their measured concentrations using the natural logarithm (ln) for linear regression (Klasson et al., 2016). Since they calculated regression functions for stationary sampling only, these functions and quality measures cannot be compared to our conversion

functions, as we focussed on personal sampling. But in the Swedish study, personal air concentrations for cobalt were measured in different departments, such as *'warehouse'* and *'periphery grinding'*. For this work area, they calculated an AM of $1.7*10^{-4}$ mg m^{-3} for the inhalable dust fraction of personal measurements. In our study, we determined an AM of 0.027 mg m^{-3}. The group *'warehouse'* of their study is formed from only two measurements and they calculated an AM of $1.8*10^{-4}$ mg m^{-3}, in our study (group 2 *'filling/transport/storage'*) AM is much higher with 0.097 mg m^{-3} ($n = 49$).

To characterize exposure among cemented tungsten carbide workers, airborne cobalt was determined by Stefaniak et al. (2009). They measured cobalt in inhalable and respirable dust using an eight-stage impactor. For *'grinding'* the GM (geometric mean) of $c_{R(Co)}$ was measured by $2.6*10^{-3}$ mg m^{-3} ($n = 7$), and $15.6*10^{-3}$ mg m^{-3} (GM $c_{I(Co)}$) was measured respectively. Kraus et al. (2001) measured in work areas of wet and dry grinding a concentration of $0.20*10^{-3}$ mg m^{-3} and $0.48*10^{-3}$ mg m^{-3} (both $n = 1$) cobalt in respirable dust with personal sampling, respectively. In our study GM $c_{R(Co)}$ is $6.4*10^{-4}$ mg m^{-3} and GM $c_{I(Co)}$ is $4.2*10^{-3}$ mg m^{-3} for the heuristic group *'grinding'*. Stefaniak et al. calculated for their work area *'powder mixing'* ($n = 14$) a GM of $c_{R(Co)}$ by $18.2*10^{-3}$ mg m^{-3} and GM $c_{I(Co)} = 15.6*10^{-3}$ mg m^{-3} respectively. In our working activity group *'filling/transport/storage'* GM $c_{R(Co)}$ can be calculated by $1.1*10^{-3}$ mg m^{-3} and GM $c_{I(Co)}$ by $1.4*10^{-2}$ mg m^{-3}. Reasons for the differences in the arithmetic means (Klasson et al. (2016)) and geometric means (Stefaniak et al., 2009) are that we excluded about 1/3 of our measured values with low concentrations due to the restriction $c_{I(Co)}$ and $c_{R(Co)} \geq$ limit of quantification and the use of an impactor might also lead to different dust concentrations than a personal sampler.

In a study of Kim et al. (2015) a total of 16 concentrations of cobalt in inhalable and 16 concentrations of cobalt in respirable dust were measured, which is a considerably low number of data. The mean concentrations in their group *'mix & weighing'* are $2.46*10^{-3}$ mg m^{-3} ($c_{R(Co)}$) and $16.12*10^{-3}$ mg m^{-3} ($c_{I(Co)}$) respectively ($n = 5$). This group can be compared to group 2 *'filling/transport/storage'* in our study. The AM of $c_{R(Co)}$ in our data is about 0.008 mg m^{-3} and the AM of $c_{I(Co)}$ 0.097 mg m^{-3} respectively. Our calculated AMs are higher in both cases. Considering the range of concentrations in both groups, the minimum concentrations in both dust fractions are lower and the maximum concentrations in both dust fractions are higher in our study. Kim et al. evaluated the influence of several factors on the airborne cobalt concentration. For this purpose, they used multilinear regression analysis for untransformed concentration values. They found no significant influence of the work type (semiautomatic/manual; $p = 0.212$), the process (equal to working activity in our study; $0.383 \leq p \leq 0.523$), the cobalt content ($p = 0.319$), the ambient temperature ($p = 0.137$) and the capture velocity ($p = 0.136$) as predictor variables. The only variable with a significant influence was relative humidity in their study ($p = 0.033$). For their multilinear regression analysis just 16 measurements were evaluated, which is a quite low number of data pairs for this kind of statistical analysis. In our study, we were not able to perform a multilinear regression analysis, since a requirement for this analysis is that the variables are independent from each other. This is the reason why we created groups and performed independent linear regression analyses. Despite that, in our study the other parameters such as work type, ambient temperature or capture velocity could not be evaluated because insufficient data.

5. Summary and conclusion

To summarize, it was possible to develop conversion functions out of 639 data pairs from cobalt in respirable and inhalable dust. It was shown that there is no linear correlation between cobalt in inhalable and in respirable dust in general and therefore no single, fixed correction factor can be used. The developed conversion functions can help occupational hygienists and risk assessors to estimate missing cobalt concentrations for retrospective analyses which are often required for the assessment of occupational diseases or for epidemiological studies. The conversion functions can also help in future discussions on legal values. The application of these conversion functions might be applicable for German exposure data in particular. In principle, we assume that the situation in other countries could be comparable, if the same sampling systems (Table 1) and sampling strategy were used as in our study. Before transferring data to other countries, studies must be performed which compare the results of this study with measurements from these countries measured with their sampling systems.

Since the stationary measurements should only be performed in exceptional cases and are not considered to be reliable to evaluate the exposure of workers, for further analysis only personal measurements were evaluated. These pairs were analyzed including different working activities and forming subgroups out of group A *'Entire dataset only personal measurements'* ($n = 515$).

The study suggests that the data should generally be evaluated using linear regression of the log-transformed data shown in equation (1) or (2) with $k \leq 1$ and $C_0 < 0$. Out of the entire dataset, specific working activity groups were formed (groups 1–3) to enable a better description of data. Group 1 *'high temperature processing'* consists mainly of different welding processes, which can be formed to the heuristic *'welding'* group (α). Slightly lower quality measures can be determined by linear regression in comparison to group 1, although the dataset is smaller and more specific. The reason for that is most probably the grinding time fraction (GTF), which influences the measured concentrations and therefore also the conversion functions, as was shown in our previous published study (Wippich et al., 2021). The GTF could not be evaluated in this study because of the insufficient amount of data.

From working group 3 '*machining/abrasive techniques*' also a more specific heuristic subgroup could be created: group β *'grinding'*. This group shows a better description of data compared to its original working activity group; it accounts for 75 % of the variance in the data (see Table 2).

For the estimation of cobalt in respirable dust, it is possible to use the conversion functions of the heuristic groups if the concerning working activity matches these groups. When an assessment does not fit into these groups, the conversion functions and confidence intervals of working activity groups 1–3 should be used. In addition to the confidence intervals of the functions, the expanded measurement uncertainty must be considered, which consist of the errors of sampling process and the analytical procedure. Errors in the sampling process have to be calculated depending on the associated dust fraction.

In the next years, more measurements of cobalt and other elements in respirable and inhalable dust will be performed and these new measurements will be used for further verification of the conversion functions found in this study. Special attention should be paid in future measurements on workplaces with no spatial separation between welding and grinding workplaces. In these cases, the GTF should be given as additional information to each dataset to evaluate its influence on the conversion function. Additionally, it would be interesting to compare the results of this study with measurements from other countries and measured with different sampling systems to guarantee the transferability of the conversion functions presented in this study.

Declaration of competing interest

The authors declare no conflict of interest relating to the material presented in this article its contents, including any opinions and/or conclusions expressed, are solely those of the authors.

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to take responsibility for the work.

Appendix A. Supplementary data

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Exposure assessment of polycyclic aromatic hydrocarbons in refined coal tar sealant applications

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ABSTRACT

Background: Refined coal tar sealant (RCTS) emulsions are used to seal the surface of asphalt pavement. Nine of the 22 polycyclic aromatic hydrocarbons (PAHs) evaluated in this study are classified as known, probable, or possible human carcinogens. Exposure assessment research for RCTS workers has not been published previously.
Objectives: The overall objective of this study was to develop a representative occupational exposure assessment of PAH exposure for RCTS workers based on worksite surveys. The specific aims were to: 1) quantify full-shift airborne occupational exposures to PAHs among RCTS workers; 2) quantify workers' dermal exposures to PAHs; 3) quantify biomarkers of PAH exposure in workers' urine; 4) identify specific job titles associated with RCTS exposure; and 5) apply these results to a biological exposure index to assess risk of potential genotoxicity from occupational exposures.
Methods: A total of twenty-one RCTS workers were recruited from three companies. Personal and area air samples were collected using a modification of NIOSH Method 5515. Dermal exposure was assessed by hand and neck wipes before and after shifts. Twenty-two PAHs were quantified via gas chromatography-mass spectrometry selected ion monitoring. Internal dose was estimated by quantifying select PAH metabolites in pre- and post-shift urine samples using on-line solid phase extraction-high performance liquid chromatography-tandem mass spectrometry.
Results: PAH levels in the worker breathing zones were highest for naphthalene, acenaphthene, and phenanthrene, with geometric means of 52.1, 11.4, and 9.8 μg/m^3, respectively. Hand wipe levels of phenanthrene, fluoranthene and pyrene were the highest among the 22 PAHs with geometric means of 7.9, 7.7, and 5.5 μg/cm^2, respectively. Urinary PAH biomarkers for naphthalene, fluorene, phenanthrene, and pyrene were detected in all workers and were higher for post-shift samples than those collected pre-shift. Urinary concentrations of the metabolite 1-hydroxypyrene were greater than the American Conference of Governmental Industrial Hygienists (ACGIH) Biological Exposure Index (BEI) for this metabolite in 89 percent of post-shift samples collected on the final day of the work week or field survey. Statistically significances were found between concentrations of fluorene, naphthalene, and phenanthrene in the breathing zone of workers and their corresponding urinary PAH biomarkers. Workers were placed in two work place exposure groups: applicators and non-applicators. Applicators had higher total PAH concentrations in personal breathing zone (PBZ) air samples than non-applicators and were more likely to have post-shift hand wipe concentrations significantly higher than pre-shift concentrations. Concentrations of post-shift urinary biomarkers were higher, albeit not significantly, for applicators than non-applicators.
Conclusions: The exposure results from RCTS worker samples cannot be explained by proximal factors such as nearby restaurants or construction. Air and skin concentration levels were substantially higher for RCTS workers than previously published levels among asphalt workers for all PAHs. PAH profiles on skin wipes were more consistent with RCTS sealant product than air samples. Last day post-shift urinary concentrations of 1-

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hydroxypyrene greatly exceeded the ACGIH BEI benchmark of 2.5 μg/L in 25 of 26 samples, which suggests occupational exposure and risk of genotoxicity. When pyrene and benzo[a]pyrene were both detected, concentration ratios from personal exposure samples were used to calculate the adjusted BEI. Concentrations of 1-hydroxypyrene exceeded the adjusted BEIs for air, hand wipes, and neck wipes in most cases. These results indicate the need to increase safety controls and exposure mitigation for RCTS workers.

1. Introduction

Coal tar pitch is a complex mixture of chemicals that includes a variety of polycyclic aromatic hydrocarbons (PAHs) and N-heterocyclic PAHs. PAHs are a class of chemicals with multiple benzene rings, while N-heterocyclic PAHs have a combination of benzene rings and N-heterocycles. Both types of PAHs are formed from incomplete combustion of organic matter, with the N-heterocycles resulting from compounds containing nitrogen. Several PAHs are classified as carcinogens, probable carcinogens, or possible carcinogens by the International Agency for Research on Cancer (IARC) (IARC, 2010, 2012). Coal tar pitch is classified as a known (Group 1) carcinogen in humans based on a combination of animal, genotoxicity, and occupational exposure studies of roofers and pavers (IARC, 2012). Research indicates that PAH carcinogenicity increases with the number of benzene rings, and therefore molecular weight (Bostrom, 2002). Tables and figures describing PAHs within this manuscript are organized by molecular weight to provide context for this carcinogenic relationship. Of the 22 PAHs addressed in this study, one is classified as Group 1 (benzo[*a*]pyrene), one is 2A, and seven are 2B (Table 1). The United States National Toxicology Program (NTP) has listed benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, and indeno[*1,2,3-cd*]pyrene as reasonably anticipated to be human carcinogens in their Fourteenth Report on Carcinogens and has expressed interest in further research on the topic (NTP, 2016).

Coal tar pitch is the residue that remains after the distillation of crude coal tar, during which specific fractions are collected and multiple products may be produced at different temperatures and processing steps (IARC, 1985). Coal tar pitch is then separated ("refined") into fractions of 12 different viscosities. RT-12 is the most viscous and is used in manufacturing pavement sealants, as specified by American Society for Testing and Materials (ASTM) D490 (ASTM, 2016).

Refined coal tar sealant (RCTS) emulsions are applied as a protective coating for asphalt pavement. RCTS emulsions are a mixture of clay, water, sand, and RT-12. The final RCTS product applied by workers contains up to 35 percent RT-12 (McClintock et al., 2005). Some products may have other components added based on use specifications (ASTM, 2017). RCTS are predominately used east of the U.S. continental divide because they are by-products of coke production and coke plants are concentrated in the eastern part of the USA.

The United States Geological Survey (USGS) performed environmental air sampling immediately after completion of pavement seal coating with RCTS and reported elevated levels of various PAHs including some of the same chemicals listed in the occupational classification "coal tar pitch volatiles" (Van Metre et al., 2012). These findings suggest the need to evaluate occupational exposures for workers applying coal tar sealants because there is currently no published occupational exposure data for PAHs in coal tar sealant. Review of the literature found only one source of occupational airborne PAH levels from a 1984 study from New Zealand. However, the study included only two data points during coal tar spraying of a chip seal road, a process rarely used then, and no longer used in the industry (Darby et al., 1986).

The general population is exposed to PAHs through consumption of food containing PAHs, breathing ambient air, smoking cigarettes, and breathing smoke from other sources, such as vehicle exhaust (NIH, 2019). Occupational exposures generally occur as a mixture of ingestion, skin contact, and inhalation (Mumtaz and George, 1995), but more recent studies of asphalt workers found that skin and inhalation exposures are equally important contributors to occupational exposures (Cavallari et al., 2012; McClean et al., 2004; Vaananen et al., 2005).

At least three groups have developed occupational exposure limits or guidelines for seven PAHs and coal tar pitch volatiles. Naphthalene(a PAH in coal tar sealants), with two benzene rings, has the lowest molecular weight and is the most volatile PAH. Airborne naphthalene has a vacated permissible exposure limit (PEL) of 50 mg/m^3 established by the Occupational Safety and Health Administration (OSHA); a full-shift recommended exposure limit (REL) of 50 mg/m^3 as a 10-h time-weighted average (TWA) established by the National Institute of Occupational Safety and Health (NIOSH); a NIOSH short-term exposure limit (STEL) of 75 mg/m^3 (NIOSH, 2007); and an American Conference of

Table 1
PAHs quantified in air, hand wipe, and neck wipe samples, and PAH biomarkers in urine samples. Abbreviations are shown in parentheses.

Analyte	IARC Classification[a]	CAS Number	Molecular Weight (g/mole)	Biomarker
PAH				
Naphthalene (NAP)[b]	2B	91-20-3	128.2	1-Hydroxynaphthalene (1-OHNAP), 2-Hydroxynaphthalene (2-OHNAP),and Sum-OHNAP
Fluorene (FLU)[b]	3	86-73-7	166.2	2-Hydroxyfluorene (2-OHFLU), 3-Hydroxyfluorene (3-OHFLU),and Sum-OHFLU
Phenanthrene (PHE)[b]	3	85-01-8	178.2	1-Hydroxyphenanthrene (1-OHPHE), 2,3-Hydroxyphenanthrene (2,3-OHPHE), and Sum-OHPHE
Pyrene (PYR)[b]	3	129-00-0	202.3	1-Hydroxypyrene (1-OHP)
Benz[*a*]anthracene (BaA)	2B	56-55-3	228.3	
Chrysene (CHR)	2B	218-01-9	228.3	
Benzo[*a*]pyrene (BaP)	1	50-32-8	252.3	
Benzo[*k*]fluoranthene (BkF)	2B	207-08-9	252.3	
Indeno[*1,2,3-cd*]pyrene (IP)	2B	193-39-5	276.3	
Dibenzo[*a,h*]anthracene (DBahA)	2A	53-70-3	278.4	
N-heterocyclic				
Quinoline (QN)	2B	91-22-5	129.2	
Carbazole (CAR)	2B	86-74-8	167.2	

[a] Group 1: Carcinogenic to humans; Group 2A: Probably carcinogenic to humans; Group 2B: Possibly carcinogenic to humans; Group 3: Not classifiable as to its carcinogenicity in humans (IARC, 2012). Refer to Supplemental Table S1 for abbreviations of PAHs that were not used for statistical modeling and not classifiable as carcinogenic, or are currently considered not carcinogenic to humans, by the IARC.

[b] Analytes have corresponding urinary metabolites or biomarkers used for statistical modeling.

Governmental Industrial Hygienists (ACGIH) Threshold Limit Value (TLV) of 50 mg/m^3. Two naphthalene derivatives, 1-methylnaphthalene and 2-methylnaphthalene, each have a TLV of 3 mg/m^3 as an 8-h TWA established by the ACGIH (ACGIH, 2019). Benzo[*a*]pyrene, benzo[*b*]fluoranthene, benz[*a*]anthracene, and chrysene do not have acceptable airborne exposure levels because they have been observed to be carcinogenic in animal studies (ACGIH, 2019). Rather, the ACGIH recommends that all exposures to these compounds be reduced to levels as low as possible (ACGIH, 2019). The ACGIH has listed chrysene as a 2A carcinogen (confirmed animal carcinogen with unknown relevance in humans) and benzo[*a*]pyrene, benzo[*b*]fluoranthene, and benz[*a*]anthracene are listed as 2B carcinogens (suspected human carcinogen).

Pyrene is present in almost every PAH mixture (Hopf et al., 2009). The ACGIH developed a biological exposure index (BEI) based on the relationship between 1-hydroxypyrene and a range of genotoxicity markers, and currently recommends assessing worker exposure to PAHs by testing urine specimens for 1-hydroxypyrene, a metabolite of pyrene (ACGIH, 2019). This metabolite is considered an index chemical that acts as a surrogate marker for the absorption of various mixtures of PAHs in occupational settings. Generally, the ACGIH considers concentrations of 1-hydroxypyrene at or above 2.5 μg/L evidence of occupational exposure and risk of genotoxicity (ACGIH, 2019).

However, the ACGIH recommends calculating an adjusted Biological Exposure Index (BEI), when specific exposure information is available. The BEI is adjusted by calculating the ratio of pyrene to benzo[*a*]pyrene collected from samples of suspected exposure routes, such as air and skin, and compared to the concentration of 1-hydroxypyrene at the end of the last shift of the work week (ACGIH, 2019). The adjusted BEI is considered the maximum acceptable urinary concentration of 1-hydroxypyrene for each worker, but due to the carcinogenicity of some PAHs, the ACGIH recommends exposures be kept as low as reasonably achievable (ACGIH, 2019).

The overall objective of this study was to develop a representative occupational exposure assessment of PAH exposure for RCTS workers based on work site surveys. This study is the first occupational exposure assessment for PAHs among refined coal tar sealant workers. The specific aims of this paper are: 1) to quantify full-shift airborne occupational exposures to PAHs for RCTS workers; 2) to assess dermal exposure to PAH among RCTS workers; 3) to quantify biomarkers of PAH exposure in workers' urine; 4) to identify specific job titles associated with RCTS work and evaluate how that affects exposure; 5) apply these results to a biological exposure index to assess risk of potential genotoxicity from occupational exposures.

2. Methods

2.1. Identifying companies and survey sites

This study focused on construction contracting companies with expertise in pavement sealing and evaluating job sites where pavement sealing with RCTS products was performed. These companies employ crews that move to different job sites as the work is completed, causing varied exposure duration within shifts ranging from five to 10 h. RCTS product samples, personal and area air samples, skin wipe samples, and spot urine samples were collected for all survey sites. A total of 22 PAHs and seven urinary metabolites were quantified in various matrices and the corresponding abbreviations were defined. Table 1 focuses on PAHs with IARC classifications which indicate potential human carcinogenicity, and PAHs whose urinary metabolites where applied to statistical modelling in this manuscript. Supplemental Table S1 includes the remaining PAHs included in this assessment, that are not suspected or known carcinogens, and were not used for statistical modelling.

2.2. Participants

The study was approved by the NIOSH Institutional Review Board. Once a company agreed to participate, individual employees were voluntarily recruited prior to the first shift of the visit. Both men and women who work with RCTS were considered eligible for this study. The study was described to workers and an informed consent was reviewed and signed by participants.

2.3. Survey sites

Three companies participated in this study, referred to here as companies A, B and C. All sites were visited between the months of July and October 2016–18 and included sampling of workers over 1 to 4 workdays. During the four-day site visit at company A, a series of small hotel and motel parking lots were sealed, along with a few small residential driveways on the first day of sampling. There were four visits to company B. Each visit lasted several days, and crews surfaced a large industrial parking lot, two commercial parking lots, an airport, and commercial and residential parking lots and driveways. Some crew members at company B participated in sampling during multiple visits because the visits occurred at different times. At company C, a very large industrial parking lot was surfaced over 2 days.

The number of workers in crews at each site ranged from two to nine. These workers performed tasks such as: site preparation (cleaning and crack repair); preparation of RCTS equipment and supplies (including mixing the product and transferring it into the trucks); manual application of sealant to difficult areas (e.g., use of brushes or other tools where overspray is not wanted); application, including use of a hand-held spray-wand application of sealant to the general area, application using a driven sealer spray-squeegee machine (a truck-mounted spray-squeegee device), and assisting with general application (e.g. handling supply hoses, moving sealant tank, and driving sealant truck); cleanup; and general oversight of work. Although work tasks varied, workers were delineated as applicator or non-applicator. Applicators were more likely to perform tasks such as mixing, applying, and handling coal tar sealant directly. In contrast, non-applicators were tasked with preparatory work (e.g., cleaning surfaces prior to application) that did not require as much direct handling of the sealant product. However, they still worked the same number of hours and were close to the sealant for most of the workday.

2.4. RCTS product sampling of sealant material

One RCTS product sample of sealant material was collected for each batch of RCTS mixed, totaling eight RCTS product samples. Samples for each batch used or mixed during the field visit were collected in pre-cleaned 120 mL amber glass jars (Thermo Scientific Cat. No. 241-0120 Waltham, MA) directly from the RCTS tank. Samples were analyzed by the NIOSH contract laboratory using a modification of Environmental Protection Agency (EPA) Method 8270D (EPA, 2014). Briefly, 1 g of RCTS product material was weighed into a 40 mL volatile organic compound analysis (VOA) vial (Thermo Scientific I-Chem™ Cat. No. 05-719-118 Waltham, MA, USA) and extracted with 10 mL of methylene chloride. The samples were placed in an ultrasonic bath with ice for 20 min. The samples were shielded from light and allowed to settle over 48 h. Next, dilutions were prepared, and an internal standard (consisting of: naphthalene-d8, acenaphthene-d10, phenanthrene-d10, chrysene-d12, and perylene-d12) was added to each vial, briefly mixed on a vortex, and PAHs were quantified using gas chromatography/mass spectroscopy (GC/MS).

2.5. Air sampling

Personal breathing zone (PBZ) and area air samples were collected by NIOSH staff at every location using a modification of NIOSH Manual of Analytical Methods (NMAM) 5515 (NIOSH, 1994). The important changes were the use of the OSHA Versatile Sampler (OVS-7 Cat. No. 226-57 SKC Inc. Eighty-Four PA) that combines the filter and sorbent in

a single glass tube to collect both vapor and aerosol (Achutan et al., 2009; Eide et al., 2010) and analysis using gas chromatography-mass spectrometry in selected ion monitoring (GC-MS SIM) rather than gas chromatography-flame ionization detector (EPA, 2014). Method validation studies examined the method performance for all analytes (Table 1 and Supplemental Table S1) as described by chapter ME of the 5th edition, NMAM (NIOSH, 2016). Validation samples were spiked with a combination of all PAHs in Tables 1 and S1 with a concentration range of 0.5–20 μg/sample for each analyte (n = 6 replicates).Samples were measured over a range of 0.5–20 μg/sample (n = 6 for each analyte).The results of this sample set gave acceptable recoveries for all the compounds. The Limits of Detection (LOD) for all compounds were 0.05–0.08 μg/sample while the Limit of Quantitation (LOQ) was 0.17–0.26 μg/sample.

To collect PBZ samples, each participant wore a personal sampling train that included an OSHA Versatile Sampler connected by flexible tubing to a sample pump. Workplace air was drawn through the sampler using a personal sampling pump operating at 1 L/min (AirChek XR-5000 SKC Inc. Eighty-Four PA). Sample collection continued over the entire work shift for each worker. Sampling pumps were pre- and post-calibrated in-line with Dry Cal Defender 521 and 520 calibrators (Bios International, Butler Park, NJ, USA). Samples were stored under refrigeration until shipped to the NIOSH contract laboratory for analysis.

Area air samples were collected each day to measure PAHs in ambient air. Sampling trains and pumps were placed approximately 5–20 feet from the edges of work areas (area air samples). The number and orientation of area samples were determined based on the size and shape of each work site. Samplers were placed across from one another on each side of roadways.

Twenty field blanks were collected to account for contaminant loadings on the sampling media that may have resulted from accumulative field and laboratory activities. Field blanks were prepared by removing the sampler caps for 30 s and then resealing the samplers. The blanks were randomly selected from the same lot of OVS-7 sorbent tubes used at each visit and submitted to the laboratory for analysis.

PBZ and area samples were analyzed by the NIOSH contract laboratory. Briefly, the OVS-7 samples were desorbed into 2 mL of methylene chloride. The filter and front section were desorbed together, and the back section was desorbed separately with the middle foam plug. Sample desorbates were placed in an ultrasonic bath with ice for 30 min, removed, and placed at room temperature for a minimum of 30 min. An aliquot was processed and analyzed using GC-MS SIM (EPA, 2014).

2.6. Skin wipe sampling

Skin wipe samples were collected from the hands and neck at the beginning and end of each worker's shift. Hand wipe samples were collected using a previously described method (Cavallari et al., 2012; Fent et al., 2014; Fent et al., 2014). Briefly, 2 mL of corn oil (Mazola, ACH Food Companies Inc. Oakbrook Terrace, IL. USA) was added to the palm of one hand. After rubbing the hands together in a washing motion for 1 min, the worker wiped the oil from their hands using an absorbent polyester wipe (AlphaWipe® 9 × 9, ITW Texwipe™ Cat. No. TX 1009 Kernersville, NC, USA). After collection, the skin wipe sample was transferred to a black opaque 50 mL centrifuge tube (Argos Technologies, Cat. No. UX-06344-35 Vernon Hills, IL, USA) and refrigerated until shipping to the laboratory for analysis. Levels of PAHs were standardized by the surface area of both hands (1070 cm^2 for males and 890 cm^2 for females) based on mean dermal exposure factor data (EPA, 2011).

Neck wipe samples were collected in a similar way to hand samples. Wearing clean gloves for each wipe sample, NIOSH personnel applied 2 mL of corn oil directly to the center of an absorbent polyester wipe. The wipe was folded such that the portion containing corn oil was facing outward and the NIOSH researcher wiped the worker's neck from behind the right ear to the left ear, between the hairline and shirt collar. A minimum of two passes were made, folding the wipe to present a clean, oiled surface with each pass. After collection, the wipe was transferred to a black opaque 50 mL centrifuge tube and refrigerated until shipped to the laboratory for analysis.

Field blank wipe samples were prepared by NIOSH staff by donning clean gloves and applying 2 mL of corn oil directly to the center of an absorbent polyester wipe. The wipe was folded at least two times and the wipe was transferred to a black opaque 50 mL centrifuge tube and samples were refrigerated until shipped to the NIOSH contract lab for analysis.

Skin wipes were analyzed by a modification of EPA 8270D method. The wipe samples were desorbed into 70 mL of methylene chloride. The sample desorbate was placed in an ultrasonic bath with ice for 30 min and then placed at room temperature for a minimum of 30 min. An aliquot was processed and analyzed using GC-MS SIM.

2.7. Urine samples

Two urine spot samples (pre-shift and post-shift) were collected from participating workers each workday. Urine samples were labeled for identification, coded for confidentiality, tested for specific gravity using a refractometer, and aliquoted in the field as follows: a glass tube for the analysis of hydroxylated PAHs, a cryovial for the analysis of cotinine, and a polypropylene vial for the analysis of creatinine. Samples were kept on ice in the field, transferred to a −20 °C freezer at the end of each workday, and stored frozen until laboratory analysis. PAH biomarkers in urine were quantified using on-line solid phase extraction-high performance liquid chromatography-tandem mass spectrometry: 1- and 2-hydroxynaphthalene, 2- and 3-hydroxyfluorene, 1-hydroxyphenanthrene and 2,3-hydroxyphenanthrene (the sum of 2- and 3-hydroxyphenanthrene isomers that could not be chromatographically resolved), and 1-hydroxypyrene. The analytical method and the quality assurance/quality control procedures have been described in depth before (Wang et al., 2017).

The concentration of cotinine, a metabolite of nicotine, in the urine samples of the workers was used to determine a worker's exposure to nicotine in tobacco and other nicotine-containing products. Cotinine was measured in urine samples using the Diagnostic Products Corporation Immulite® 2000 analytical platform (Siemens Healthineers Malvin, PA). The Immulite 2000 cotinine assay is an Food and Drug Administration (FDA) waived assay that is capable of differentiating passive from active tobacco users (Rodriguez et al., 2010). Cotinine values of 200 ng/mL or greater were selected to classify workers as smokers (Kim, 2016). Creatinine in each urine sample was quantified with the Vitros Autoanalyzer (Ortho Clinical Diagnosis, Raritan, NH). Urinary creatinine was used to normalize the urinary PAH biomarker concentrations for urine dilution. .

2.8. Data analysis and statistical methods

In calculating the summary statistics, non-detectable air, hand wipe, and neck wipe concentrations were assigned values using the β-substitution method (Ganser and Hewett, 2010). Median, geometric mean (GM), and geometric standard deviation (GSD) are presented for air, hand wipe post-shift, neck wipe post-shift, and urine pre-shift and post-shift concentrations. Median differences of urine pre-shift and post-shift concentrations are also provided. These summary statistics were computed for concentrations of twelve PAHs in air, hand wipe, and neck wipe samples, and for concentrations of seven PAH metabolites in urine samples. A Tukey-Kramer test was used to compare the mean concentration between each pairwise combination of PAHs in air, hand wipe, and neck wipe samples. Additionally, univariate linear regression models of RCTS product were conducted to determine unadjusted associations between molecular weight of individual PAHs and 1) logarithmic PBZ air PAH concentration, and 2) assemblage of PAHs in products and hand wipe post-shift concentrations.

Differences of creatinine adjusted urinary pre-shift and post-shift concentrations for each metabolite and summation of relevant metabolites for phenanthrene, fluorene, and naphthalene were calculated. These metabolites were summed because they come from the same parent compound to create three additional biomarkers: Sum-hydroxynaphthalene, Sum-hydroxyfluorene, and Sum-hydroxyphenanthrene. A marginal median regression model incorporating an exchangeable working correlation structure was used to account for the statistical correlation among repeated measurements from the same worker (Chen et al., 2021). The estimated correlation parameter of the exchangeable working structure represented a correlation coefficient between responses of any two samples from the same worker. The use of median regression was not only for log-normally exposure data, but for asymmetric logged exposure data. After adjusting for company, multivariable models with relevant PAH concentrations in PBZ air samples, and post-shift hand wipe and neck wipe samples as the dependent variables were conducted for testing the job title (applicator vs. non-applicator). Models adjusting for company were also carried out with urinary biomarker concentration difference as the dependent variable, in which covariates including corresponding PAH concentrations in PBZ air samples, and post-shift hand wipe and neck wipe samples, and job title (applicator versus non-applicator) were evaluated. Statistical tests were two-sided at the 0.05 significance level. All analyses were performed in R version 4.0.4 (R Core Team, 2021).

2.9. Biological exposure index (BEI)

The ACGIH considers urinary 1-hydoxypyrene a surrogate marker for carcinogenic PAHs (ACGIH, 2017). Presence of 1-hydroxypyrene was assessed by using the ACGIH adjusted BEI (ACGIH, 2017). The adjusted BEI requires calculation of the ratio of pyrene to benzo[*a*]pyrene present in suspected routes of exposure. Workers' post-shift 1-hydroxypyrene results from the final day of sampling were compared to the BEI adjusted for the particular ratio of pyrene to benzo[a]pyrene in thei air, and hand and neck wipe samples. Therefore, adjusted BEIs were calculated for 26 PBZ air, hand wipe post-shift, and neck wipe post-shift samples, then compared to individual post-shift, end of work week, 1-hydroxypyrene urine results to assess the BEI for each suspected exposure route. For example, if a participant's post-shift 1-hydroxypyrene results were higher than their adjusted BEI for the exposure route in question (PBZ, hand or neck wipe), this was an indication of chronic occupational exposure and risk of genotoxicity.

Smoking status does not effect BEI considerations. The ACGIH has determined that smoking is very unlikely to elevate urinary concentrations of urinary 1-hydroxypyrene high enough to exceed the benchmark concentration of 2.5 μg/L, which they consider evidence of occupational exposure and risk of genotoxicity (ACGIH, 2019).

3. Results

3.1. Demographics

Twenty-one RCTS workers from three companies consented to participate in this study. Their corresponding environmental and biological data were used in the analyses (Table 2). Most workers were male (95%), non-applicator (71%), and non-smoking (52%). Among the six applicators, five of them were smokers. Only one worker was female, non-applicator, and non-smoker. Note that, because of different biology, the results we provided in the manuscript were for male workers only.

3.2. RCTS product results

Eight RCTS product samples were collected for this study, one from company A, six from company B, and one from company C. The distributions of RCTS product values (μg/g) of 12 PAHs and their corresponding molecular weights (g/mole) are presented in Fig. 1. Overall, phenanthrene and pyrene had the highest concentrations. The third sealant products supplied by company A had higher PAH concentrations relative to the other two companies.

Table 2
Characteristics of study participants or workers by company, N = 21.

Company	A	B	C	Total
Characteristic	(N = 4)	(N = 8)	(N = 9)	(N = 21)
	No. (%)	No. (%)	No. (%)	No. (%)
Gender				
Male	3 (75)	8 (100)	9 (100)	20 (95)
Female	1 (25)	0	0	1 (5)
Age, years				
Mean ± SD	26 ± 6	41 ± 13	–	36 ± 13
Median	25	44	–	33
Range	21–33	25–54	–	21–54
Missing	1 (25)	2 (25)	9 (100)	12 (57)
Job Title				
Non-Applicator	3 (75)	5 (63)	7 (78)	15 (71)
Applicator	1 (25)	3 (38)	2 (22)	6 (29)
Smoking[a]				
No	3 (75)	4 (50)	4 (44)	11 (52)
Yes	1 (25)	4 (50)	5 (56)	10 (48)
Worked ≥20 days on coal tar sealant jobs during the prior 30 days				
No	4 (100)	1 (13)	–	5 (24)
Yes	0	7 (87)	–	7 (33)
Missing	0	0	9 (100)	9 (43)
Number of PBZ Air Samples	15	39	18	72
Number of Wipe Samples	4	25	9	38
Number of Urinary Samples	15	42	18	75

[a] Smoking is defined based on cotinine values of 200 ng/mL or greater.

3.3. Air results

A total of 68 PBZ samples were collected from 20 workers and the median number of samples collected from each worker was two, ranging from two to eight. Eleven of 12 analytes were detected in more than 50% of PBZ air samples in all companies (Table 3; results of the remaining ten analytes not selected are in Supplemental Table S2). Airborne naphthalene level was at least two orders of magnitude below occupational exposure limits. The three PAHs listed as carcinogens by ACGIH (benz[*a*]anthracene, chrysene, and benzo[*a*]pyrene) were detected in 69, 75, and 69%, respectively, of the workers' PBZ air samples. Naphthalene had significantly higher GM concentrations (all p-values < 0.001) than the other PAHs. Applicators had higher phenanthrene, benz[a]anthracene, chrysene, and benzo[k]fluoranthene median concentrations in PBZ air samples than non-applicators (p-value < 0.05) (Table 4). Detailed summary PAH concentrations in PBZ air samples for applicators and non-applicators across all three companies are in Supplemental Table S3. Summary concentrations of area air samples are also provided (Supplemental Table S4). The PAH GM concentrations in area air samples were significantly lower than in PBZ air samples (all analytes with p-values < 0.001). Note that PAH concentrations of all field blank air samples were below the LOD. We also found that, through the use of GM- and mean-oriented data, logarithmic GM concentrations of PAHs in PBZ air significantly decreased with increasing mean molecular weights of the PAHs (p-value $= 0.004$). This result was consistent with the finding in Achten and Andersson (2015).

3.4. Hand and neck wipe results

A total of 38 hand and neck wipe samples were collected from 20 workers and the median number of samples collected from each worker was one, ranging from one to four. Hand wipe post-shift GM concentrations of phenanthrene and pyrene were significantly higher than those of the other PAHs for all companies combined (p-values < 0.05) but were not significantly different from one another (Table 3). Among neck wipe post-shift samples, phenanthrene and pyrene had the greatest

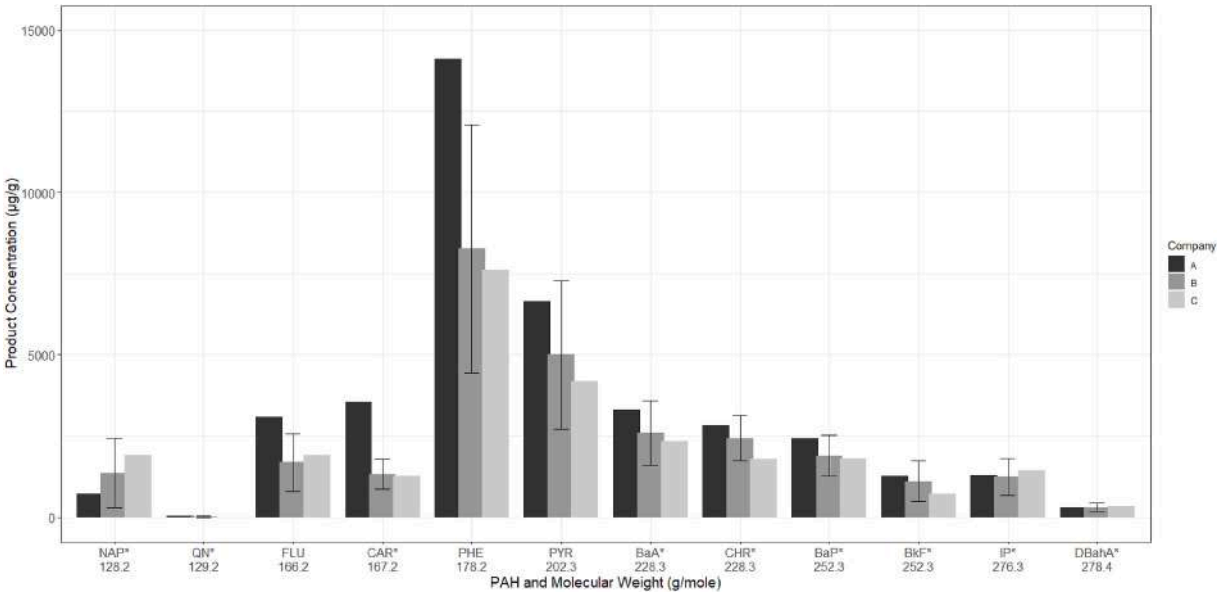

Fig. 1. RCTS product results (µg/g) by PAHs with corresponding molecular weights (g/mole) for three companies. Companies A and C had one RCTS product sample each. Company B had six samples, and values shown are arithmetic means with standard deviation. Asterisks were used to indicate the IARC Group 1 (carcinogenic to humans), Group 2A (probably carcinogenic to humans), and Group 2B (possibly carcinogenic to humans) PAHs.

Table 3
PBZ air (µg/m^3), hand wipe post-shift (µg/cm^2), and neck wipe post-shift (µg/sample) concentrations of PAHs for all companies.

Analyte	PBZ Air Concentration (Number of Samples = 68)				Hand Wipe Post-Shift Concentration (Number of Samples = 37)				Neck Wipe Post-Shift Concentration (Number of Samples = 37)			
	K < LOD[a] (%)	Medain[b]	GM[b]	GSD[b]	K < LOD[a] (%)	Medain[b]	GM[b]	GSD[b]	K < LOD[a] (%)	Medain[b]	GM[b]	GSD[b]
NAP [e]	0 (0)	61.81	55.81	2.76	5 (14)	0.28	0.17	7.82	9 (24)	1.10	0.43	11.27
QN [e]	1 (1)	1.03	0.84	3.15	8 (22)	0.17	0.05	13.64	27 (73)	0.00	0.00	114.2
FLU	0 (0)	6.66	6.79	2.48	0 (0)	0.77	0.98	3.89	16 (43)	1.00	0.14	40.49
CAR [e]	4 (6)	0.36	0.29	3.61	1 (3)	1.31	1.44	6.62	23 (62)	0.00	0.02	64.45
PHE	0 (0)	11.26	10.09	2.34	0 (0)	6.07	7.96	3.76	1 (3)	5.60	6.22	6.05
PYR	0 (0)	0.98	0.96	2.89	1 (3)	5.79	5.32	9.41	11 (30)	3.00	1.00	30.55
BaA [e]	21 (31)	0.16	0.06	15.54	1 (3)	2.99	2.90	7.70	13 (35)	1.50	0.44	34.02
CHR [e]	17 (25)	0.20	0.10	11.19	1 (3)	3.55	3.50	7.86	13 (35)	1.60	0.53	35.35
BaP[c]	21 (31)	0.15	0.05	15.13	1 (3)	2.62	2.52	7.36	12 (33)	1.90	0.52	30.27
BkF [e]	29 (43)	0.07	0.01	35.90	1 (3)	1.40	1.32	6.14	19 (51)	0.00	0.08	54.12
IP [e]	24 (35)	0.11	0.03	20.92	1 (3)	1.87	1.78	6.43	14 (38)	1.30	0.28	35.74
DBahA [d]	54 (79)	0.00	0.00	533.7	1 (3)	0.46	0.48	4.54	27 (73)	0.00	0.00	82.36

[a] Non-detected values replaced using β-substitution (Ganser and Hewett, 2010). The PBZ air LOD for all analytes is 0.02 µg/m^3. Exposure levels of PAHs were standardized by the surface area of both hands based on mean dermal exposure factor data (1070 cm^2 for males (EPA, 2011)). The hand wipe LODs are 0.0009 µg/cm^2. The neck wipe LODs are 0.01 µg/sample.
[b] Reported medians, GMs, and GSDs for analytes with less than 50% detection rate may not be reliable.
[c] IARC Group 1: Carcinogenic to humans.
[d] IARC Group 2A: Probably carcinogenic to humans.
[e] IARC Group 2B: Possibly carcinogenic to humans.

median and GM concentrations (Table 3). Applicators were more likely to have higher hand wipe and neck wipe post-shift median concentrations of most PAHs than non-applicators (Table 4). Medians and GMs of PAH concentrations in neck wipe samples were much lower than those in hand wipe samples (results not shown). In addition, through the use of GM- and mean-oriented data, GM concentrations of PAHs in post-shift hand wipes increased with increasing mean compositions of PAHs in the products (p-value < 0.001) (Supplemental Fig. S1). Note that PAH concentrations of all field blank wipe samples were below the LOD.

3.5. Urine results

A total of 75 urinary samples were collected from 20 workers. The median number of samples collected from each worker was three, ranging from two to four. Differences in post- and pre-shift urinary PAH biomarker concentrations were generally greatest for company B (Table 5). Medain differences for urinary biomarkers, 2-hydroxyfluorene, 3-hydroxyfluorene, Sum-hydroxyfluorene, 1-hydroxyphenanthrene, Sum-hydroxyphenanthrene, and 1-hydroxypyrene were significantly higher for company B than company C, and median concentration differences of 1-hydroxynaphthalene and 1-hydroxypyrene were higher for company B than company A (p-values < 0.05) (Table 5). The concentrations of Sum-hydroxyfluorene and Sum-hydroxyphenanthrene were dominated by 2-hydroxyfluorene and 2,3-hydroxyphenanthrene, respectively. In addition to the results analyzing adjusted urinary samples, the summary results of unadjusted urinary biomarkers are presented in Supplemental Table S5.

Urine biomarker concentration differences (i.e., pre- and post-shift) were significantly and positively related to naphthalene, fluorene, and phenanthrene PBZ air concentrations (p-values < 0.001, 0.04 and < 0.001, respectively) (Table 6). Urine concentration differences were also significantly associated with increased neck wipe post-shift fluorene

Table 4
Multivariable analysis using PBZ air (μg/m^3), hand wipe post-shift (μg/cm^2), and neck wipe post-shift concentrations (μg/sample) as the outcomes of interest (dependent variable) and comparing PAH concentrations of applicators with non-applicators (predictor)[a], N of workers = 20.

Dependent Variable	PBZ Air		Hand Wipe Post-Shift		Neck Wipe Post-Shift	
Analyte	Difference[b] (SE)	P-Value	Difference[b] (SE)	P-Value	Difference[b] (SE)	P-Value
NAP [e]	13.46 (10.86)	0.233	0.14 (0.07)	0.068	0.20 (0.15)	0.196
QN [e]	0.34 (0.33)	0.312	0.01 (0.03)	0.717	–	–
FLU	3.57 (1.79)	0.064	1.32 (0.13)	<0.001	1.70 (0.93)	0.086
CAR [e]	0.25 (0.18)	0.195	2.30 (0.12)	<0.001	1.40 (0.07)	<0.001
PHE	6.44 (2.42)	0.017	16.82 (0.50)	<0.001	8.90 (0.61)	<0.001
PYR	0.75 (0.75)	0.331	12.52 (0.15)	<0.001	7.20 (0.76)	<0.001
BaA [e]	0.10 (0.04)	0.021	5.33 (0.11)	<0.001	5.70 (0.50)	<0.001
CHR [e]	0.21 (0.04)	<0.001	8.04 (0.08)	<0.001	6.40 (0.42)	<0.001
BaP[c]	0.07 (0.11)	0.503	4.95 (0.38)	<0.001	4.00 (0.31)	<0.001
BkF [e]	0.06 (0.03)	0.043	1.59 (0.13)	<0.001	2.90 (0.15)	<0.001
IP [e]	0.08 (0.04)	0.081	2.15 (0.16)	<0.001	3.10 (0.44)	<0.001
DBahA [d]	–	–	0.97 (0.09)	<0.001	–	–

[a] Median regression models adjusting for company were used for the analyses. Models not convergent were marked as a dash.
[b] Difference of PAH median concentrations of applicators and non-applicators. Non-applicators were the reference group.
[c] IARC Group 1: Carcinogenic to humans.
[d] IARC Group 2A: Probably carcinogenic to humans.
[e] IARC Group 2B: Possibly carcinogenic to humans.

concentrations. Job title was not significantly related to concentration differences. Summary statistics of environmental and biological data including the female are provided in supplemenal tables (Tables S6 and S7).

3.6. Biological exposure index (BEI) results

The ACGIH BEIs were adjusted by calculating the ratios of pyrene to benzo[*a*]pyrene (Table 7 and Supplemental Table S8). Unadjusted urinary last-day post-shift 1-hydroxypyrene concentrations, ranging from 0.5 to 377 μg/L, exceeded the adjusted BEI in almost every case. Of 18 end-of-week urine 1-hydroxypyrene sample results that could be compared to airborne pyrene to benzo[*a*]pyrene ratios (applied as the adjusted BEI), 17 were above the adjusted BEIs. Workers' end-of-week urine 1-hydroxypyrene concentrations also exceeded the BEI when using hand wipe and neck wipe samples for calculation.

4. Discussion

4.1. Composition of RCTS products

The chemical composition of RCTS product samples from companies indicate which exposures to expect. We found little difference in overall composition of PAHs present in RCTS between companies and batches (Fig. 1 and Supplemental Fig. S1). Company A had the highest summed PAH levels among the three companies. The small differences observed between companies could relate to differences in the chemical composition of the crude coal tar starting product or variance between batches mixed on job sites. One batch may have contained more water or filler agents than another. Depending on the size of a project, it may also be necessary to re-mix or rehydrate a batch of RCTS, potentially further altering the final product. Despite these small differences, our results suggest that the composition was similar among all companies (Fig. 1). All analytes found in RCTS product samples were found in PBZ samples or post-shift hand wipe samples.

Asphalt is a product containing the most comparable PAH profile and application environment, with published research, that could be identified for comparison of this data. Results from previous RCTS and asphalt product sampling conducted by the IARC indicate that concentrations of 13 PAHs included in this manuscript are almost all at least one thousand times higher in RCTS than asphalt (IARC, 2013). For example, the IARC monograph reported that the benzo[*a*]pyrene concentration in asphalt product samples had a range of 0.22–1.8 μg/g, whereas the range of benzo[*a*]pyrene concentration present in coal-tar pitch samples without filler agents was 11,360 to 15,170 μg/g (IARC, 2013). The concentrations of benzo[*a*]pyrene in the RCTS sealants, after adding filler agents, in the present study were 2,436, 1,896, and 1817 μg/g for companies A, B, and C, respectively.

4.2. PBZ and area air samples

PBZ samples were included in this exposure assessment to help determine the primary exposure route that effects RCTS workers. Area air samples represent the environment immediately surrounding work areas and PBZ samples illustrate personal airborne occupational exposures. All nine PAHs classified by IARC as possible human carcinogen (Group 2B) to known human carcinogen (Group 1) were detected in PBZ samples at all companies, except for dibenzo[*a,h*]anthracene, which was not found in PBZ samples from company A (Table 3). In this exposure assessment, the GM concentration for workers exposed to benzo[*a*]pyrene was 0.05 μg/m^3. For context, the GESTIS International Limit Value database, which reports international occupational exposure limits by country, reports airborne concentration limits ranging from 0.07 to 2.0 μg/m^3 for an 8-h workday (IFA, 2021).

Workers in this study were exposed to atmospheric PAHs that are known or suspected carcinogens at levels at least an order of magnitude higher than published exposures of asphalt workers. McClean et al. (2012) reported GMs of airborne pyrene and naphthalene concentrations of 0.06 and 0.83 μg/m^3. In this exposure assessment, GMs for pyrene and naphthalene were 0.96 μg/m^3 and 55.81 μg/m^3, respectively.

Of the twelve PAHs considered potentially carcinogenic or used in our staticistal modelling, only seven were detected on area samples at work sites (Supplemental Table S4). PAH concentrations in area air samples that were detected were an order of magnitude lower than PAH concentrations found in the PBZ results (Supplemental Tables S2, S3, & S4), despite close proximity of area air sampling to the surfaces being treated. The comparison of the area air sample results to PBZ concentrations suggests the primary source of cumulative airborne exposure is occupationally derived.

4.3. Implications of skin wipe concentrations

Skin wipe samples were included in this exposure assessment to help determine the primary exposure route that effects RCTS workers. Mid-molecular weight PAHs phenanthrene, pyrene, and chrysene, in that order, were measured in the highest concentrations in post-shift hand wipes (Table 3). The lower molecular weight PAHs, such as

Table 5
Urinary biomarker pre-shift and post-shift concentration (μg/g creatinine), and difference of pre- and post-shift concentrations by company.

	Pre-Shift			Post-Shift			Difference
	Median	GM	GSD	Median	GM	GSD	Median
All Companies (Number of Samples = 71)							
1-OHNAP	8.35	8.07	2.36	14.75	16.13	2.18	6.35
2-OHNAP	10.28	10.51	2.32	18.27	20.57	2.03	7.88
Sum-OHNAP	20.57	20.31	2.16	34.23	39.27	1.94	13.39
2-OHFLU	13.17	11.79	2.57	31.42	27.71	2.31	14.06
3-OHFLU	5.00	4.77	2.66	8.30	8.19	2.37	2.51
Sum-OHFLU	17.88	16.83	2.53	37.84	36.55	2.27	15.99
1-OHPHE	6.45	5.04	2.71	10.37	9.79	2.53	3.40
2,3-OHPHE	6.85	6.26	2.50	17.77	15.73	2.48	9.71
Sum-OHPHE	15.05	11.53	2.53	27.02	25.91	2.46	13.11
1-OHP	15.11	10.10	3.91	20.02	14.72	3.70	2.25
Company A[a] (Number of Samples = 11)							
1-OHNAP	6.25	7.01	1.66	8.90	11.29	1.53	3.90
2-OHNAP	6.06	7.88	2.09	13.13	13.78	1.66	4.90
2-OHFLU	8.83	9.34	2.42	30.29	23.80	2.31	14.37
3-OHFLU	3.59	3.51	2.68	5.98	5.84	2.42	2.04
1-OHPHE	4.10	3.58	2.98	8.76	6.38	2.87	2.41
2,3-OHPHE	4.53	4.34	2.87	16.02	11.04	2.94	4.77
1-OHP	9.60	4.41	6.88	13.42	5.27	6.66	0.08
Company B[a] (Number of Samples = 42)							
1-OHNAP	7.81	7.67	2.35	14.30	15.58	2.43	7.18
2-OHNAP	11.13	11.93	2.58	23.20	23.66	2.27	9.13
2-OHFLU	15.14	14.11	2.59	35.95	35.77	2.15	17.77
3-OHFLU	6.33	5.78	2.52	11.28	10.52	2.26	3.40
1-OHPHE	7.61	6.23	2.57	14.19	13.37	2.21	6.28
2,3-OHPHE	7.29	7.06	2.37	18.85	19.17	2.35	11.01
1-OHP	17.52	14.59	2.83	27.96	24.96	2.45	4.35
Company C[a] (Number of Samples = 18)							
1-OHNAP	9.58	9.89	2.81	21.95	21.73	1.77	9.41
2-OHNAP	10.05	9.33	1.77	18.21	18.97	1.46	8.24
2-OHFLU	7.46	8.93	2.49	13.58	16.76	2.20	6.36
3-OHFLU	3.59	3.67	2.85	4.87	5.62	2.19	1.42
1-OHPHE	3.48	3.78	2.68	6.85	6.15	2.43	1.86
2,3-OHPHE	6.65	5.91	2.56	13.14	12.32	2.33	5.24
1-OHP	8.44	7.11	4.05	8.36	8.04	3.04	0.66

Abbreviations of biomarkers: 1-Hydroxynaphthalene (1-OHNAP), 2-Hydroxynaphthalene (2-OHNAP), 2-Hydroxyfluorene (2-OHFLU), 3-Hydroxyfluorene (3-OHFLU), 1-Hydroxyphenanthrene (1-OHPHE), 2,3-Hydroxyphenanthrene (2,3-OHPHE), 1-Hydroxypyrene (1-OHP).

[a] 1 and 2 workers had 3 and 4 samples, respectively, in company A; 3, 8, and 3 workers had 2, 3, and 4 samples, respectively, in company B; 9 workers had 2 samples in company C.

naphthalene, quinoline, and acenaphthene, also were detected on hand wipes but at much lower concentrations, and at lower concentrations than most of the higher molecular weight PAHs (benz[*a*]anthracene – benzo[*g,h,i*]perylene). This finding contrasts with that reported for asphalt workers, for whom lower molecular weight, more volatile PAHs contributed the most to skin exposure (McClean et al., 2012).

There are no occupational exposure limits for skin exposures to PAHs; however, all nine PAHs classified as possible human carcinogens (Group 2B) or known human carcinogens (Group 1) were detected in post-shift hand wipes. The post-shift hand wipe GM of pyrene for all companies and visits was 5.32 μg/cm^2 (Table 3). These results are considerably higher than levels reported in a previous study of asphalt workers, that reported GMs of post-shift hand wipe levels of pyrene to be 0.285 ng/cm^2 (Cavallari et al., 2012). Naphthalene, classified as possibly carcinogenic to humans (Group 2B) by the IARC, is commonly measured in asphalt worker exposure research. Cavallari et al. reported a range of 0.23–1.2 ng/cm^2 of naphthalene, with a detection rate too low to calculate the GM on participants' hands. The hand wipe results in this study for RCTS workers for all companies for naphthalene had a GM of 0.17 μg/cm^2.

Benzo[*a*]pyrene is the only PAH identified in refined coal tar that is classified as a known carcinogen (McClean et al., 2004). The post-shift hand wipe GM of benzo[*a*]pyrene in this study was 2.52 ± 7.36 μg/cm^2. Recent research found that benzo[*a*]pyrene is continually absorbed and metabolized by human skin over 48 h, meaning repeated occupational exposures throughout the workweek have a cumulative effect that likely increases risk of genotoxicity (Bourgart et al., 2018).

4.4. PAH biomarkers in urine

Urinary metabolites were assessed in this manuscript to support the corresponding exposure data. By pairing exposure data and urine results, we were able to identify the likely source of PAH exposure. For further context, RCTS workers' urinary PAH biomarkers are compared to those of the general population. Average urinary metabolite concentrations for the general population are reported by NHANES.

Table 7
Summary results of unadjusted urinary 1-OHP last-day post-shift concentrations (μg/L) and corresponding BEI values for 26 PBZ air, hand wipe post-shift, and neck wipe post-shift samples.

	N	BEI[a] Mean (PYR/BaP) (SD)	BEI Median (PYR/BaP) (Range)	N of 1-OHP > BEI[a] (%)
Air	18[b]	6.76 (4.61)	5.31 (2.43–17.62)	17 (94.4)
Hand Wipe	25[b]	2.19 (0.30)	2.20 (1.64–2.79)	25 (100)
Neck Wipe	21[b]	2.04 (1.11)	1.94 (0.68–6.20)	21 (100)
		Mean (μg/L) (SD)	**Median (μg/L) (Range)**	
Urinary 1-OHP	26[‡]	92.72 (94.22)	55.54 (0.53–377.0)	

[a] BEI: Biological exposure index; this index was calculated using ratio of PYR to BaP for each corresponding sample (ACGIH, 2019).

[b] BaP was not detected for eight air samples, one hand wipe sample, and four neck wipe samples. Also, PYR was not detected for one hand wipe sample and three neck wipe samples. Therefore, 18 air BEIs, 25 hand wipe BEIs, and 21 neck wipe BEIs were used to compare with the urinary 1-OHP data. Some workers were sampled for more than one week.

Table 6
Multivariable analysis using urine biomarker concentration difference between pre-shift and post-shift (μg/g creatinine) as the outcome of interest[a], N or workers = 20.

Biomarker	Analyte	PBZ Air PAH		Hand Wipe Post-Shift		Neck Wipe Post-Shift	
		Difference[b] (SE)	P-Value	Difference[b] (SE)	P-Value	Difference[b] (SE)	P-Value
Sum-OHNAP	NAP [c]	0.23 (0.05)	<0.001	7.46 (3.62)	0.056	4.85 (16.88)	0.777
Sum-OHFLU	FLU	1.87 (0.84)	0.040	0.16 (0.78)	0.839	0.95 (0.39)	0.028
Sum-OHPHE	PHE	1.00 (0.16)	<0.001	0.04 (0.04)	0.304	−0.04 (0.12)	0.737
1-OHP	PYR	1.70 (0.92)	0.082	0.03 (0.02)	0.261	0.01 (0.05)	0.803

[a] Median regression models adjusting for company were used for the analyses.

[b] Difference from median pre-shift to median post-shift values.

[c] IARC Group 2B: Possibly carcinogenic to humans.

NHANES data includes both occupationally and non-occupationally exposed people.

The metabolites of naphthalene (1- & 2-hydroxynaphthalene) can be used to indicate other airborne exposures to PAHs, due to their similar volatility. According to an NHANES survey conducted in 2013–2014, the unadjusted GM metabolite concentrations of 1-hydroxynaphthalene and 2-hydroxynaphthalene are 1.71 and 4.24 μg/L in the general population for people over the age of 20 (CDC, 2021). RCTS workers in the current study had unadjusted post-shift urinary GM concentrations of 43.26 and 55.18 μg/L for 1-hydroxynaphthalene and 2-hydroxynaphthalene, respectively (Supplemental Table S5), indicating substantially higher exposures to PAHs than the representative population sampled by NHANES.

The metabolite of pyrene (1-hydroxypyrene) can be used as a surrogate for skin exposures among higher molecular weight PAHs in RCTS. According to the 2013-2014 NHANES survey, the unadjusted GM metabolite concentration of 1-hydroxypyrene is 128 ng/L for people over the age of 20 (CDC, 2021). Pesch et al. reported medians of unadjusted post-shift urinary concentrations for non-smoking asphalt pavers of 419, and 793 ng/L for pavers who smoked (Pesch et al., 2011). Urinary concentrations of RCTS workers in this study had a GM of over 39,000ng/L 1-hydroxypyrene for smokers and nonsmokers combined. The urinary 1-hydroxypyrene concentrations for RCTS workers in this study were approximately 49 times higher than concentrations reported for asphalt workers that smoked, and over 300 times higher than the population sampled by NHANES (Supplemental Table S5).

Urinary 1-hydroxypyrene concentrations, for all workers, was above the BEI recommended by ACGIH when the pyrene to benzo[*a*]pyrene ratio for skin wipe samples were used to adjust the the BEI (Table 7). When the BEI was adjusted using PBZ values of pyrene to benzo[*a*] pyrene ratio, urinary 1-hydroxypyrene exceeded the BEI in 89% of workers. In many cases, worker 1-hydroxypyrene levels were orders of magnitude above the BEI (Supplemental Table S8). The BEI results indicate that PAH exposures are occupationally derived and highlight the need to be reduce workplace exposures to minimize risk of genotoxicity for RCTS workers.

The relationships between urinary biomarkers and potential explanatory variables, including exposures and job title, were not statistically significant between non-applicators and applicators. This could be because both groups have long-term, daily exposures to RCTS. The urinary metabolite 1-hydroxypyrene did not have a significant correlation with explanatory variables, consistent with a much lower airborne concentration of pyrene relative to the three other volatile PAHs found in the highest concentrtions in PBZ samples (naphthalene, phenanthrene, and fluorene) (Table 3). There was no correlation between urinary biomarkers and PAHs in hand wipes, despite hand wipes having much higher levels of PAHs than neck wipes.

However, there was a correlation between urinary biomarkers and PAHs in neck wipes (Table 6). A significant correlation was only found for the sum of both urinary metabolites of fluorene (2- and 3-hydroxyfluorene). The difference between hand wipe and neck wipe associations with urinary biomarkers could be related to differences in PAH exposures at different locations on the body.

The hands are more transient than the neck. For example, the hands were likely washed or wiped at least once during the shift, and therefore produced more variable results than the neck, which may remain untouched for most of a work day. Hand wipe results could reflect cumulative exposures over a shift or reflect an acute exposure immediately before sampling occurred. Meanwhile, the neck represents potential cumulative exposures via vapor deposition and is a less transient part of the body. The neck also absorbs PAHs more quickly than hands, with relative absorption index values of 1.41 and 0.68, respectively (VanRooij et al., 1993), which likely contributed to the correlation between urinary biomarkers and PAH concentrations found on the neck wipe samples.

4.5. Job task and personal protective equipment

Applicators had significantly higher PBZ and post-shift hand wipe concentrations than non-applicators (Table 4). These results are likely related to differences in work-related tasks between the two subgroups. Applicators conducted work that always required direct contact with RCTS such as mixing and application, while non-applicators were more likely to conduct ancillary tasks conducted further from the RCTS product.

There were statistical significances in phenanthrene median concentration levels between applicator status for all three sample types (Table 4). As a mid-molecular weight PAH, phenanthrene is more likely to be found in the air and on the skin, than more, or less volatile PAHs found in RCTS. Phenanthrene was identified as the most abundant PAH in samples of the starting product and was reported in much higher concentrations on PBZ and skin wipe samples than any other PAH, except airborne naphthalene. Due to the combination of its relative abundance in the starting product and the higher concentrations present on all sample media (Table 3), phenanthrene may be a suitable surrogate for cumulative PAH exposures in future RCTS worker exposure assessments.

Workers did not wear personal protective equipment (PPE) consistently. Many workers wore long pants and work boots, while others wore shorts and shoes. There was intermittent use of gloves, booties, dust masks, and splash-protective suits. No difference in PPE was observed between applicators and non-applicators, except that applicators wore gloves when conducting certain tasks, such as mixing. One applicator was observed in a full Tyvek suit and face covering when operating the boom sprayer/squeegee apparatus on the back of a truck. Some workers were observed wearing the same clothes every day, which likely contributed to chronic and take-home exposures. Although there is currently no research specific to RCTS safety controls, providing employees with PPE and developing company policies for guidance could reduce RCTS workers' risk of genotoxicity.

4.6. Limitations

As a result of the difficulty in finding companies to participate, the study had a low sample size and one company was visited multiple times. More detailed data on PPE, demographics, and post-shift cleaning practices (i.e., hand washing methods) could have provided additional insight. Analysis of additional metabolites that can't be assessed via urinalysis, such as 3-hydroxybenzo[*a*]pyrene and 6-hydroxychrysene, may have yielded useful information.

5. Conclusions

The exposure results from RCTS worker samples cannot be explained by proximal factors such as nearby restaurants or construction. Air and skin concentration levels were substantially higher for RCTS workers than previously published levels among asphalt workers for all PAHs. PAH profiles on skin wipes were more consistent with RCTS sealant product than air samples. Last day post-shift urinary concentrations of 1-hydroxypyrene greatly exceeded the ACGIH BEI benchmark of 2.5 μg/L in 25 of 26 samples, which suggests occupational exposure and risk of genotoxicity. When pyrene and benzo[*a*]pyrene were both detected, concentration ratios from personal exposure samples were used to calculate the adjusted BEI. Concentrations of 1-hydroxypyrene exceeded the adjusted BEIs for air, hand wipes, and neck wipes in most cases. These results indicate the need to increase safety controls and exposure mitigation for RCTS workers.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.113971.

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Firefighters' urinary concentrations of VOC metabolites after controlled-residential and training fire responses

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Introduction: Firefighters are exposed to volatile organic compounds (VOCs) during structural fire responses and training fires, several of which (e.g., benzene, acrolein, styrene) are known or probable carcinogens. Exposure studies have found that firefighters can absorb chemicals like benzene even when self-contained breathing apparatus (SCBA) are worn, suggesting that dermal absorption contributes to potentially harmful exposures. However, few studies have characterized VOC metabolites in urine from firefighters.

Objectives: We quantified VOC metabolites in firefighters' urine following live firefighting activity across two field studies.

Methods: In two separate controlled field studies, spot urine was collected before and 3 h after firefighters and firefighter students responded to simulated residential and training fires. Urine was also collected from instructors from the training fire study before the first and 3 h after the last training scenario for each day (instructors led three training scenarios per day). Samples were analyzed for metabolites of VOCs to which firefighters may be exposed.

Results: In the residential fire study, urinary metabolites of xylenes (2MHA), toluene (BzMA), and styrene (MADA) increased significantly (at 0.05 level) from pre- to post-fire. In the training fire study, MADA concentrations increased significantly from pre- to post-fire for both firefighter students and instructors. Urinary concentrations of benzene metabolites (MUCA and PhMA) increased significantly from pre- to post-fire for instructors, while metabolites of xylenes (3MHA+4MHA) and acrolein (3HPMA) increased significantly for firefighter students. The two highest MUCA concentrations measured post-shift from instructors exceeded the BEI of 500 μg/g creatinine.

Conclusions: Some of the metabolites that were significantly elevated post-fire are known or probable human carcinogens (benzene, styrene, acrolein); thus, exposure to these compounds should be eliminated or reduced as much as possible through the hierarchy of controls. Given stringent use of SCBA, it appears that dermal exposure contributes in part to the levels measured here.

1. Introduction

Firefighters, firefighter students, and instructors may be exposed to volatile organic compounds (VOCs) during a variety of combustion events, including structure, vehicle, dumpster, vegetation, and training fires. The types of VOCs previously reported in personal air sampling on the exterior of gear of firefighters, firefighter students, and instructors responding to fires include benzene, toluene, ethylbenzene, xylenes,

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styrene (BTEX + S), formaldehyde, acrolein, 1,3-butadiene, and naphthalene along with many other organic compounds (Jankovic et al., 1991; Austin et al. 2001a, 2001b; Fent et al. 2018, 2019a). While the composition and magnitude of VOCs will vary depending on the type of materials being burned, VOCs will always be present in smoke and several of the most common constituents have been classified as known (e.g., benzene, formaldehyde, 1,3-butadiene) and probable (e.g., acrolein, styrene) carcinogens by the International Agency for Research on Cancer (IARC) (IARC 2012a; IARC 2012b; IARC 2019; IARC 2021).

In 2010, IARC evaluated the occupation of firefighting and based on evidence at that time, classified it as "possibly carcinogenic to humans" (IARC 2010). In 2019, IARC listed the occupation of firefighting as a high priority for re-evaluation as new evidence has become available, including several epidemiologic studies that have found increased risk of certain types of cancer (Daniels et al., 2014; Pukkala et al., 2014; Tsai et al., 2015; Glass et al., 2016; Muegge et al., 2018; Jalilian et al., 2019; Soteriades et al., 2019; Casjens et al., 2020; Lee et al., 2020; Pinkerton et al., 2020) as well as biomarker studies that have shown genetic and epigenetic changes in firefighters (Andersen et al., 2017; Zhou et al., 2019; Jung et al., 2021).

A number of exposure studies have also characterized the biological uptake of combustion byproducts for firefighters, firefighter students, and instructors, including polycyclic aromatic hydrocarbons (PAHs) and benzene (Feunekes et al., 1997; Caux et al., 2002; Laitinen et al. 2010, 2012; Oliveira et al., 2016; Keir et al., 2017; Fent et al. 2019b, 2020). Some of these studies were also able to confirm that self-contained breathing apparatus (SCBA) was worn consistently throughout the fire event, suggesting that dermal absorption contributed to the PAH metabolites measured in urine and benzene measured in exhaled breath of the firefighters, firefighter students, and instructors (Fent et al. 2019b, 2020). Mayer et al., (2020) employed stationary mannequins in a smoke exposure chamber to show that benzene can come through or around turnout jackets. It is plausible, then, that benzene vapor is contacting firefighters' skin during live-fire events and a small percentage is being absorbed through skin, as has been shown in animal studies (Franz and McFarland, 1984, Wester et al., 1993; Thrall et al., 2000; Wester and Maibach 2000).

Compounds with lower vapor pressure that contact skin should have an increased propensity for being dermally absorbed; although, the specific properties of the compounds, such as octanol/water coefficient, also play an important role (Frasch 2002; Rauma et al., 2013). It's also possible that turnout gear could off-gas VOCs after SCBA has been removed, contributing to firefighters' exposure through inhalation and dermal absorption (Fent et al., 2015). Regardless, given the overall abundance of VOCs in the fire environment, an understanding of the extent to which those VOCs may be absorbed by firefighters is crucial. However, few studies have attempted to quantify VOC metabolites in firefighters after live-fire events. Of the studies that have measured VOC biological burden in firefighters, most have collected exhaled breath (Fent et al. 2019b, 2020) or only measured urinary metabolites of benzene (Caux et al., 2002; Laitinen et al., 2010; Rosting and Olsen 2020).

We conducted two field studies: one involving controlled residential fires (firefighters) and a second involving three types of training environments (firefighter students and instructors), each with different fuel packages and structures. These two studies allowed us to investigate the biological uptake of PAHs (urine) and VOCs including benzene (exhaled breath) and compare these values to airborne measurements as well as dermal exposure monitoring results for PAHs (Fent et al. 2017, 2018, 2019a, 2019b, 2020). The purpose of this paper is to quantify and compare specific VOC metabolites in urine across the two studies and collection time points. Specifically, firefighters and firefighter students responded to a single fire scenario and a spot urine sample was collected before and 3 h after the fire responses. Urine was also collected from instructors each day before they led three training scenarios and 3 h after the training scenarios.

2. Methods

2.1. Study design

This paper reports biomonitoring results from two separate live-fire exposure studies: 1) residential fire study and 2) training fire study, both of which took place at the Illinois Fire Service Institute. The residential fire study was approved by the Institutional Review Boards (IRBs) at the University of Illinois and the National Institute for Occupational Safety and Health (NIOSH). The training fire study was also approved by the University of Illinois IRB, with reliance out by NIOSH IRB. The methodology used to recruit and consent participants has been described previously (Fent et al. 2017, 2019a). Firefighters with any known cardiovascular disease, who used tobacco products, were younger than 18 or older than 55 years of age, or pregnant were excluded from participating in the studies. Details about each study design, including the fire structures and fuels, are provided in Table 1.

For the residential fire study, participating firefighters included in this analysis were assigned to either fire attack, where they advanced the hoseline and suppressed all active fires, or search and rescue, where they

Table 1
Summary of the two studies where urine samples were collected before and after live-fire training and subsequently analyzed for VOC metabolites.

	Residential fire study	Training fire study
Design	Repeated measures	Repeated measures
Fire structure and fuels	Fires ignited in two rooms of a 111 m^2 wood frame residential structure containing residential furnishings, including double bed with polyurethane foam topper, stuffed chair, wood side table and dresser, lamp, flat screen TV, and polyester carpet with recycled polyurethane foam padding oriented as in a residential bedroom common in the 21st century United States.	Training fires involved obscuring visibility by 1) Burning of **pallet and straw** in a ground-based rack inside single story concrete training building, 2) Burning of oriented strand board (**OSB**) attached to the training structure ceiling, along with pallet and straw in a ground-based rack in each of two rooms of a T-shaped metal shipping container. Two types of OSB were used denoted as Alpha and Bravo (see supplemental files for more details), 3) Generation of simulated smoke and electronic flame inside series of metal shipping containers.
Participants	12 firefighters per response team, 3 response teams, each team fought 4 fires (1 per day), firefighters assigned to new positions after two fires. Positions included: attack (2), search (2), outside vent (2), overhaul (4), incident command and pump operator (2). There were 41 total participants as five firefighters did not complete all 4 fires and were replaced.	24 firefighter students total. Six crews of 4 firefighter students. Each crew fought 1 training fire per day over 3 non-consecutive days. 10 instructors total (two groups of 5 instructors) who oversaw 3 training fires per day over 3 non-consecutive days.
Sampling	Spot urine collected pre-fire and 3-hr post fire.	For the firefighter students, spot urine collected pre-fire and 3-hr post fire. For the instructors, spot urine collected pre-fire and 3-hr post third fire (end of shift).
Total number of samples	Only urine samples collected from firefighters assigned to interior firefighting ("Attack" and "Search") were analyzed for VOC metabolites. 24 pre and 24 3-hr post fire urine samples were analyzed from both the attack and search firefighters.	Only urine samples collected during OSB and pallet and straw scenarios were analyzed for VOC metabolites. For students, 36 pre and 36 3-hr post fire urine samples were analyzed. For instructors,12 pre and 12 end-of-shift urine samples were analyzed.

conducted forcible entry and then searched for and rescued two simulated victims (75 kg manikins). Of the 36 firefighters included in the analysis (median age = 36), 32 were male and 4 were female.

For the training fire study, two instructors acted as stokers to light the fires and control ventilation for fire and smoke development, two instructors were assigned as company officers and supervised the attack team, and the remaining instructor was the officer in charge of the search and rescue team. Meanwhile, firefighter students undergoing the training performed either fire attack or search and rescue and performed very similar activities as the firefighters in the residential fire study, including hose advance and suppression and rescuing two simulated victims. Twenty-four firefighter students (median age = 40; 22 male and 2 female) and 10 instructors (median age 35; 9 male and 1 female) participated in this study.

All participants (firefighters, firefighter students, and instructors) wore National Fire Protection Association (NFPA) 1971-compliant protective ensembles (NFPA 2018), and NFPA 1981-compliant self-contained breathing apparatus (SCBA). The participants were required to wear SCBA (mask-up) before entering any of the fire structures. The SCBA masks were not removed until the fire was suppressed, and participants were upwind of the fire structures. The participants removed their turnout gear before reporting to the climate-controlled data collection areas. In the residential fire study, the attack and search firefighters continued to breath from their SCBA until they reached the data collection area, thus further minimizing the potential to inhale any airborne contaminants from the fires or off-gassing from their gear. In the training fire study, the participants removed their turnout gear before reporting to the climate-controlled data collection areas.

Participants cleaned their skin using commercial skin-cleansing wipes soon after entering the data collection area. All participants other than the instructors then subsequently (within about 30 min) showered and put on clean station gear. Instructors waited until the end of their shift (after the third fire of the day) to shower, which is common practice. After showering, all participants reported to a climate-controlled area of the research facility to await additional biological sample collection. Personal and area air sampling for VOCs was also conducted for both studies and reported previously (Fent et al. 2018, 2019a).

2.2. Urine sampling and analysis

Participants provided spot urine samples pre-firefighting. All participants other than the instructors also provided spot urine samples 3-hr post firefighting. For the instructors, urine samples were collected 3-hr after the last fire of the day (end-of-shift).

Specific gravity was measured in the field by using a manual hand-held specific gravity refractometer (Atago USA., Inc, Bellevue, WA). The manual refractometer was calibrated by placing 2 drops deionized room temperature (RT) distilled water on the faceplate and holding the refractometer to light and setting the indicator line to 0.000 on the specific gravity or UG scale. The specific gravity of urine (RT) was determined by reading the indicator line on the UG scale after similarly placing 2 drops of urine on the faceplate and holding the refractometer to light. Calibration was rechecked throughout and after reading urine samples.

The urine samples were then aliquoted into labeled tubes and stored on dry ice while in the field. Samples pending creatinine and cotinine analyses were then stored at −80 °C and those pending VOC metabolite analyses at −20 °C. Creatinine and cotinine analyses took place within a few months of the sample collections. The residential fire and training fire urine samples were stored for approximately 3 years and 2 years, respectively, before VOC metabolite analyses.

Creatinine was measured using a Vitros Autoanalyzer (Johnson & Johnson, New Brunswick, NJ) with a Vitros CREA slide. Cotinine, a metabolite of nicotine, was measured using Diagnostic Products Corporation (Siemens Corporation, Washington, DC) Immulite® 2000 analytical platform. Cotinine concentrations were used to confirm current non-tobacco use status of the participants and to quantify possible exposure to environmental tobacco smoke (ETS), which can be a source of VOC exposure (Jain 2015). The vast majority of urine samples (92% and 96% in the residential and training fire, respectively) had cotinine levels consistent with non-tobacco use status and no ETS exposure (<10 ng/mL).

The urine samples were analyzed for VOC metabolites using ultra-performance liquid chromatography-tandem mass spectrometry. Benzene metabolites (MUCA and PhMA) were measured using the analytical method by Bhandari et al. (2019). 2MHA, 3MHA+4MHA, MADA, 4HBeMA, 3HPMA, and BzMA were measured using a different method published by Alwis et al. (2012). Table 2 provides a list of the VOC metabolites that were measured and the associated parent compounds.

Urinary VOC metabolite concentrations (ng/mL) were corrected by creatinine (by unit conversion and division, resulting in units of μg/g creatinine in mg/dL urine) and by specific gravity using the following equation (described in Sauve et al., 2015):

$$C_{corr} = \frac{C_i(SG_{ref} - 1)}{(SG - 1)}$$

C_{corr} = Corrected concentration (μg/L urine)
C_i = Measured concentration of the biological indicator (μg/L urine)
SG_{ref} = Reference specific gravity value, here we used 1.016
SG = Specific gravity of the urine analyzed

2.3. Data analysis

Summary statistics were presented as mean, median, and range for VOC metabolite creatinine-adjusted concentrations, stratified by collection period (pre and 3-hr post-fire) and, for the training fire study, firefighter type (student and instructor). Median and 95th percentile urinary concentrations of VOC metabolites were calculated from the National Health and Nutrition Examination Survey (NHANES), a nationally representative sample of the U.S. general population, from 2013 to 2014 for individuals aged 18–55 years and stratified by smoking status. In calculating the descriptive statistics, non-detectable VOC metabolite concentrations below the limits of detection (LOD) were assigned values using the β-substitution method (Ganser and Hewett 2010) that adjusts each non-detectable value based on the uncensored data. Box and whisker plots with minimum, 25th percentile, median, 75th percentile, and maximum were performed for the detailed stratifications with respect to position (attack and search) or fuel type (Alpha OSB, Bravo OSB, and pallet and straw).

A mixed model that accounted for left censoring was fit using maximum likelihood estimation with individual firefighter as a random effect was utilized to account for the statistical correlation among

Table 2
VOC metabolites and associated parent compounds.

Acronym	Analyte	Detection Limit, in μg/L	Parent Compound
3HPMA	N-Acetyl-S-(3-hydroxypropyl)-L-cysteine	13	Acrolein
MUCA	trans, trans-Muconic acid	9.81	Benzene
PhMA	N-Acetyl-S-(phenyl)-L-cysteine	0.6	Benzene
MADA	Mandelic acid	12	Styrene
BzMA	N-Acetyl-S-(benzyl)-L-cysteine	0.5	Toluene or benzyl alcohol
3MHA+4MHA	3-Methylhippuric acid + 4-Methylhippuric acid	8	Xylenes
2MHA	2-Methylhippuric acid	5	Xylenes
4HBeMA	N-Acetyl-S-(4-hydroxy-2-buten-1-yl)-L-cysteine	0.6	1,3-Butadiene

repeated measures from the same firefighter and to examine whether the change in urinary VOC metabolites between pre-firefighting and 3-hr post firefighting was significantly different from zero in the residential fire study and by firefighter type in the training fire study (Jin et al., 2011). A median regression model for skewed exposure data with repeated measures and non-detects, incorporating an exchangeable working correlation structure, was used to investigate whether the median in post-fire urinary VOC metabolites among firefighter types was significantly different from zero (Chen et al., 2021). All tests were two-sided at the 0.05 significance level. Statistical analyses were conducted in SAS version 9.4 (SAS Institute, Cary, NC).

3. Results

3.1. Urinary VOC metabolites for firefighters participating in the residential fire study

Table 3 summarizes creatinine-adjusted VOC metabolite concentrations for 24 firefighters participating in the residential fire study assigned to fire attack and search and rescue. The VOC metabolites that increased significantly from pre- to post-fire are summarized in Fig. 1 and include 2-Methylhippuric acid (2MHA), N-Acetyl-S-(benzyl)-L-cysteine (BzMA), and mandelic acid (MADA) (p-values 0.005, <0.001, and <0.001, respectively). Benzene metabolites trans, trans-Muconic acid (MUCA) and N-Acetyl-S-(phenyl)-L-cysteine (PhMA) did increase from pre- to post-fire, but that increase was not significant.

Compared to the general population (Table 3), median post-fire BzMA levels (7.23 μg/g creatinine) were higher than median concentrations for general population smokers (6.06 μg/g creatinine) and non-smokers (6.32 μg/g creatinine). Pre-fire MADA median concentrations (122 μg/g creatinine) were similar to median non-smoker general population concentrations (116 μg/g creatinine), while median post-fire MADA concentrations (177 μg/g creatinine) were elevated to near median levels reported in general population smokers (182 μg/g creatinine).

VOC metabolite concentrations adjusted for specific gravity for the same firefighters are provided in Supplemental Materials (Table S1). Overall, specific gravity-adjusted VOC metabolite concentrations were similar to the creatinine-adjusted results, as 2MHA, BzMA, and MADA increased significantly from pre- to post-fire.

3.2. Urinary VOC metabolites for firefighter students and instructors participating in the training fire study

Creatinine-adjusted VOC metabolite concentrations for training fire participants stratified by participant type (firefighter students vs. instructors) are summarized in Table 4. MADA concentrations increased significantly from pre- to post-fire for both firefighter students and instructors (p-values <0.001 and < 0.0001, respectively). Concentrations of benzene metabolites including MUCA and PhMA also increased significantly from pre- to post-fire for instructors (p-values 0.038 and 0.016, respectively), while 3-methylhippuric acid + 4-methylhippuric acid (3MHA+4MHA) and N-acetyl-S-(3-hydroxypropyl)-L-cysteine (3HPMA) increased significantly for firefighter students (p-values 0.011). On the other hand, 4HBeMA and MUCA results significantly decreased for firefighter students (p-values 0.008 and 0.015, respectively).

Fig. 2 shows creatinine-adjusted results for metabolites that increased significantly from pre- to post-fire, stratified by fuel type. Post-fire MUCA concentrations were significantly elevated for instructors responding to Alpha OSB scenarios. 3HPMA and MADA concentrations, on the other hand, were elevated for firefighter students and instructors responding to Bravo OSB scenarios. Post-fire PhMA concentrations were elevated for both Alpha and Bravo OSB scenarios.

Median post-fire MADA concentrations for both firefighter students (227 μg/g creatinine) and instructors (356 μg/g creatinine) were higher than the median smoker (182 μg/g creatinine) and non-smoker (116 μg/g creatinine) general population concentrations (Table 4). In fact, median post-fire MADA concentrations for instructors were higher than the 95th percentile of the non-smoking general population (299 μg/g creatinine). Median post-fire BzMA concentrations for firefighter students (8.65 μg/g creatinine) and instructors (9.31 μg/g creatinine) were also above median smoker (6.06 μg/g creatinine) and non-smoker (6.32 μg/g creatinine) general population levels. Median post-fire 3HPMA concentrations for firefighter students (211 μg/g creatinine) and instructors (322 μg/g creatinine) were well above median concentrations for the non-smoking general population (174 μg/g creatinine) but below levels found in smokers (508 μg/g creatinine). The two highest post-shift concentrations of MUCA measured from instructors (750 and 631 μg/g) exceeded the American Conference of Governmental Industrial Hygienists (ACGIH) biological exposure indices (BEI) of 500 μg/g creatinine (ACGIH, 2021). It's important to note that instructors completed

Table 3
VOC metabolite results (μg/g creatinine) for the residential fire participants (attack and search firefighters).

Analyte	Collection Period	N (N of non-detects)	Mean	Median	Min-Max	P-value[A]	General Population (Non-Smoker/Smoker)[B]		Biological Exposure indices (BEI)[C]
							Median	95th Percentile	
3HPMA	Pre	48 (0)	207	182	68.1–739	0.871	175/508	835/2,579	Not Available
	3 h	48 (0)	209	196	92.2–665				
MUCA	Pre	48 (5)	46.9	27.3	14.1–259	0.257	Not Available	Not Available	500
	3 h	48 (10)	53.4	38.4	20.1–266				
PhMA	Pre	48 (39)	0.04	0.01	0.00–0.37	0.078	Not Available	Not Available	25
	3 h	48 (38)	0.07	0.02	0.00–0.75				
MADA	Pre	48 (0)	129	122	56.5–235	**<0.001**	116/182	299/600	400,000
	3 h	48 (0)	184	177	80.9–526				
BzMA	Pre	48 (0)	7.07	5.68	2.01–36.5	**<0.001**	6.32/6.06	38.9/33.1	Not Available
	3 h	48 (0)	8.85	7.23	2.53–29.3				
3MHA+4MHA	Pre	48 (0)	145	84.7	23.4–1,550	0.498	149/488	872/2,026	1,500,000
	3 h	48 (0)	158	134	46.1–1,130				
2MHA	Pre	48 (2)	29.1	19.2	2.42–157	**0.005**	26.4/68.5	141/354	1,500,000
	3 h	48 (1)	44.0	40.4	12.7–105				
4HBeMA	Pre	48 (0)	5.41	5.32	1.77–10.7	0.136	4.02/15.5	14.9/87.9	Not Available
	3 h	48 (3)	4.83	4.64	2.01–7.65				

A. Test of significant mean difference between pre and 3-hr post-fire concentrations.
B. National Health and Nutrition Examination Survey (NHANES) (2018). 2013–2014 data documentation, codebook, and frequencies. Volatile Organic Compounds & Metabolites - Urine (UVOC_H). Available at https://wwwn.cdc.gov/Nchs/Nhanes/2013-2014/UVOC_H.htm.
C. American Conference of Governmental Industrial Hygienists (ACGIH) (2021). "2021 TLVs and BEIs with 9th Edition Documentation". Cincinnati, OH: ACGIH.

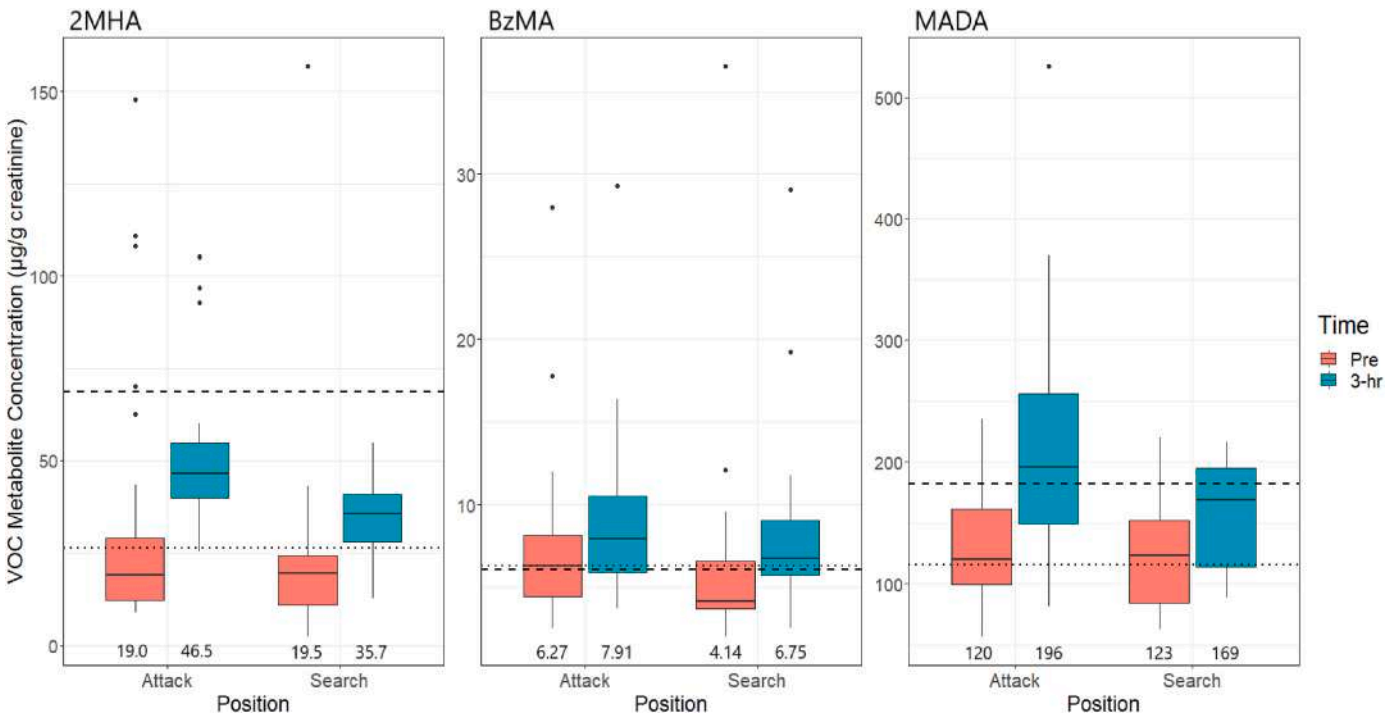

Fig. 1. Summary of urinary VOC metabolites (µg/g creatinine) with medians included at the bottom of each plot for the residential fire study participants stratified by position for analytes that increase significantly post-fire. The number of firefighters in each position (attack and search) is 24. Dashed and dotted lines represent VOC medians of smoking and non-smoking general population, respectively.

three fire exercises each day while firefighter students completed one.

VOC metabolite concentrations adjusted for specific gravity for the firefighter students and instructors are in Supplemental Materials (Table S2). Like the results from the residential fire study, the specific gravity-adjusted VOC metabolite concentrations for firefighter students and instructors were similar to the creatinine-adjusted results. Specifically, MADA concentrations increased significantly from pre- to post-fire for both firefighter students and instructors (p-values <0.001 and 0.011). 3MHA+4MHA and 3HPMA increased significantly for firefighter students (p-values 0.039 and 0.022), while MUCA and PhMA concentrations increased significantly from pre- to post-fire for instructors (p-values 0.037 and 0.016).

4. Discussion

Although many studies have documented firefighters' airborne exposures to VOCs, to our knowledge, this is the first study to quantify the biological uptake of numerous types of VOCs measured as metabolites in urine following live fire suppression or training. Exposures to combustion byproducts will vary due to a variety of factors, including the materials in and orientation of products being burned, the duration of the fires, and the ventilation conditions. Nevertheless, the live fires described in this paper represent fire conditions that are likely to be encountered during responses to residential fires that involve household furnishings and during typical types of live-fire training scenarios.

4.1. Urinary VOC metabolites for firefighters participating in the residential fire study

The urinary VOC metabolite results from the residential fire study indicate that firefighters assigned to attack and search absorbed xylenes (2MHA), toluene (or benzyl alcohol) (BzMA), and styrene (MADA) during the live-fire scenarios. The metabolites of these compounds were all significantly elevated in the 3-hr post fire urine samples. The metabolites of benzene (MUCA and PhMA) were not significantly elevated. Benzene is often one of the most abundant combustion byproducts produced during fires (Jankovic et al., 1991; Austin et al., 2001b), which was the case for this study (Fent et al., 2018). Studies have also reported that firefighters can have significant increases of benzene in breath and benzene metabolites in urine post firefighting (Caux et al., 2002; Laitinen et al., 2010; Fent et al., 2020). In fact, firefighters in this study had significant increases of exhaled breath concentrations of benzene after firefighting (Fent et al., 2019b). While the post-fire increase of the benzene metabolites in urine did not meet the level of significance, it does suggest that some biological absorption took place.

Although typically not present in as high of concentrations as benzene, styrene and toluene are also commonly produced during structure fires. For example, when we measured the personal air concentrations of VOCs for attack firefighters during the residential fire study, we found median levels of benzene, styrene, and toluene of 40, 2.7, and 2.4 ppm, respectively. For search firefighters, we found similar median air concentrations (38, 2.6, 2.6 ppm, respectively) (Fent et al., 2018). When we stratified the urinary concentrations by job assignment (Fig. 1), we found a larger increase in MADA concentrations for attack firefighters than search firefighters, suggesting absorption of styrene might be higher for those assigned to attack even though personal air concentrations of styrene were similar.

4.2. Urinary VOC metabolites for firefighter students and instructors participating in the training fire study

For the training fire study, we found differences among the urinary VOC metabolites between firefighter students and instructors. This is not unexpected, because the instructors participated in three training exercises per day and their post-fire urines were collected at the end of their shift (~9 h after first scenario and ~3 h after last scenario). In comparison, the firefighter students only participated in one training scenario per day and had their post-fire urine samples collected 3-h after that single scenario. Hence, the timing of specimen collection and duration of the exposures differ between these two groups. Additionally,

Table 4
VOC metabolite results (μg/g creatinine) for the training fire participants, stratified by participant type.

Analyte	Firefighter Type	Collection Period	N (N of non-detects)	Mean	Median	Min-Max	P-value	General Population[A] (Non-Smoker/Smoker)		Biological Exposure indices (BEI)[B]
								Median	95th Percentile	
3HPMA	Firefighter	Pre	36 (0)	172	146	92.7–403	**0.011**[C]	175/508	835/2,579	Not Available
	Student	3 h	36 (0)	342	211	96.3–1660				
	Instructor	Pre	12 (0)	231	168	97.7–764	0.052			
		3 h	12 (0)	439	322	179–1230				
MUCA	Firefighter	Pre	36 (7)	74.0	34.0	8.03–499	**0.015**[D]	Not Available	Not Available	500
	Student	3 h	36 (11)	42.8	28.6	14.9–222				
	Instructor	Pre	12 (3)	28.7	27.4	13.6–49.9	**0.038**[C]			
		3 h	12 (1)	186	74.2	29.9–750				
PhMA	Firefighter	Pre	36 (33)	0.03	0.01	0.00–0.38	0.121	Not Available	Not Available	25
	Student	3 h	36 (31)	0.05	0.01	0.00–0.44				
	Instructor	Pre	12 (11)	0.02	0.01	0.00–0.16	**0.016**[C]			
		3 h	12 (4)	0.22	0.24	0.01–0.51				
MADA	Firefighter	Pre	35 (0)	141	134	48.7–259	**<0.001**[C]	116/182	299/600	400,000
	Student	3 h	36 (0)	244	227	124–549				
	Instructor	Pre	12 (0)	114	97.2	51.2–178	**<0.001**[C]			
		3 h	12 (0)	393	356	188–873				
BzMA	Firefighter	Pre	36 (1)	9.02	5.85	1.26–36.3	0.634	6.32/6.06	38.9/33.1	Not Available
	Student	3 h	36 (0)	9.68	8.65	0.77–28.7				
	Instructor	Pre	12 (0)	5.47	5.01	2.38–10.3	0.080			
		3 h	12 (0)	20.6	9.31	4.32–94.6				
3MHA+ 4MHA	Firefighter	Pre	36 (0)	100	77.4	54.0–293	**0.002**[C]	149/488	872/2,026	1,500,000
	Student	3 h	36 (0)	133	104	69.1–298				
	Instructor	Pre	12 (0)	117	89.5	60.7–377	0.619			
		3 h	12 (0)	131	121	59.4–271				
2MHA	Firefighter	Pre	36 (8)	21.2	19.0	5.50–69.9	0.106	26.4/68.5	141/354	1,500,000
	Student	3 h	36 (8)	26.7	18.9	9.33–92.7				
	Instructor	Pre	12 (0)	23.8	15.8	4.43–101	0.227			
		3 h	12 (0)	33.7	31.9	14.7–63.5				
4HBeMA	Firefighter	Pre	36 (1)	6.65	6.24	1.52–13.7	**0.008**[D]	4.02/15.5	14.9/87.9	Not Available
	Student	3 h	36 (2)	5.25	4.40	1.61–13.5				
	Instructor	Pre	12 (0)	5.22	5.16	2.68–7.74	0.292			
		3 h	12 (0)	4.62	4.66	1.81–7.35				

A. National Health and Nutrition Examination Survey (NHANES) (2018). 2013–2014 data documentation, codebook, and frequencies. Volatile Organic Compounds & Metabolites - Urine (UVOC_H). Available at https://wwwn.cdc.gov/Nchs/Nhanes/2013-2014/UVOC_H.htm.
B. American Conference of Governmental Industrial Hygienists (ACGIH) (2021). "2021 TLVs and BEIs with 9th Edition Documentation". Cincinnati, OH: ACGIH.
C. 3-hr post-fire mean concentrations were significantly higher than pre-fire mean concentrations.
D. 3-hr post-fire mean concentrations were significantly lower than pre-fire mean concentrations.

instructors did not doff their gear and shower until after the third training exercise each day, potentially contributing to their exposure relative to firefighter students. This design is consistent with real-life scenarios where instructors often participate in multiple training exercises in a day. This difference between instructors and students might also help elucidate the differences among the VOC metabolites that were measured.

For the firefighter students, we saw significant increases in metabolites of xylenes (2MHA), acrolein (3HPMA), and styrene (MADA). For the instructors, we saw significant increases in metabolites of benzene (MUCA, PhMA) and styrene (MADA). When comparing the level of increase for the metabolite of styrene (MADA, the only metabolite found to increase significantly in both groups), we found that instructors had a significantly larger post-fire increase than firefighter students (p-value <0.001), most likely due to their repeated exposures. For the metabolites of xylenes and acrolein, the inconsistent findings of significance between firefighter students and instructors may have more to do with lower statistical power for the instructors (i.e., study included three times the number of firefighter students compared to instructors). Fig. 2 provides some visual evidence that the firefighter students and instructors had similar trends in the excretion patterns of these metabolites despite different post-fire collection periods. Still, because firefighter students had increased urinary levels of 3MHA+4MHA and 3HPMA after just one fire, we might expect an even bigger increase in these metabolites among instructors after three consecutive fires than what we found.

However, there are numerous factors that may influence the urinary VOC metabolite levels, including different activities and proximity to the fires between instructors and firefighter students (i.e., students were generally closer to the seat of the fires), as well as the rate of metabolism after absorption of the parent compounds and differences among the analyte elimination half-lives. These factors are further complicated by the different routes of exposure (inhalation and dermal).

We previously showed a statistically significant post-fire increase in exhaled breath concentrations of benzene among the participants of both studies, indicating some absorption of benzene (Fent et al. 2019b, 2020). However, for benzene or the other VOCs to be measured in urine after absorption into the bloodstream, they must first be metabolized and then excreted in urine. The elimination half-lives of the mercapturic acid metabolites of acrolein and benzene have been estimated at ~9 h, the elimination half-life of MUCA has been estimated at ~5 h, and it is safe to assume that all the VOC metabolites in this study have elimination half-lives of several hours (van Sittert et al., 1993, Boogard and van Sittert, 1995, Watzek et al., 2012; St Helen et al., 2020). That post-fire benzene metabolites were not elevated in the firefighter students' urine but were elevated in the instructors' urine may suggest that not enough time had transpired after benzene exposure to show up as metabolites in the students' urine. In fact, the post-shift urine sample collected from the instructors might only be reflecting the peak excretion from exposures encountered during the first scenario that day, even for MUCA that theoretically would be eliminated faster than PhMA.

Another factor that may impact the urinary levels of VOCs is the type and orientation of fuel that was burned and the structure layout that created the training fire environment. For the training fire study, we

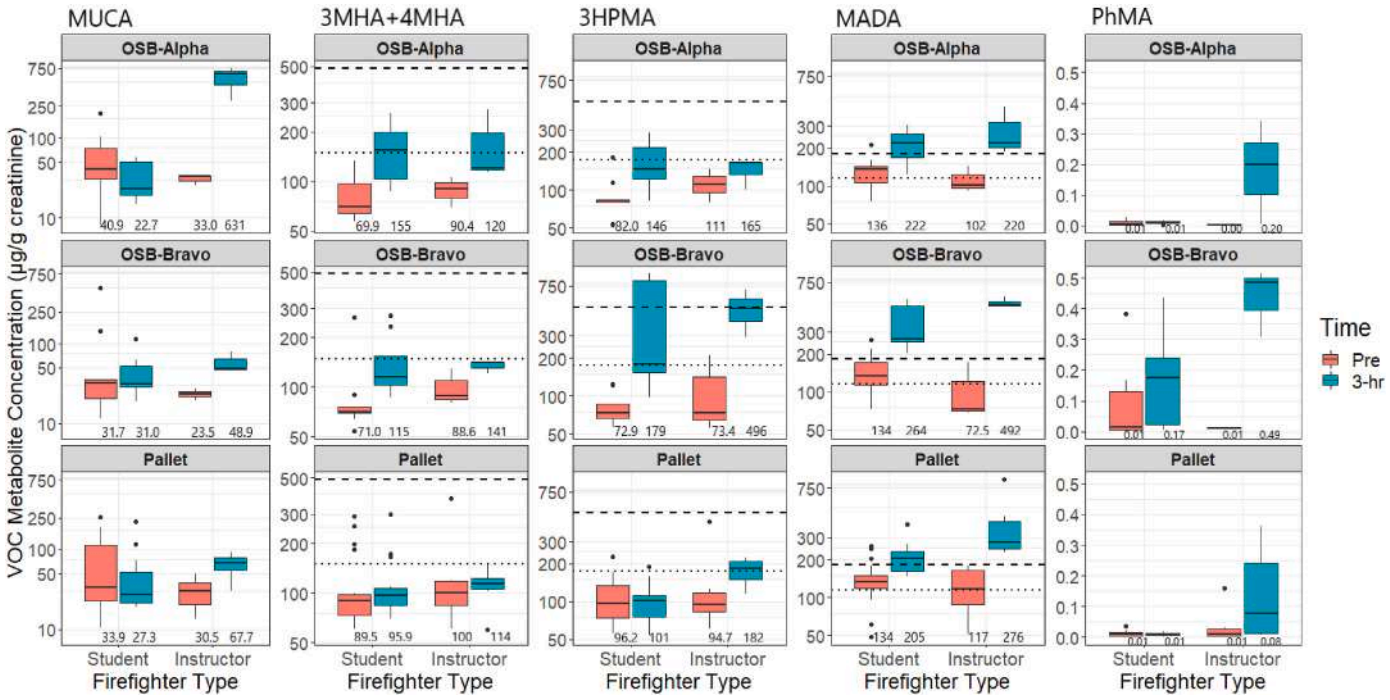

Fig. 2. Summary of urinary VOC metabolites (µg/g creatinine) with medians included at bottom of each plot for the training fire participants stratified by participant type and fuel type for the analytes that increase significantly post-fire. The number of firefighter students for OSB-Alpha, OSB-Bravo, and Pallet are 9, 9, and 18, respectively, and the number of instructors is 3, 3, and 6. Dashed and dotted lines represent VOC medians of smoking and non-smoking general population, respectively.

were able to compare the urinary metabolites measured after training scenarios involving two types of OSB (in a metal container with some fuels on the ceiling) and pallet and straw (in a concrete structure with fuel located in a hopper, slightly elevated off the floor) (Fig. 2). Instructors' post-fire levels of MUCA (metabolite of benzene) were most elevated during the Alpha OSB scenario; conversely, 3HPMA (acrolein) and MADA (styrene) were most elevated during the Bravo OSB scenario. The urinary benzene metabolite findings are surprising because personal air concentrations of benzene were far higher during the Bravo OSB scenario (median = 20 ppm) than the Alpha OSB scenario (median = 5.8 ppm) or the pallet and straw scenario (median = 3.9 ppm) (Fent et al., 2019a). Hence, we would have expected instructors overseeing the Bravo OSB scenarios to have the highest post-shift urinary benzene metabolite concentrations. Styrene air concentrations were also higher during the Bravo OSB scenario (median = 2.3 ppm) compared to the Alpha OSB (median = 0.79 ppm) or pallet and straw scenarios (median = 0.39 ppm) (Fent et al., 2019a), which was consistent with the instructors' MADA urinary results reported here. Additionally, area air sampling from this study found acrolein concentrations during Bravo OSB scenarios (median- 60.6 mg/m^3) were significantly higher than Alpha OSB (median = 4.85 mg/m^3) and pallet and straw scenarios (median = 5.38 mg/m^3), which is consistent with the 3HPMA results in the current study.

4.3. Comparison of urinary VOC metabolites between residential fire and training fire participants

The burning materials for the residential fire study included a variety of furnishings and synthetic materials (e.g., stuffed chair, innerspring mattress with foam topper, carpet, and padding, etc.). Thus, we might expect more pronounced increases in urinary VOC metabolites for firefighters in that study compared to firefighter students in the training fire study. Post-fire urine was collected 3 h after the burns in both studies, so comparisons can be made. When we compared the 3-hr post-fire median concentrations between the firefighters and firefighter students for the urinary metabolites that increased significantly in at least one of the study populations (i.e., 2MHA, 3MHA+4MHA, 3HPMA, BZMA, and MADA), we found that firefighters had significantly higher 2MHA, 3MHA+4MHA, and MADA concentrations compared to firefighter students.

Much of the increase in urinary metabolites that were observed in the training fire study can be attributed to the OSB scenarios (Fig. 2). We previously published findings that showed significant and more pronounced increases in urinary metabolites of PAHs in firefighter students after the OSB training fires than the pallet and straw fires (Fent et al., 2019b). In fact, the students' median 3-hr post-fire concentration of 1-hydroxypyrene following the Bravo-OSB scenario (0.78 µg/g) was near what we measured 3-hr post fire from firefighters in the residential fire study (median = 0.81 µg/g) (Fent et al. 2019b, 2020). As such, the use of OSB in the upper layer of training fires should be minimized when it is possible to achieve the same training objective with pallet and straw in a more well ventilated configuration.

It appears that some types of training fires can result in VOC exposures that are on par with those experienced during residential fires involving a variety of synthetic materials. This is an especially important finding for instructors who may participate in several training fires per day. The instructors in our study participated in three consecutive training fires each day and had post-shift concentrations of MUCA (metabolite of benzene) that were significantly higher than the students who only participated in one fire. As previously published, the instructors' repeated exposures also resulted in concentrations of 1-hydroxypyrene well above the levels measured from students in the same study or from firefighters in the residential fire study (Fent et al. 2019b, 2020). While we did not (and cannot) measure exposure to all possible combustion byproducts, many of the parent compounds that were quantified as metabolites (e.g., benzene, styrene, acrolein) are known and probable carcinogens (IARC, 2012a, IARC 2019; IARC 2021), and exposures should be reduced as much as possible.

The firefighters, firefighter students, and instructors in both studies were instructed to wear SCBA throughout the duration of firefighting

operations that occurred inside of the structures. Still, some inhalation exposure likely occurred; for example, after responding to the structure prior to donning SCBA or when the firefighters were doffing their contaminated gear, which has been shown to off-gas VOCs for several minutes after firefighting (Fent et al., 2015). It is even possible that instructors who oversaw the Alpha OSB scenarios (compared to the Bravo OSB scenarios) were not as careful about wearing SCBA throughout the entire training exercises and had more inhalation exposure to benzene (hence higher levels of MUCA). Another possibility is that the Alpha OSB instructors unintentionally had looser fitting gear, which could have resulted in more skin exposure to benzene. However, all three groups wore identical PPE that was similarly fit based on participants chest and waist size.

We have previously shown using mannequins that turnout gear provides very little attenuation (<43%) against the ingress of benzene vapors (Mayer et al., 2020, 2022). Previous research has also shown that small amounts of benzene vapor (<1%) can be absorbed dermally, and that skin absorption increases with increasing temperature and humidity (Franz and McFarland, 1984, EPA 1992; Thrall et al., 2000). Dermal absorption, in addition to inhalation, likely contributed to the urinary levels that were measured at least for some of the VOCs. Skin permeation coefficients can be used to estimate the potential for dermal absorption. According to the CDC skin permeation calculator and the Frasch Model (Frasch 2002), xylenes, styrene, and toluene have similar skin permeation coefficients (6.3 E^{-2}, 5.8 E^{-2}, and 5.6 E^{-2} cm/h), followed by benzene (3.4 E^{-2} cm/h) and then acrolein (4.0 E^{-4} cm/h). The ability for acrolein to be absorbed transdermally is estimated to be very low compared to the other compounds (which are not especially high themselves). That urinary 3HPMA (acrolein) was elevated ~3 h after firefighting for the training firefighter students provides evidence of the inhalation route of absorption. Urinary 3HPMA did not increase in the 3-hr collection for the residential fire study participants. However, in the residential fire study, we instructed the attack and search firefighters to breath from their SCBA until right before breath collection (Fent et al., 2020), which was not required for the training fire participants. The fact that we still found increased urinary metabolites of some VOCs post-firefighting among the residential fire study participants, as well as increased exhaled breath concentrations of benzene (Fent et al., 2020), indicates contribution from the dermal route of absorption. Both routes likely contributed to the biological levels we found, but the inhalation route was probably more predominant in the training fire study. This finding is further evidence of the importance of wearing SCBA throughout the response.

4.4. Comparison of urinary VOC concentrations among participants of the two studies to general U.S. Population and BEI concentrations

To put the biological levels (i.e., urinary concentrations) into perspective, we provided comparisons to the general population levels for smokers and non-smokers. Note that, according to the cotinine analysis, nearly all of the firefighters in these two studies were not exposed to tobacco smoke (94%). The majority of the VOC metabolites that were found to be significantly elevated post-fire were measured at levels between the median non-smoking and median smoking general population levels (where the data exist). The exceptions to this were for MADA (styrene) and BzMA (toluene). The post-fire median MADA and BzMA concentrations for firefighter students and instructors who participated in the training fire study (as well as the residential firefighters' post-fire BzMA concentrations) exceeded the smoking general population levels.

Both firefighter students and instructors had median post-fire 3HPMA concentrations that were well above the non-smoking general population levels after having median pre-fire 3HPMA concentrations that were near or below median non-smoking general population levels (Table 4). When we further stratified the training fire study participants by type of fuel, we found that instructors participating in Bravo OSB scenarios had median post-shift urinary 3HPMA (acrolein) concentrations well above smoking general population levels (Fig. 2).

NHANES has not yet reported general population levels of MUCA or PhMA measured using the current assay. However, exposure studies have measured urinary MUCA in firefighters and other workers. Laitinen et al., (2010) measured urinary MUCA in fire instructors following three training fires in a day and reported concentrations as molar volume, thereby complicating our comparisons. However, assuming urinary creatinine of 200 mg/dL (middle of the normal range), the mean post-fire urinary levels following training fires involving plywood as fuel was ~100 μg/g creatinine (with pre-exposure ~40 μg/g creatinine). The mean post-fire levels we measured from instructors across all types of live-fire training (186 μg/g creatinine) was greater than this, and for Alpha OSB (558 μg/g creatinine), it was substantially greater. A study of gas station workers in Brazil measured mean post-shift MUCA concentrations of 204 μg/g creatine (Geraldino et al., 2020), which is within the range of post-shift concentrations we measured for instructors across all types of training and substantially lower than what we found for instructors following the Alpha OSB training.

Comparisons were also made to any applicable ACGIH BEIs. Although median urinary VOC metabolite concentrations were below all applicable BEIs, two post-shift urine samples collected from fire instructors had concentrations of MUCA that exceeded the BEI. This suggests that the amount of benzene absorbed for a few of the participants were above the levels ACGIH considers to be acceptable for safe working conditions. What is most striking about this finding is that the actual exposure or fire periods were relatively short (most fire responses were 10–15 min in duration), and participants were generally very good about wearing their SCBA inside and even outside the structure. This warrants further investigation into firefighters' VOC metabolite concentrations after longer duration emergency fires where SCBA usage may not be as tightly monitored.

Often biomarker exposure studies correct for hydration status by using creatinine (hence the comparisons made in this paper). However, specific gravity of urine can also be used and has even been suggested by some researchers to be the preferred approach because creatinine may be more affected by physiological differences in the study population (Suwazono et al., 2005; Sauve et al., 2015). We have provided specific gravity corrected urinary metabolite concentrations in the supplemental materials. In general, the results did not differ in that significant pre- to post-fire differences observed using creatinine correction were also observed using specific gravity correction. It is important to note that the participants in both the residential fire and training fire studies were strongly encouraged to hydrate both before and after the fires. As a result, most of the participants were well hydrated, especially at the 3-hr or post-shift urine collections.

The studies in this paper are not without limitations. Other sources of VOCs may have been present at the fire training institute; although, we attempted to control these exposures by keeping participants in a climate-controlled facility following the fire scenarios. By collecting only one post-fire urine sample (3 h after firefighting) for the firefighters and firefighter students, we may not have captured the peak excretion period for some VOCs. Our urine sampling regimen (3 h after the last of three fires) may have even underestimated the cumulative exposures for the fire instructors. However, a recent study found that firefighter instructors' peak PMA and HPMA urinary concentrations occurred 3 h after the training exercise (Rossbach et al., 2022). The possibility also exists that non-occupational factors like diet (e.g., sorbic acid in food) and application of beauty of products (e.g., benzyl alcohol in cosmetics) could impact the urinary concentrations reported here (e.g., MUCA and BzMA, respectively). However, participants were provided the same breakfast and lunch during study days and thus differences in urinary concentrations of MUCA by job title were unlikely due to ingestion of sorbic acid.

Another potential limitation is that the residential and training fire urine samples were stored for 3 years and 2 years, respectively, prior to

the VOC metabolite analysis. However, because the samples were kept at a temperature well below freezing (−20 °C), we do not expect the extended storage time to greatly impact the results reported here. Additionally, we did not analyze urine for metabolites of some other hazardous compounds (e.g., acetaldehyde, formaldehyde, isocyanates) that were measured in area air samples taken during the training fires (Fent et al., 2019a). Future exposure studies of firefighters are warranted, including quantifying urinary metabolite concentrations of hazardous combustion products not analyzed here while also validating the findings from this study.

5. Conclusions

We have reported and compared the urinary VOC metabolite concentrations among firefighter participants from two separate studies focusing on residential fire environments and training fire environments. The participants in both studies were instructed to wear SCBA throughout the response and to not remove their SCBA until the fire was suppressed and they were upwind of the fires. Some participants (i.e., attack and search firefighters in the residential fire study) continued to breathe from their SCBA until they reached the climate-controlled biological collection area. It is unlikely that firefighters would maintain such stringent SCBA protocols under normal circumstances. Still, we found post-fire urine metabolite levels of a variety of VOCs increased from pre-fire levels. In some cases, the post-fire VOC metabolite concentrations were above smoking general population levels. Because many of the VOC metabolites have elimination half-lives of several hours, it is likely that we did not capture the peak excretion levels. Some of the compounds that were significantly elevated post-firefighting are metabolites of known or probable carcinogens (benzene, styrene, acrolein) and exposure to them should be eliminated or reduced as much as possible through the hierarchy of controls (NIOSH et al., 2016). Further research is needed to better understand the exposure pathways for these compounds (dermal vs. inhalation) and interventions that can be implemented to reduce biological uptake. These results suggest firefighters should wear SCBA on scene, especially in the presence of smoke or combustion products, and remove contaminated gear and clean skin as soon as possible post-fire to further reduce their exposures.

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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.2022.113969.

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High burden and diversity of carbapenemase-producing *Enterobacterales* observed in wastewater of a tertiary care hospital in Germany

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ABSTRACT

Hospitals are one of the main reservoirs of multi-resistant *Enterobacterales* (MRE). As MRE are resistant to the most frequently used antibiotics, therapy for patients with MRE infections is challenging. It has been previously described that MRE from hospital wastewater can pass into municipal wastewater and even surface water. In this study, we investigated the diversity and epidemiology of MRE in the wastewater of a large tertiary care hospital. Wastewater samples were collected for a four-day period and tested for the presence of *Enterobacterales* resistant to 3rd gen. cephalosporins. Representative isolates were further characterized by whole genome sequencing. In 120 β-glucuronidase-producing isolates, 68 *Escherichia coli* and, interestingly, also 52 *Citrobacter freundii* were identified. In 120 β-glucosidase-producing isolates 45 *Serratia marcescens*, 34 *Klebsiella oxytoca*, 32 *Enterobacter cloacae* and 9 *Klebsiella pneumoniae* were observed. For all species various MLST sequence types and different clusters of resistance genes were determined, showing a great diversity within the different *Enterobacterales*, further corroborated by clonal analysis performed by cgMLST. The most prominent clone was wastewater associated *E. coli* ST635, which accounted for 47.1% of all *E. coli* isolates. Interestingly, 45.6% of *E. coli*, 88.5% of *C. freundii*, 95.6% of *S. marcescens*, 91.2% of *K. oxytoca*, 96.9% of *E. cloacae* and 88.9% of *K. pneumoniae* isolates carried a carbapenemase gene, indicating a high burden with carbapenemase-producing *Enterobacterales*. Comparison with clinical isolates from the same hospital displayed few clonal matches. One wastewater isolate of *K. pneumoniae* was identified to be closely related compared to a clone that had been introduced into the hospital during an outbreak four years earlier. One *E. coli* isolate was identified as identical to an isolate from a patient, with inpatient stay during the sampling period. The data obtained in this study highlight the problem of antibiotic resistance of *Enterobacterales* in hospital wastewater. In particular, the clustered occurrence of carbapenemase genes is of great concern and underscores the problem of increasingly scarce antibiotic options against these bacteria.

1. Introduction

Multidrug-resistant *Enterobacterales* have become more important in the last years. Especially since these bacteria continuously develop strategies to become resistant to frequently used antibiotics, they represent a serious health risk (Davido et al., 2018). Next to 3rd generation cephalosporin-resistant *Enterobacterales* (3GCRE), carbapenem-resistant (CRE) and carbapenemase-producing *Enterobacterales* (CPE) are of particular concern in healthcare (Elsa et al., 2020; Hoffman et al., 2021), since therapeutic options are significantly limited and last resort antibiotics are not used in the first line in the calculated therapy of even life-threatening infections. Such isolates can also spread into the community from healthcare settings (Kelly et al., 2017). Infections caused by CRE can be community- as well as hospital-acquired and are associated with significant mortality up to 72% (Borer et al., 2009; Hirsch and Tam, 2010).

A simple and effective way to detect multi-resistant bacteria in environmental or clinical samples is growth on selective media. Selective chromogenic media can achieve high sensitivity to 3GCRE (Göttig et al., 2020) and simultaneously a simple separation of clinically

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relevant *Enterobacterales* from other bacteria by chromogenic reaction for β-glucuronidase and β-glucosidase-producing *Enterobacterales*. The presence of β-glucuronidase is associated with *E. coli*, whereas genera within the KESC group (*Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter*) are characterized by β-glucosidase production (Huang et al., 2010; McLellan et al., 2001).

Within the carbapenemases in *Enterobacterales* the most important as well as clinically relevant ones are KPC, OXA-48, NDM, VIM and IMP, with worldwide variances between different regions (Bonnin et al., 2021; Cantón et al., 2020; Lasko and Nicolau, 2020; Tan et al., 2021). The most prominent carbapenemase-producing *Enterobacterales* in Germany are *K. pneumoniae*, *E. coli* and *E. cloacae* (Mischnik et al., 2015; Pfennigwerth and Schauer, 2017). Of all species, carbapenem-resistant *K. pneumoniae* is the most clinically relevant representative of CRE and has been highlighted as the fastest-growing resistance threat in Europe (David et al., 2020). The predominant carbapenemases in Europe in the different species vary: In *K. pneumoniae* the most prominent carbapenemase is OXA-48 (43%), followed by KPC-2 (23%) and KPC-3 (23%). In *E. coli* it is also OXA-48 (44%), but followed by VIM-1 (28%), whereas in *E. cloacae* VIM-1 (71%) is the predominant carbapenemase (Cantón et al., 2012; David et al., 2019). In Germany, the carbapenemase OXA-48 is particularly common among all *Enterobacterales*. Carbapenemase genes are often located on plasmids in the bacteria and can be transferred between different strains of *Enterobacterales*. One plasmid can carry multiple resistance genes at once (Kopotsa et al., 2019).

Carbapenemases can belong to class A, B, or D of the Ambler classification system of β-lactamases, depending on their molecular structure. Class A includes KPC, GES, IMI, NMC-A, SME and SFC, of which KPCs are the most abundant in *Enterobacterales* worldwide (Logan and Weinstein, 2017). KPC has been originally reported in *K. pneumoniae*, but later also in other *Enterobacterales* and *P. aeruginosa* (Bassetti and Peghin, 2020). Class B β-lactamases are metallo-β-lactamases (MBLs), including IMP, VIM and NDM. Class D are OXA β-lactamases that can be divided into *A. baumannii*-derived OXA-β-lactamases (Evans and Amyes, 2014; Logan and Weinstein, 2017) and *Shewanella*-derived OXA-48-type carbapenemases (Tacão et al., 2018). Not all β-lactamases of the OXA family are carbapenemases as well. Carbapenemases have the ability to render a wide range of β-lactams, including carbapenems, cephalosporins and penicillin, harmless by hydrolysis (Halat and Moubareck, 2020).

Carbapenemase genes occur in the environment on almost all continents. Reservoirs of carbapenemase-producing bacteria and resistance genes can be located in hospital and community wastewater, drinking water and natural waterways (Mills and Lee, 2019). Although the highest amount of CRE continues to be found in hospital effluents, previous studies have already shown an alarming trend of CRE transport into municipal wastewaters and even surface waters. Exner et al. demonstrated an impact of CPE contaminated hospital wastewater on the water environment in Germany. The load of CRE found in urban wastewater with the influence of hospital effluents was significantly higher (28.4%) compared to rural wastewater (0.4%) with no hospital influence. Those CRE could also not completely be removed by wastewater treatment plants, resulting in the release of CRE into the environment (Exner et al., 2018). Similar results were shown by Müller et al. finding 134 CPE in urban and only eight in rural wastewater analyzed by real-time PCR, detecting CRE even in the effluent of wastewater treatment plants (WWTPs) (Müller et al., 2018). Similar issues have also been reported in the USA, where Hoelle et al. demonstrated that 62% of all collected carbapenem-resistant *E. coli* from the wastewater treatment plant effluent were even positive for more than one carbapenemase, highlighting a risk not only for environmental spread but also for gene exchange in WWTPs (Hoelle et al., 2019).

The impact of hospitals on the spread of CRE and CPE in Germany becomes more evident by a direct comparison of urban wastewater and hospital effluent. Sib et al. could demonstrate, that influent hospital wastewater accounted for 6% of the total wastewater in the system but 90% of the carbapenemase genes (Sib et al., 2020). This situation is reflected in several European countries, proving that the burden of multi-resistant *Enterobacterales*, especially CPE, is higher in hospitals and in hospital wastewater than in mixed urban wastewater (Cahill et al., 2019; Hocquet et al., 2016; Huijbers et al., 2020). To improve the understanding of the significance of the introduction of widely resistant *Enterobacterales* into the urban wastewater system, the clonal relationship of clinically relevant species was further investigated in this study. Detailed genetic characterization by whole genome sequencing of 240 bacterial isolates selected for 3rd gen. cephalosporin resistance from wastewater and comparison with clinical isolates was performed.

2. Material and methods

2.1. Sampling and identification of wastewater isolates

Representative 24 h wastewater samples were taken with a Buehler 3010 Stationary Automatic Sampler (Hach, Düsseldorf, Germany) for four days from 05th to 08th November 2019. The sampler was programmed to take 50 mL water samples every 10 min, resulting in a 7.2 l mixed wastewater sample per 24 h. 100 μL of the mixed wastewater of each day was spread out onto chromID® ESBL agar (bioMérieux, Marcy l'Etoile, France) and incubated at 37 °C overnight. 120 β-glucuronidase-producing (red) and 120 β-glucosidase-producing (green) colonies were picked and isolated on chromID® ESBL agar. The plates with subcultures from glucuronidase producing strains were incubated at 42 °C overnight, to support the selection for *E. coli*. Plates with subcultures from glucosidase producing strains were incubated at 37 °C. The species of all strains were determined by mass spectrometry with MALDI Biotyper® MBT smart instrument (Bruker, Bremen, Germany) and the associated MALDI Biotyper® database. Since MALDI is unable to definitively distinguish between the different species of the *Enterobacter cloacae* complex and the *Klebsiella oxytoca* complex, all isolates belonging to the *E. cloacae* complex were subsequently designated as *E. cloacae* and all isolates belonging to the *K. oxytoca* complex were designated as *K. oxytoca*.

2.2. Whole genome sequencing and genomic analysis

The DNA of all isolates was extracted with a QIASymphony SP Instrument and the QIAsymphony DSP Virus/Pathogen Mini Kit (Qiagen, Venlo, Netherlands). The DNA concentration was measured with the Qubit dsDNA High Sensitivity Kit (ThermoFisher Scientific, Waltham, USA) and adjusted to 5–8 ng/μL. 60 μL of the DNA were sheared by ultrasound in a bioruptor pico (diagenode, Seraing, Belgium) 10 times for 30 s with 30 s pausing in between. The library preperation was performed using the NEBNext Ultra DNA Library Prep Kit for Illumina and the NEBNext Multiplex Oligos for Illumina (NEB, Ipswich, USA). The PCR was set with 6 cycles.

Concentration of the library pool was measured again with Qubit and fragment length distribution of the final libraries was analyzed with the DNA High Sensitivity Chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). The library pool was normalized to 2 nM DNA and sequenced on the NextSeq500 (Illumina, San Diego, USA) with 2x151 bp in the mid output format. Samples had 1.3 to 4.7 mio reads per sample. The resulting FASTQ files were uploaded to the pipeline of Ridom SeqSphere+ (Ridom, Münster, Germany), assembled with Velvet (version 1.1.04) and analyzed for their sequence type and their relationship to other isolates. For this purpose, core genome Multi Locus Sequence Typing (cgMLST) was performed for all species with the published cgMLST schemes (*E. coli* and *K. pneumoniae*) or *ad hoc* cgMLST schemes calculated in Ridom SeqSphere+ with reference strains *C. freundii* FDAARGOS_61 (Accession nr.: NZ_CP026045.1; core genome: 2527 alleles) *S. marcescens* CAV1492 (Accession nr.: NZ_CP011642.1; core genome: 3177 alleles), *K. oxytoca* AR_0028 (Accession nr.: NZ_CP026715.1; core genome: 3175 alleles) and *E. cloac*ae ATCC13047

(Accession nr.: CP001918.1; core genome: 523 alleles). The threshold for identical isolates for each species was set at 15 different alleles for *K. pneumoniae* and at 10 different alleles for all other species according to the guidelines of Ridom SeqSphere+ (Francisco et al., 2009). To ensure international comparability, the sequence types of classical MLST schemes were used for strain designation, if available. For *E. coli* the Warwick MLST scheme was used for analysis (Wirth et al., 2006). Information about quality sequencing and typing is displayed in Supplementary Table 1. Sequences of all isolates are available in BioProject Nr. PRJNA764050 at NCBI BioProject. The sequences were uploaded to the bacterial pipeline of the Center for Genomic Epidemiology (CGE; Thomsen et al., 2016) to identify the presence of resistance and virulence genes in all isolates (Bortolaia et al., 2020; Camacho et al., 2009; Joensen et al., 2014; Malberg Tetzschner et al., 2020; Zankari et al., 2017). Additional to the designation by MLST sequence types the presence of one or more carbapenemase genes was used to distinguish within clonally related isolates, if applicable. Descriptive statistics were performed using R (version 3.6.2) and RStudio (version 1.2.5033) with the library *dplyr* activated (Wickham et al., 2019).

2.3. Selection of clinical isolates for comparative analysis

The data of wastewater samples were compared to genomes of relevant clinical isolates sequenced as part of the hospital's resistance surveillance strategy (3GCRE with additional resistance against fluoroquinolones from ICUs and oncology wards or isolates causing outbreaks in other wards, as well as CPE from all wards) during a defined comparison period of 12 months (1st February 2019 to 31st January 2020; Supplementary Table 2). Additionally, clonally related sequenced MRE isolates associated with former outbreaks were included regardless of time period. In total 988 *E. coli*, 50 *C. freundii*, 25 *S. marcescens*, 18 *K. oxytoca*, 306 *E. cloacae* and 253 *K. pneumoniae* clinical isolates were used for the comparison. For the defined time period a patient-adjusted relative rate of CRE within all detected *Enterobacterales* with phenotypic resistance against cefotaxime (CTX-R) was calculated for comparison with the wastewater samples cultivated on selective media.

2.4. Biofilm assay

To investigate the principal capability of the strains to attach to solid surfaces and to express intercellular adhesion, a biofilm assay was performed, modified from the method described by Fiamengo et al. (2020) (Fiamengo et al., 2020). In brief, one colony of each isolate was mixed into 5 mL LB medium (TH Geyer, Renningen, Germany) and incubated overnight at 37 °C. 20 µL of the culture were transferred into 2 mL DMEM medium (1% glucose; TH Geyer, Renningen, Germany). After mixing the solution thoroughly, 200 µL were transferred into wells of a 96 well NUNClon delta plate (ThermoFisher Scientific, Waltham, USA) and incubated overnight at 37 °C. Subsequently, plates were inverted and tapped firmly to remove the media and were incubated for an additional 1 h at 37 °C to fix remaining cells or biofilms. 50 µL Gentiana violet (Roth, Karlsruhe, Germany) were added to each well, incubated for 15 min, and washed out by carefully draining water over the plate surface. The stain absorbed by adhering bacteria was dissolved in 100 µL of 70% isopropanol for 15 min before analysis using a Spark (Tecan, Männedorf, Switzerland) with an adjusted wavelength of 570 nm and a reference wavelength of 405 nm. One permanent positive and one permanent negative wastewater strain were used as positive and negative controls, respectively, for biofilm forming bacteria on each plate. For each isolate, the biofilm assay was performed in two independent experiments with four replicates per isolate. The mean value of the negative control was subtracted from the mean value of all eight wells to obtain the final value. A value of <0.1 was defined as biofilm-negative, 0.1 to 0.5 as weak biofilm-positive and >0.5 as biofilm-positive.

3. Results

The 120 β-glucuronidase-producing isolates from hospital wastewater were identified as *E. coli* (n = 68), and *C. freundii* (n = 52). The 120 β-glucosidase-producing isolates were identified as *S. marcescens* (n = 45), *K. oxytoca* (n = 34), *E. cloacae* (n = 32), and *K. pneumoniae* (n = 9). Out of the 240 isolates, all except two *S. marcescens* isolates (99.17%) were β-lactamase-positive (Fig. 1) and 191 isolates (79.58%) were also carbapenemase-positive. Additionally, resistance genes against aminoglycosides, trimethoprim, sulfonamides, fluoroquinolones, tetracyclines and phenicols were present in all species. MLS (macrolides, lincosamides, streptogramines) resistance genes occurred only in *E. coli*, *C. freundii* and *E. cloacae*, whereas fosfomycin resistance genes occurred in all *E. cloacae* and *K. pneumoniae*, but in no other species. Rifampicin resistance occurred in all species except *K. oxytoca* and *K. pneumoniae*.

The most prominent carbapenemase gene was $bla_{\text{OXA-48}}$ (n = 112) detected in *E. coli* (n = 20), *C. freundii* (n = 36), *S. marcescens* (n = 11), *K. oxytoca* (n = 18), *E. cloacae* (n = 21) and *K. pneumoniae* (n = 6). In *E. coli* 29 isolates (45.6%) were positive for at least one carbapenemase

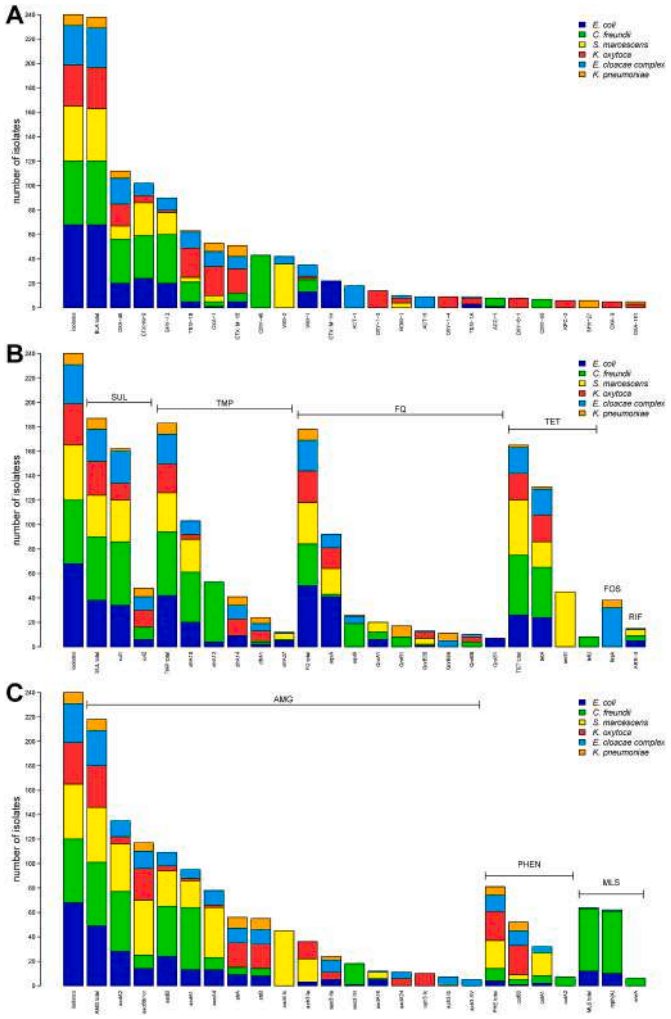

Fig. 1. Resistance genes detected individual species. Genes encoding resistance against β-lactams (**A**), sulfonamides (SUL), trimethoprim (TMP), fluoroquinolones (FQ), tetracyclines (TET), fosfomycin (FOS), and rifampicin (RIF) (**B**) as well as aminoglycosides (AMG), phenicols (PHEN) and MLS (macrolides, lincosamides, streptogramines) (**C**) are displayed. Colors represent the different species. The first bar represents the total number of analyzed isolates. Only genes that occurred at least five times are displayed. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

gene with two isolates displaying each two carbapenemase genes (Supplementary Table 3). Beside *bla*$_{OXA-48}$ in *E. coli* only *bla*$_{VIM-1}$ (n = 13) could be detected (supplementary figure 1.2). 88.5% of the *C. freundii* isolates were carbapenemase-positive, with 45 isolates containing one and one isolate containing two carbapenemase genes (Supplementary Table 3). The genes *bla*$_{VIM-1}$ (n = 10) and *bla*$_{OXA-232}$ (n = 1) were detected in this species beside the dominant *bla*$_{OXA-48}$ gene (Supplementary Fig. 2B). Out of the *S. marcescens* isolates 95.6% were carbapenemase-positive. 33 isolates contained one and ten isolates contained each two carbapenemase genes (Supplementary Table 3). In *S. marcescens* *bla*$_{VIM-2}$ was the most often identified carbapenemase gene (n = 36). In this species *bla*$_{OXA-48}$ was the second most frequent carbapenemase gene and *bla*$_{NDM-1}$ (n = 4), *bla*$_{GES-5}$ (n = 1) and *bla*$_{VIM-1}$ (n = 1) genes were also observed in this species (Supplementary Fig. 3B). *K. oxytoca* includes 91.2% carbapenemase-positive isolates with 29 isolates containing one and two isolates containing each two carbapenemase gene (Supplementary Table 3). Beside the dominant *bla*$_{OXA-48}$ gene, *bla*$_{KPC-2}$ (n = 6), *bla*$_{NDM-1}$ (n = 4), *bla*$_{OXA-181}$ (n = 3), and *bla*$_{VIM-1}$ (n = 2) were observed (Supplementary Fig. 4B). Out of the *E. cloacae* isolates 96.9% were carbapenemase-positive, of which 24 isolates contained one and seven isolates contained two carbapenemase genes (Supplementary Table 3). Next to *bla*$_{OXA-48}$, *bla*$_{VIM-1}$ (n = 9), *bla*$_{VIM-2}$ (n = 6) and *bla*$_{NDM-1}$ (n = 1) were detected (Supplementary Fig. 5B). 88.9% of the *K. pneumoniae* isolates were also carbapenem-resistant, each of them containing one single carbapenemase gene (Supplementary Table 3), either a *bla*$_{OXA-48}$ or a *bla*$_{OXA-181}$ (n = 2) (Supplementary Fig. 6B).

Clonal analysis using cgMLST was performed for all species as well as automated determination of classical MLST sequence types, except of *S. marcescens*. For *E. coli* 15 MLST sequence types (STs) were observed with eight singleton STs identified only once (SSTs; Supplementary Table 1). In *E. coli* ST635 was the most prominent ST (n = 32), constituting 47.1% of all isolates in this species. Using cgMLST five clonally distinct subgroups were identified (Fig. 2A). Even within clonally closely related strains, as detected by cgMLST, different presence of carbapenemase genes was observed (supplementary fig. 1.2). The next most frequent STs were ST1722 (n = 10) and ST23 (n = 8). Both revealed to be identical or closely related within the group as detected by cgMLST. The remaining 10 non SST *E. coli* isolates were identified as STs ST38 (n = 4), ST501 (n = 2), ST744 (n = 2) and ST2967 (n = 2). Except for ST744, all isolates of the respective sequence types were clonally identical or closely related according to cgMLST. For *C. freundii* four MLST STs with two SSTs observed only once were identified (Supplementary Fig. 2A). The predominant ST22 (n = 43) could be further divided into three subgroups by cgMLST. While in the largest subgroup (n = 34) all isolates carry a *bla*$_{OXA-48}$ gene locus, the two smaller clonally related groups displayed variable carbapenemase gene carriage. In *C. freundii* ST91 (n = 7) all isolates carried a *bla*$_{VIM-1}$ gene. However, one isolate displayed 18 differing alleles in cgMLST, indicating a separate clone. *S. marcescens* is divided into seven genotypes (GT) depending on the cluster threshold, as MLST for this species is not available (Supplementary Fig. 3A). The only clonally related group of strains in cgMLST (n = 39) displayed different carriage of carbapenemase genes (Supplementary Fig. 3B). *K. oxytoca* splits into six STs, which could not be further subdivided by cgMLST (Supplementary Fig. 4A). ST98 (n = 14) was the most prominent ST with all isolates carrying *bla*$_{OXA-48}$. ST170 (n = 6) and ST143 (n = 4) displayed a variable carriage of carbapenemase genes, with three ST170 isolates lacking the presence of a carbapenemase gene and three isolates carrying *bla*$_{OXA-181}$, whereas ST143 isolates carried *bla*$_{OXA-48}$ with two strains displaying an additional *bla*$_{NDM-1}$ gene. ST371 (n = 6), ST183 (n = 2) and ST319 (n = 2) carried *bla*$_{KPC-2}$, *bla*$_{NDM-1}$ and *bla*$_{VIM-1}$, respectively. *E. cloacae* displayed six STs, of which ST24 (n = 18) and ST54 (n = 2) are each divided into two, subgroups according to cgMLST (Supplementary Fig. 5A). The other three STs were SSTs. *K. pneumoniae* splits into three STs with one SST and no further cgMLST division of ST45 (n = 6) and ST147 (n = 2) into subgroups (Fig. 2B).

Out of the wastewater isolates 131 displayed the capacity to form a strong biofilm under the investigated condition while another 47 were weak biofilm-positive, constituting 74.2% of the cultivated wastewater samples. In *E. coli* 33 wastewater isolates were biofilm-positive, 14 weak biofilm-positive and 21 biofilm-negative (supplementary figure 1.3). Two of the biofilm-positive *E. coli* wastewater isolates (one ST635, one ST501) could be determined as typical enteroaggregative *E. coli* (tEAEC) carrying the marker gene *aggR* (Supplementary Table 1) as well as other virulence factors of tEAEC. *C. freundii* had 13 biofilm-positive, four weak biofilm-positive and 35 biofilm-negative isolates (Supplementary Fig. 2C). In *S. marcescens* 24 isolates were biofilm-positive, 16 were weak biofilm-positive and five were biofilm-negative (Supplementary Fig. 3C). There were no biofilm-negative and only two weak biofilm-positive isolates in *K. oxytoca* (Supplementary Fig. 4C). *E. cloacae* had 20 biofilm-positive, 11 weak biofilm-positive and one biofilm-negative isolate (Supplementary Fig. 5C). All *K. pneumoniae* displayed a strong biofilm-positive phenotype.

The cefotaxime-resistant (CTX-R) clinical isolates from the selected time period contained 0.6% CRE in *E. coli*, 6.0% in *C. freundii*, 4.0% in *S. marcescens*, 0% in *K. oxytoca*, 3.3% in *E. cloacae* and 7.1% in *K. pneumoniae* (Table 1). A comparison of wastewater and selected

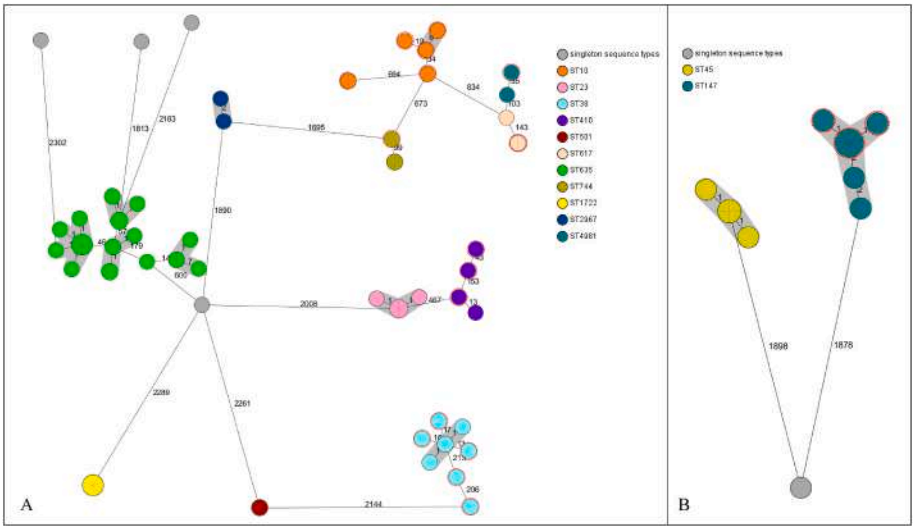

Fig. 2. cgMLST analysis of clonal relations in *E. coli* (**A**) and *K. pneumoniae* (**B**) wastewater isolates and related clinical isolates. Isolates are colored depending on their sequence type. Grey shaded clusters show clonally related subgroups according to cgMLST. Clinical isolates are marked with a dotted red circle. Numbers represent the allele differences (*E. coli*: 2513 alleles; *K. pneumoniae*: 2358 alleles). Additional information about carbapenemase genes, biofilm formation and clinical isolates are displayed in the supplementary figures. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
comparison of wastewater CPE and clinical CRE.

	wastewater isolates (CPE/3GCRE)	Clinical CTX-R isolates (CRE/3GCRE)
E. coli	31/68 (45.6%)	6/988 (0.6%)
C. freundii	46/52 (88.5%)	3/50 (6.0%)
S. marcescens	43/45 (95.6%)	1/25 (4.0%)
K. oxytoca	31/34 (91.2%)	0/18 (0.0%)
E. cloacae	31/32 (96.9%)	10/306 (3.3%)
K. pneumoniae	8/9 (88.9%)	18/253 (7.1%)

sequenced clinical isolates displayed matches for *E. coli* (Fig. 2A and supplementary figure 1.1) and *K. pneumoniae* (Fig. 2B and Supplementary Fig. 6A). For all other species there was no genetic accordance with clinical isolates.

4. Discussion

In the presented study the diversity of 3rd generation cephalosporin resistant *E. coli* and other *Enterobacterales* in hospital wastewater were characterized. The *E. coli* isolates were highly diverse with 15 different STs and showed resistance against nine different antibiotic groups, including carbapenems. Since all strains were isolated from selective agar containing β-lactams, the presence of β-lactamases in all species was expectable. Interestingly 45.6% of the *E. coli* isolates were positive for at least one carbapenemase gene too. This is of particular concern, because usually less than 0.1% of pathogenic *E. coli* are CPE (Schulz--Stübner, 2017). These data are confirmed by the fact that an additional carbapenem-resistant phenotype only occurred at low frequency (0.6%) in the clinical CTX-R *E. coli* isolates examined. The most prominent *E. coli* ST635 found in this study is known to be adapted to wastewater and is described as wastewater treatment resistant, but has already been found in hospital sinks as well (Constantinides et al., 2020; Zhi et al., 2019). In our study all except four of the carbapenemase-positive *E. coli* isolates belong to ST635, resulting in 87.5% of the ST635 isolates being CPE. In *E. coli* the high proportion of CPE in wastewater is therefore mainly justifiable by the presence of this wastewater adapted clone. Even clonal identical isolates, lacking different alleles in cgMLST, carried different carbapenemase genes (Supplementary Table 1). This led to the assumption, that ST635 might frequently underlie horizontal transfer of plasmids.

Bla$_{OXA-48}$-type carbapenemase genes (including *bla*$_{OXA-48}$, *bla*$_{OXA-181}$ and *bla*$_{OXA-232}$) were the most common carbapenemase type, which were detected in this study. It must be noted that the selected medium contains cefpodoxime as selective agent. Since carbapenemases of the OXA-48-type have low hydrolytic activity against 3rd generation cephalosporins (Pitout et al., 2020), isolates lacking an additional β-lactamase could be missed. Therefore, despite the already very high rate of carbapenemase positive isolates under 3GCRE in this study, there may still be an underestimation of the presence of carbapenemase positive isolates in the investigated wastewater.

The principle ability to attach to solid surfaces and to express intercellular adhesion may contribute to the persistence of this clone in wastewater. However, due to the differences between environmental conditions in wastewater and experimental verification, it is still not sure that ST635 persists in the drainage system fixed in biofilms. Interestingly, one ST635 was identified to carry plasmid encoded virulence factors present in tEAEC, suggesting that this strain acquired a virulence plasmid, indicating that a primary environmentally adapted clonal lineage can turn to be pathogenic for humans.

In a direct comparison of wastewater and clinical isolates one single *E. coli* ST10 was also found in the wastewater and was determined by cgMLST to be clonal identical to a clinical isolate from the same day. *E. coli* ST10 is a known pathogen for humans as well as animals and the third most common extra intestinal pathogenic *E. coli* worldwide (García-Meniño et al., 2018; Manges et al., 2019), that has already been described as multidrug-resistant (dos Anjos et al., 2019; Mohsin et al., 2018). The fact that this clone was detected in wastewater only once and simultaneously with the presence of a carrier of this clone in the hospital, indicates that an incidental finding of a clinical isolate was observed and a constant presence of this clone in wastewater is no assumed. The other less common *E. coli* STs underline the microbial diversity of *E. coli* in hospital wastewater.

On chromID ESBL agar, one expects β-glucuronidase-producing species to be primarily *E. coli*. In this study, interestingly, we identified almost half (n = 52) of the β-glucuronidase-producing isolates as *C. freundii*. There are already single descriptions of β-glucuronidase-producing *C. freundii* (McDaniels et al., 1996; Tapsall and McIver, 1995) and one *C. freundii* was also observed to grow in an unexpected color on chromID ESBL agar (Réglier-Poupet et al., 2008). Usually, *C. freundii* are described as β-glucosidase-producing strains, but no *C. freundii* were found in the 120 strains examined in this study, what is a remarkable observation. However, the lack of β-glucosidase-positive *C. freundii* might still be caused by a sampling bias. One possible explanation would be the chosen selective medium primarily manufactured for the use in human diagnostics.

The *C. freundii* isolates also split into different clonal groups, meaning there is not just one single β-glucuronidase-producing clone present in the investigated wastewater. In contrast to CTX-R clinical isolates the vast majority (88.5%) of *C. freundii* isolates carried a carbapenemase gene. The two most common STs (ST22 and ST91) have been previously described as carbapenemase-producing strains in hospitals (Villa et al., 2017). As we observed different carbapenemase genes present even in clonally closely related strains (cgMLST) the diversity of this species is underlined. Under the investigated artificial conditions for most *C. freundii* no intercellular adhesion could be observed. As it has been demonstrated, that environmental stress can trigger biofilm formation in different species (Chu et al., 2018; Knobloch et al., 2001; Knobloch et al., 2004; Nickerson and Faherty, 2018; Yin et al., 2019), strains tested biofilm-negative under laboratory conditions might still be capable of biofilm formation in the stressful environment of wastewater.

Two *S. marcescens* isolates lacked the identification of a known β-lactamase gene, but *S. marcescens* can also display a resistant phenotype against β-lactams by differential expression of outer membrane porins (Weindorf et al., 1998). In contrast to only 4.0% phenotypic resistance against carbapenems in CTX-R clinical *S. marcescens* isolates, 95.6% of the wastewater isolates were carbapenemase-producing. For *S. marcescens* no classic MLST scheme is published, but using cgMLST the isolates could be split into seven different clonal groups. In the only major clonally related group in this species, several different carbapenemase genes were observed indicating a high diversity in *S. marcescens* with the potential capability of horizontal transfer of plasmids as well.

The high amount of carbapenemase-positive *K. oxytoca* isolates in the wastewater is especially concerning as there were no carbapenem-resistant clinical *K. oxytoca* isolates detected in the investigated hospital during the study period (Table 1). As upstream of the sampling point only buildings of the hospital drain into the wastewater system and backflow of wastewater is very unlikely due to the existing slope, the presence of carbapenemase-producing *K. oxytoca* isolates indicates long term residence of individual clones or acquisition of plasmids from other species inside the wastewater system. Again the amount of carbapenem-resistant phenotypes among the CTX-R clinical isolates (3.3%; Table 1) was strikingly lower compared to wastewater isolates with 96.9%. *E. cloacae* ST24 as the most prominent *E. cloacae* ST found in this study has earlier been described as the cause for a CPE outbreak in an Australian hospital (Marmor et al., 2020). In our study the 18 ST24 isolates could be divided in two clonal groups by cgMLST. However, all isolates of this ST carried at least one carbapenemase gene.

K. pneumoniae was the species with the lowest number of isolates identified in this study, but still could be divided into three clonal groups. Even though CTX-R clinical isolates had the highest amount of

phenotypic carbapenem-resistance in *K. pneumoniae* (7.1%), while wastewater isolates displayed again a clearly higher rate (88.9%) of isolates being positive for a carbapenemase gene. According to cgMLST, *K. pneumoniae* was the second species in our study where a clonally relationship between wastewater and clinical isolates was observed. During winter season 2014/2015, there was a noticeable outbreak of *K. pneumoniae* ST147, with isolates closely related to the ST147 wastewater isolates (2 of 2358 alleles difference in cgMLST). *K. pneumoniae* ST147 has earlier been reported as the cause for a hospital outbreak in Portugal (Guerra et al., 2020) and also could be detected in wastewater isolates (Nüesch-Inderbinen et al., 2018). This ST is also known to carry an OXA-48-type carbapenemase and is designated as a high risk clone (Peirano et al., 2020; Pitout et al., 2020). In our study, we observed an $bla_{\text{OXA-181}}$ gene in this genetic background. As this strain is known to be associated with medical care, a repeated introduction into the hospital sewage system by different patients over time is possible. However, the very low number of different alleles in cgMLST also raises the possibility of long-term persistence of this clone without significant chromosomal variation in the wastewater system. Thereby, it is unclear in which part of the hospital drainage system this clone might reside, including the possibility of persistence in sink siphons with the potential re-transmission to patients. In this study we observed the loss of some resistance genes compared to the original outbreak isolates (Supplementary Tables 1 and 3), but the presence of the $bla_{\text{OXA-181}}$ gene didn't change.

Despite the cultural selection only for resistance against 3rd generation cephalosporins in this study, the proportion of carbapenemase-positive *Enterobacterales* was significantly higher compared to CTX-R clinical samples and clearly indicates an unexpected high burden of carbapenemase genes in the hospital wastewater. The high diversity of carbapenemase genes even within clonally related strains supports the hypothesis of horizontal transfer of plasmids, which might occur also in the wastewater system. Our study indicates that individual clones might persist for long periods in the wastewater, leading to a continuous release of these carbapenemase-positive strains into the communal wastewater system. Because *Enterobacterales* could not be completely removed from community wastewater, and individual isolates are released to natural water reservoirs, resistance genes liberated from hospitals might reenter human beings by the food and water chain (one health). Further studies should be conducted to understand the relative enrichment of carbapenemase-producing *Enterobacterales* compared to the expected release from the patients' flora.

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.2022.113968.

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Insights into the genetic diversity, antibiotic resistance and pathogenic potential of *Klebsiella pneumoniae* from the Norwegian marine environment using whole-genome analysis

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ABSTRACT

Klebsiella pneumoniae (Kp) can cause hospital- and community acquired infections. Although, Kp is widespread in the environment, very little is known about the genetic diversity and pathogenicity of Kp from the marine environment. The aim of our study was to understand the genetic diversity, resistome and pathogenic potential of 87 Kp isolates from the Norwegian marine environment, using whole-genome sequencing. We identified 50 sequence types, including globally disseminated sequence types associated with multidrug resistance or hypervirulence. Ten isolates carried the yersiniabactin loci. Acquired antibiotic resistance genes were identified in six Kp isolates. Heavy metal resistance genes were widespread among the isolates, with 71% carrying genes encoding resistance to copper, silver, arsenic, nickel and/or mercury. Co-occurrence of antibiotic resistance genes and heavy metal resistance genes was seen in five Kp isolates. Phylogenetic analysis revealed a close genetic relationship between Kp 2016-1200 ST25 isolated from blue mussels (*Mytilus edulis)* and a clinical isolate reported in Germany. To the best of our knowledge, this study provides the first comprehensive account of genetic diversity among Kp from the marine environment. Our study reveals high diversity of Kp in the Norwegian marine environment and seafood, including globally disseminated pathogenic sequence types carrying clinically relevant antibiotic resistance genes and virulence factors, as well as several heavy metal resistance genes.

1. Introduction

Klebsiella pneumoniae (Kp) can cause nosocomial as well as community acquired infections (Paczosa and Mecsas, 2016). In addition to the clinical environment, Kp is widespread in nature and can be found in surface waters, soil, on plants and in the gut of healthy humans and animals (Brisse et al., 2006; Bagley, 1985; Podschun et al., 2001). However, the primary reservoirs of Kp are not well understood (Davis and Price, 2016).

Recently, whole-genome sequencing has revealed the existence of five closely related species, of which two include subspecies, that together constitute the *Klebsiella pneumoniae* species complex (KpSC). The KpSC consists of *K. pneumoniae* sensu stricto, *K. quasipneumoniae* subsp. *quasipneumoniae*, *K. variicola* subsp. *variicola*, *K. quasipneumoniae* subsp. *similipenumoniae*, *K. variicola* subsp. *tropica, K. quasivariicola*, and *K. africana* (Wyres et al., 2020a). Of the KpSC members, Kp is responsible for the majority of human infections (Wyres et al., 2020a).

Kp is well known for its ability to acquire genetic material through horizontal gene transfer (Wyres and Holt, 2018), and the acquisition of mobile genetic elements have led to the development of two Kp groups, hypervirulent Kp (hvKp) and multidrug resistant Kp (MDR-Kp) (Russo and Marr, 2019). The hvKp carry plasmids and integrative conjugative elements (ICEs) encoding siderophores (*iro, iuc* and *ybt*), the colibactin toxin (*clb)* and/or genes responsible for a mucoid phenotype (*rmpA/rmpA2*) and are able to cause infections in otherwise healthy

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Abbreviations

ARGs	antibiotic resistance genes
BSI	blood stream infection
clb	colibactin
GI	gastrointestinal
HMRGs	heavy metal resistance genes
hvKp	hypervirulent *Klebsiella pneumoniae*
ICE	integrative conjugative element
iro	salmochelin
iuc	aerobactin
K	capsule
KL	capsule locus
Kp	*K. pneumoniae*
KpSC	*Klebsiella pneumoniae* species complex
MDR	multidrug resistant
MLST	multilocus sequence typing
SNP	single nucleotide polymorphism
ST	sequence type
ybt	yersiniabactin

individuals (Russo and Marr, 2019). In most cases *ybt* is chromosomally encoded and mobilised by ICEs, whereas the remaining virulence factors associated with hvKp are normally carried on plasmids (Wyres et al., 2020a). MDR-Kp is a common cause of hospital acquired infections (Pomakova et al., 2012; Russo and Marr, 2019). Both groups are associated with specific sequence types (STs), but recently convergence between the two groups has been observed (Wyres et al., 2020a).

Kp is a frequent coloniser of the human gastrointestinal (GI) tract and colonisation represents a significant risk for subsequent development of infections in immunocompromised individuals (Martin et al., 2016; Podschun and Ullmann, 1998; Martin and Bachman, 2018). Large variations in GI carriage rates of Kp have been reported worldwide. It has been found to be 16% in Norway and 6% in Australia, while in Asia, carriage rates as high as 88% in healthy adults have been reported (Gorrie et al., 2017; Lin et al., 2012; Raffelsberger et al., 2021).

Although not a classic foodborne pathogen, food has been identified as a risk factor for GI colonisation with Kp (Huynh et al., 2020; Lepuschitz et al., 2020; Raffelsberger et al., 2021). Kp has been isolated from several food sources, such as meat, street food, vegetables and seafood (Sanjit Singh et al., 2017; Guo et al., 2016; Davis et al., 2015; Falomir et al., 2013). Furthermore, it has been shown that strains isolated from food and the environment resemble clinical strains (Davis et al., 2015; Struve and Krogfelt, 2004).

Since the 1960s, the consumption of seafood has more than doubled worldwide (FAO, 2018). Consumption of contaminated seafood is a possible cause of GI infections. Seafood can be contaminated with pathogenic microorganisms in the environment, or it can be contaminated during transport and/or processing (Elbashir et al., 2018). Bivalve molluscs are filter feeders that retain and concentrate particles, including bacteria and viruses of both marine and terrestrial origin (Bernard, 1989). As a result, bivalves are well known to cause foodborne disease, and species traditionally consumed raw or lightly conserved, such as oysters (*Crassostrea gigas*), frequently cause food borne infections (Potasman et al., 2002; Elbashir et al., 2018). Due to the active accumulation of microorganisms and exposure to chemical pollutants, bivalves are also good indicators of faecal and chemical contamination in a given marine environment (Kibria et al., 2016; Grevskott et al., 2017).

Kp is extensively studied in clinical settings but the prevalence in the environment, especially the marine environment, is not well known (Manges, 2015). There are numerous transmission routes of pathogenic bacteria like Kp to the marine environment, *e.g.* through run-off from land and wastewater (Baquero et al., 2008; Marathe et al., 2017). Although we have shown the presence of Kp in marine bivalve molluscs collected along the Norwegian coast (Håkonsholm et al., 2020), there is a lack of knowledge on the genetic diversity and pathogenic potential of Kp isolated from the marine environments. The aim of this study was to understand the diversity, resistome and pathogenic potential of Kp strains isolated from the marine environment using whole-genome sequencing. We further examined the genetic relatedness of marine isolates of specific STs to isolates of human origin, including clinical isolates.

2. Materials and methods

2.1. Sampling, isolation and identification of presumptive Klebsiella pneumoniae

All samples included in the study were collected in 2016, and 2019–2020. In total, 578 batch samples of bivalve molluscs were examined. Of these, 563 samples covering production locations, depurated bivalves and wild populations were collected from 79 locations through the national surveillance programme of bivalve molluscs conducted by the Norwegian Food Safety Authority (NFSA), while 15 batch samples were collected from six locations not covered by the national surveillance programme.

The bivalve samples comprised 476 blue mussels (*Mytilus edulis*), 58 oysters (*Crassostrea gigas*), 31 scallops (*Pecten maximus*), five horse mussels (*Modiolus modiolus*), three ocean quahogs (*Arctica islandica)*, two carpet shells (*Politapes rhomboides*), two cockles (*Cerastoderma edule*) and one sand gaper (*Mya arenaria*). Although not bivalves, the samples also included seven batch samples of sea urchins (*Strongylocentrotus droebachiensis*) from two locations. A total of 53 fish samples were examined, 40 herring (*Clupea harengus)* and five mackerel (*Scomber scombrus*) collected by commercial fishing vessels in the North- and Norwegian Sea, three pollack (*Pollachius pollachius*), two cusk (*Brosme brosme*), two ling (*Molva molva)* and one hake (*Merluccius merluccius*) caught from coastal waters. Additionally, 17 samples of surface water from 13 different locations collected using a Van Dorn water sampler (KC Denmark, Denmark), and 24 sediment samples from nine locations were collected using a Van Veen Grab (KC Denmark, Denmark) were included. All samples were collected in sterile plastic containers (VWR, USA) or sterile plastic bags (VWR, USA) and kept at 4 °C until analysis.

Isolation of Kp from bivalve molluscs was performed as previously described (Håkonsholm et al., 2020). From each seawater sample, 1–5 l water was filtered through three separate 0.45 μm filters (Merck Millipore, Germany) using the EZ-fit Manifold 3-place system (Merck Millipore, Germany). The three filters used per sample were folded with sterile forceps and transferred to 100 ml buffered peptone water (BPW) (VWR, USA). From fish, 10 g of intestinal contents were weighed into sterile plastic bags (VWR, USA), homogenised for 2.5 min, diluted 1:10 with BPW and homogenised for 30 s. Sediment samples were diluted 1:10 in BPW in sterile plastic bags and homogenised for 30 s. Incubation conditions for all samples and further processing of enrichment cultures followed the methods described previously (Håkonsholm et al., 2020). All presumptive Kp isolates were identified using MALDI-TOF MS (Bruker, Germany). A complete list of isolates is provided in Supplementary Table S1.

2.2. Antibiotic susceptibility testing

Antibiotic susceptibility profiles for 70 Kp isolates included in the study have been reported previously (Håkonsholm et al., 2020). Antibiotic susceptibility testing of the additional isolates included in the present study was done with disk diffusion following the protocol described previously (Håkonsholm et al., 2020). The inhibition zones were interpreted following EUCAST breakpoints for Enterobacterales (https://www.eucast.org/clinical_breakpoints/). For tetracycline (TET), no breakpoints were available, and no inhibition zone was used to

classify the isolates as resistant. Measured inhibition zones for all isolates are presented in Supplementary Table S2.

2.3. DNA extraction and whole-genome sequencing

DNA was extracted from freshly grown isolates using MagNA Pure 96 and Viral Small volume kit with the Pathogen Universal 200 4.0 purification protocol (Roche Applied Science, Germany). Genomic libraries were prepared using Illumina Nextera DNA Flex library prep and sequenced using the Illumina MiSeq system and the Illumina MiSeq Reagent Kit V3 (600 cycle) to obtain 2 × 300 bp paired end reads.

2.4. Whole-genome sequence analysis

Raw short reads were adapter- and quality trimmed using Trim Galore v0.6.4 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and *de novo* assembled with Unicycler v0.4.8 (Wick et al., 2017). Species identification, multilocus sequence typing (MLST) and identification of the key virulence factors yersiniabactin (*ybt*), salmochelin (*iro*), aerobactin (*iuc*), colibactin (*clb*) and the regulator genes of a mucoid phenotype (*rmpA* and *rmpA2)*, and antibiotic resistance genes (ARGs) was done using Kleborate v2.1.0 (Lam et al., 2021), while serotype prediction was done with Kaptive v0.7.3 (Wyres et al., 2016). Plasmid replicons were identified with Plasmid Finder v.2.1(Carattoli et al., 2014). Further identification of ARGs, heavy metal resistance genes (HMRGs) and virulence genes was done using AMRFinderPlus v3.9.8 (Feldgarden et al., 2019), the BIGSdb-Kp database (https://bigsdb.pasteur.fr/klebsiella) and VFDB v2021-4-8 (Chen et al., 2016) via ABRicate v1.0.1 (https://github.com/tseemann/abricate). All bioinformatic tools were run using default settings. Novel STs were assigned by submitting sequence data to the Kp MLST database (https://bigsdb.pasteur.fr/klebsiella). The assembled genomes were annotated with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). In isolates where intrinsic virulence genes were not identified in the assemblies, the annotated files were manually searched. A complete list of identified ARGs, virulence genes and HMRGs are provided in Supplementary Table S3.

2.5. Colistin MIC determination

Isolates with substitutions in the *pmrB* gene were subjected to MIC testing by broth microdilution using the Sensititre EUVSEC panel (Thermo Scientific, USA) following the protocol described earlier (Grevskott et al., 2021) and results interpreted according to EUCAST breakpoints for Enterobacterales (https://eucast.org/clinical_breakpoints/).

2.6. String test

All isolates possessing the yersiniabactin locus or belonging to serotypes associated with invasive infections were subjected to the string test to identify the hypermucoid phenotype associated with systemic infections (Catalán-Nájera et al., 2017). The isolates were grown on MacConkey agar (Sigma-Aldrich, USA) over night at 37 °C. A 10 μm loop was used to stretch a single colony, and a hypermucoviscous phenotype was defined as the formation of a string ≥ 5 mm (Catalán-Nájera et al., 2017).

2.7. Phylogenetic analysis

The RedDog pipeline v1beta.11 (https://github.com/katholt/RedDog) was used to create a core genome single nucleotide polymorphism (SNP) phylogeny of Kp isolated from the marine environment. Isolates belonging to other species of the KpSC were also included in the analysis. The raw reads were aligned to the Kp ST11 HS11286 chromosome (NC_016845.1) using BowTie2 v2.2.9 (Langmead and Salzberg, 2012) and SNPs identified with SAMtools v1.9 (Danecek et al., 2021). A core chromosomal SNP phylogeny was inferred with FastTree v2.1.10 (Price et al., 2010).

To examine the genetic relatedness between isolates belonging to selected Kp STs (ST17, ST20, ST25, ST29 and ST37) isolated from the marine environment and isolates of human origin, including clinical isolates, isolates from a hospital outlet and from waste water treatment plant, were used for ST specific core genome SNP analysis, performed as described above. The following genomes were used as references, NZ_CP056275.1 (ST17), NZ_CP056432.1 (ST20), NZ_CP033777.1 (ST25), NZ_CP065167.1 (ST29) and NZ_CP021960.1 (ST37). Gubbins v2.4.1 (Croucher et al., 2014) was used to remove SNPs in recombination sites. The total number of SNPs in the aligned core genomes were extracted with SNP-sites (Page et al., 2016), and SNP-dists (https://github.com/tseemann/snp-dists) was used to create pairwise SNP distance matrices. The SNP matrices are presented in Supplementary Table S4. RAxML v8.2.12 (Stamatakis, 2014) was used to infer maximum likelihood phylogenies from the core SNP alignments. The public available Kp genomes included in the core genome SNP analyses were downloaded from the European Nucleotide Archive and are listed in Supplementary Table S5.

3. Results

3.1. Prevalence and genetic diversity of Klebsiella pneumoniae in the marine environment

In total, 99 isolates from all samples were identified as Kp using MALDI-TOF MS and were whole-genome sequenced. The sequenced genomes were *de novo* assembled into an average of 133 contigs (35–343) with a mean genome length of 5 423 501 bp (5 009 383–5 854 074) and an average GC content of 57.35% (56.68%–58.07%).

Based on Kleborate analysis of whole-genome sequences, 87 of these isolates were identified as Kp, nine as *K. quasipneumoniae* subsp. *similipenumoniae*, one isolate was identified as *K. quasipneumoniae* subsp. *quasipneumoniae*, one isolate as *K. variicola* subsp. *variicola* and one isolate was identified as *K. quasivariicola*. Kp was recovered from 81 (14%) bivalve samples collected from 43 locations. Of these, 34 locations were used for commercial production of bivalves for human consumption. Kp was isolated from 74 samples of *M. edulis*, four batch samples of *C. gigas* and three *P. maximus* samples. Six isolates were found in water samples from six different locations (35%). For the other members of the KpSC, *K. quasipneumoniae* subsp. *similipenumoniae* was recovered from nine (2%) bivalve samples collected from eight locations, of which seven were used for commercial production of bivalves, one *K. variicola* subsp. *variicola* and one *K. quasivariicola* isolate was isolated from bivalves from two separate locations (0.2%), and the single *K. quasipneumoniae* subsp. *quasipneumoniae* isolate was recovered from a water sample (6%). No isolates were recovered from bivalves cleared for market, fish or sediment samples.

The Kp isolates belonged to 50 different STs, of which 34 were only represented by one single isolate. The most common STs were ST20 (n = 8), ST10 (n = 7), ST200 (n = 5) and ST643 (n = 5). ST200 was the only ST isolated from both bivalve molluscs and seawater (Fig. 1). Four isolates belonged to novel STs (ST4675, ST4676, ST5676 and ST5696). The nine *K. quasipneumoniae* subsp. *similipenumoniae* isolates belong to eight different STs.

Among all Kp isolates, 34 different capsule loci (KL) were identified, with KL28 (n = 10), KL102 (n=7) and KL62 (n = 6) being the most common. For six isolates no KL was assigned. ST specific combinations of KL and O types were seen in the ST20 (n = 8), ST10 (n = 7), ST416 (n = 3), ST110 (n = 2), ST1867 (n = 2), ST1966 (n = 2), ST2441 (2), ST27 (n = 2) and ST29 (n = 2) isolates. The remaining STs with more than one isolate differed in KL and/or O-type. One *K. quasipneumoniae* subsp. *similipneumoniae* isolate belonged to KL1.

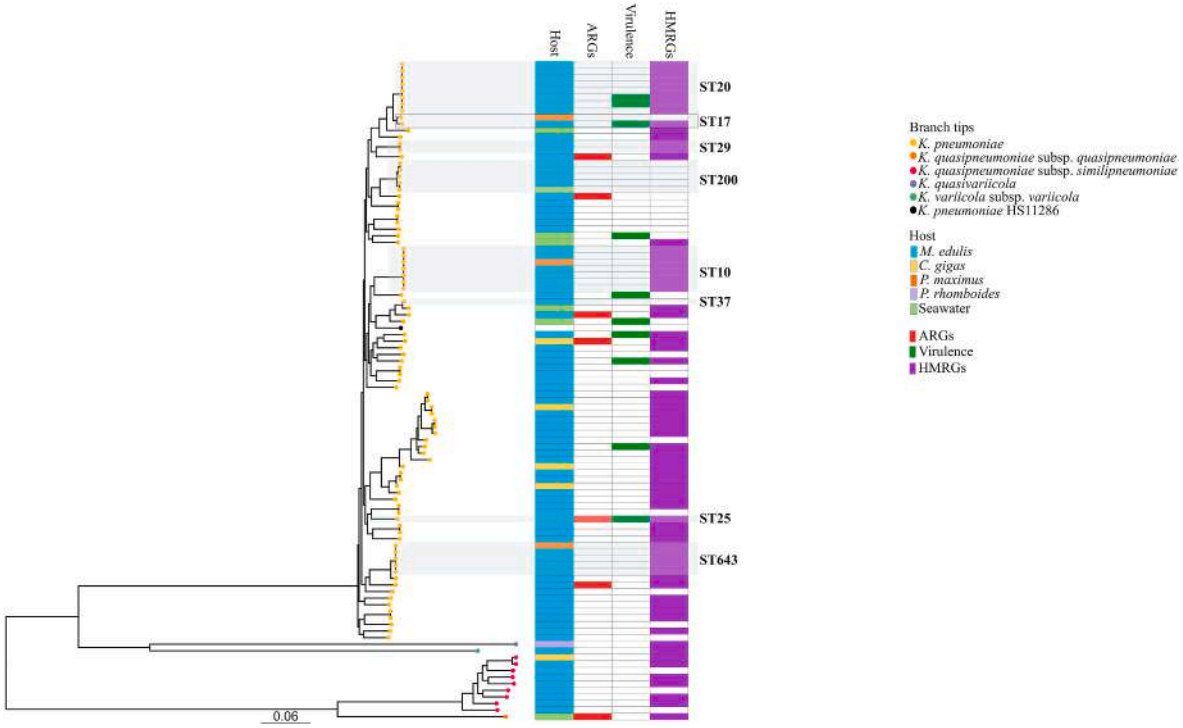

Fig. 1. Midpoint-rooted core genome phylogeny of 87 *Klebsiella pneumoniae,* nine *K. quasipneumoniae* subsp. *similipenumoniae*, one *K. quasipneumoniae* subsp. *quasipneumoniae*, one *K. quasivariicola* and one *K. variicola* subsp. *variicola* isolated from the marine environment. In total, 218 281 SNPs were identified in the aligned core genome of the marine Kp isolates. Branch tips are coloured according to the species the isolates belong to. The phylogeny is visualised alongside the marine host the isolates were recovered from, acquired antibiotic resistance genes (ARGs), virulence factors (yersiniabactin) and heavy metal resistance genes (HMRGs). Sequence types (STs) that are frequently reported in clinical settings and the most common STs isolated in this study are highlighted.

3.2. Phenotypic antibiotic resistance

Among the isolated Kp, we observed phenotypic resistance to tetracycline (~3%, n = 3), chloramphenicol (~2%, n = 2), nitrofurantoin (~2%, n = 2), trimethoprim-sulfamethoxazole (~2%, n = 2), ciprofloxacin (~1%, n = 1), cefotaxime (~1%, n = 1) and cefuroxime (~1%, n = 1). In total, ~3% (n = 3) of the isolates were susceptible to ampicillin. Resistance to amoxicillin-clavulanic acid was observed in three isolates (~3%) according to breakpoints for intravenous administration. However, these isolates remained susceptible while applying breakpoints for oral administration. No phenotypic resistance to agents other than ampicillin was seen among other species within the KpSC.

3.3. Acquired antibiotic resistance genes, heavy metal resistance genes and plasmid replicons

Among the 87 Kp genomes, 17 different acquired ARGs were identified. The ARGs were detected in six isolates, of which three were MDR as defined by Magiorakos et al. (2012). The identified ARGs included five genes encoding resistance to aminoglycosides (*aph(3″)-Ib, aph(3')-Ia, aph(6)-Id, aadA1* and *aadA2*) and three genes encoding resistance to sulphonamides (*sul1, sul2* and *sul3*), while the most prevalent ARGs was $bla_{\text{TEM-1}}$ (n = 3) and *tet*(D) (n = 3) (Table 1). As previously described, Kp 2016-1400 carried $bla_{\text{CTX-M-3}}$ and $bla_{\text{TEM-1}}$ on a non-conjugative plasmid and lacked the chromosomal $bla_{\text{SHV-1}}$ gene (Håkonsholm et al., 2020). Further, the three ampicillin susceptible

Table 1
Sequence types (STs), acquired antibiotic resistance genes (ARGs), heavy metal resistance genes (HMRGs) and plasmid replicons identified in antibiotic resistant *Klebsiella pneumoniae* and *K. quasipneumoniae* subsp. *quasipneumoniae* isolated from the marine environment.

Isolate	Species	ST	ARGs	HMRGs	Plasmid replicons
2016–1200	*K. pneumoniae*	ST25	*dfrA14, sul1, sul2, aph(3″)-lb, aph(6)-ld, aph(3')-la, tet(D),* $bla_{\text{TEM-1}}$	*silABCEFPRS, pcoABCDRS, arsABDR*	IncFIB(K), IncFII(K)
2016–1400	*K. pneumoniae*	ST1035	$bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-3}}$	*silABCEFPRS, pcoABCDERS, arsABDR*	IncFIB(K), IncFII (pKP91)
2016–1198[a]	*K. pneumoniae*	ST2196	*sul2, tet(D), catA2*	*silABCEFPRS, pcoABCDERS, merDEFPRT*	IncFIB(K)(pCAV1099-114), IncHI1B(pNDM-MAR)
2016–319	*K. pneumoniae*	ST556	*tet(D)*	*silABCEFPRS, pcoABCDERS, arsABDR*	IncFIB(K)
2019–1792[b]	*K. pneumoniae*	ST4267	*tet(A)*	*silABCEFPRS, pcoABCDRS, arsABCDR*	IncFIB(K), IncFII(K)
2019–1764	*K. pneumoniae*	ST292	*dfrA12, sul3,* $bla_{\text{TEM-1}}$*, cmlA1, qnrS1, aadA1, aadA2*	–	IncFIB(pKPHS1)
2019–1836	*K. quasipneumoniae* subsp. *quasipneumoniae*	ST5648	*aph(3″)-Ib, aph(6)-Id,*	*silABCEFPRS, pcoABCDRS*	IncFIB(K), IncR

Note; bla_{SHV}, *fosA* and *oqxAB* are intrinsic and therefore not presented in the table. a; Two copies of *merP, merR* and *merT* identified on separate contigs, b; : Two copies of *arsA, arsB, arsD* and *arsR* identified on separate contigs.

isolates all carried the intrinsic bla_{SHV}-gene. Additionally, one *K. quasipneumoniae* subsp. *quasipneumoniae* isolate carried the *aph(3″)-Ib* and *aph(6)-Id* genes encoding resistance to aminoglycosides (Table 1). A single amino acid substitution in the *pmrB* gene (R256G) associated with colistin resistance (Xiaoliang et al., 2019) was identified in six Kp isolates. However, MIC for colistin was <1 μg/ml for these isolates.

HMRGs were widespread in isolates from the marine environment, with 71% (n = 62) of the Kp isolates carrying genes encoding resistance to silver (*sil*), copper (*pco*), mercury (*mer*), nickel (*ncr*) and/or arsenic (*ars*). Among the nine *K. quasipneumoniae* subsp. *similipenumoniae* isolates, 44% (n = 4) carried *sil* and *pco* genes, 33% (n = 3) harboured genes conferring resistance to arsenic while 1% (n = 1) carried *ncr* or *mer* genes. Silver and copper resistance genes were identified in the single *K. quasipneumoniae* subsp. *quasipneumoniae* isolate, while the *K. quasivariicola* isolate carried mercury resistance genes. Both HMRGs and ARGs were present in five Kp isolates and one *K. quasipneumoniae* subsp. *quasipneumoniae* isolate.

Plasmid replicons were found in 84% (n = 83) of the isolates with 22 different replicon types identified. The most common plasmid replicon was IncFIB(K) (n = 59) followed by IncFII(K) (n = 48) and IncR (n = 23). More than one replicon type was found in 72 (73%) isolates. IncFIB(K) or IncFIB(K)(pCAV1099-114) replicons were found in all strains carrying both ARGs and HMRGs (Table 1).

3.4. Virulence genes

The type 3 fimbriae (*mrk*) cluster was present in all except one Kp isolate, while the type 1 fimbriae (*fim*) cluster was present in all except two isolates. The intrinsic enterobactin (*ent)* siderophore was identified in all isolates. The previously described CTX-M producing Kp 2016-1400 lacked both the *mrk* and *fim* clusters.

The yersiniabactin locus (*ybtAEPQSTUX-fuyA-irp1-irp2*) was detected in 11% (n = 10) of the Kp isolates. Five distinct integrative conjugative elements (ICEKps) and six *ybt* lineages were identified among the 10 ybt positive isolates, of which ICEKp5 (n = 5) and *ybt*14 (n = 4) were the most common. One *ybt* positive isolate (2019-1349), carried a novel *ybt* lineage (*ybt*18) and a new structural variant of ICEKp (ICEKp15). No other complete siderophore loci or hypermucoidity-encoding genes were identified in the marine Kp isolates. One isolate carried partial *iro*N and *iro*C genes on the same contig, this may be due to a deletion of the locus as described previously (Lam et al., 2018). Two of the Kp isolates carried the KL2 and KL57 locus, while one *K. quasipneumoniae* subsp. *similipenumoniae* isolate harboured the KL1 locus. These KLs encode capsule types associated with hypervirulence or invasive infections (Russo and Marr, 2019). All examined isolates were negative for the hypermucoviscous phenotype (Table 2). Genes encoding allantoinase (*all*) was present in two Kp isolates, one isolate carried *allABCDRS*, while Kp 2016-1400 harboured *allARS*. Genes involved in ferric iron uptake (*kfu*) and/or capsule formation (*kvg*) were found in 5% (n = 4) of the isolates. *kfu* genes were common in *K. quasipneumoniae* subsp. *similipenumoniae*, present in 78% (n = 7) of the isolates, while *all* genes were identified in four (44%) of the *K. quasipneumoniae* subsp. *similipneumoniae* isolates. *kfu* genes were also present in *K. variicola* subsp. *variicola* (n = 1) and *K. quasipneumoniae* subsp. *quasipneumoniae* (n = 1).

Table 2
Strain characteristics of *Klebsiella pneumoniae* with yersiniabactin isolated from the marine environment.

Isolate	ST	*ybt*	ICEKp	KL	String test
2016–1200	ST25	*ybt* 6	ICEKp5	KL2	–
2016–637	ST17	*ybt* 15	ICEKp11	KL25	–
2019–604	ST111	*ybt* 9	ICEKp3	KL63	–
2019–1349	ST866	*Ybt* 18	ICEKp15	KL46	–
2019–1394	ST20	*ybt* 14	ICEKp5	KL28	–
2019–1497	ST45	*ybt* 10	ICEKp4	KL43	–
2019–1897	ST20	*ybt* 14	ICEKp5	KL28	–
2019–1898	ST3403	*ybt* 16	ICEKp12	KL43	–
2019–2010	ST1307	*ybt* 14	ICEKp5	KL127	–
2020–749	ST704	*ybt* 14	ICEKp5	KL31	–

ST: Sequence type, *ybt*: yersiniabactin lineage, ICEKp: *Klebsiella pneumoniae* integrative conjugative element variant, KL: capsule (K) locus, -: negative string test.

3.5. ST specific phylogenetic analyses

The ST specific phylogenetic analyses identified 2 131, 3 700, 938, 3 010 and 2 988 SNPs in the aligned recombination-free core genomes of ST17, ST20, ST25, ST29 and ST37 isolates, respectively.

The marine isolates of ST17 and ST20 were intermingled with isolates of human origin, while the two ST29 isolates from bivalves clustered together with only two core genome SNPs between them (Fig. 2A, B, D). The single ST37 isolate clustered closest to a clinical urine isolate (232 SNPs) (Fig. 2E). Comparison of the MDR and *ybt* positive Kp 2016-1200 ST25 isolate to clinical isolates revealed a close genetic relationship to Kp ERR1217000 isolated from a patient with blood stream infection (BSI) in Germany in 2013, differing by only 24 core genome SNPs (Fig. 2C), with 94.5% of the Kp 2016-1200 genome and 95.9% of the ERR1217000 genome mapped to the ST25 NZ_CP033777 reference chromosome. Further, Kp 2016-1200 ST25 and ERR1217000 shared the same ARGs, HMRGs, virulence genes and plasmid replicons (*aph(3′)-Ia, aph(3″)-Ib, aph(6)-Id,* bla_{TEM-1}*, dfrA14, sul1, sul2, tet(D), silABCEFPRS, arsABDR, pcoACDRS, ybt,* IncFIB(K) and IncFII(K)), suggesting that these isolates are clonally related.

4. Discussion

During recent years, the environment has emerged as a potential reservoir for transmission of Kp and antibiotic resistance to humans (Wyres et al., 2020a). To the best of our knowledge, this study provides the first comprehensive account of genetic diversity among Kp from the marine environment. Our results show high genetic diversity of Kp and the presence of Kp carrying clinically relevant ARGs and virulence genes in the marine environment. Further, phylogenetic analysis of globally disseminated STs revealed a close genetic relationship between Kp isolated from blue mussels (*M. edulis*) and a clinical isolate, suggesting a potential transmission route for Kp from the marine environment to humans via seafood.

A high ST diversity of Kp was observed in Norwegian coastal waters and bivalve molluscs, including globally disseminated STs, like ST17, ST20, ST25, ST29 and ST37, associated with MDR or hypervirulence (Wyres et al., 2020a; David et al., 2019). Carbapenem resistant Kp ST17, ST20 and ST29 have been reported from a range of geographical locations, including Africa (Strydom et al., 2020), Asia (Safavi et al., 2020) and Europe (Aires-de-Sousa et al., 2019). High genetic diversity is also frequently reported from studies on Kp carriage in healthy individuals (Lepuschitz et al., 2020; Huynh et al., 2020), among clinical isolates (Fostervold et al., 2021) as well as studies on Kp in animals (Runcharoen et al., 2017; Gibbon et al., 2021; Paulin-Curlee et al., 2007), potentially indicating various sources of origin for the isolates from the marine environment. Interestingly, a recent study on Kp carriage in humans in Norway also found ST20 as the most common ST (Raffelsberger et al., 2021), possibly indicating exchange of Kp between the human population and the marine environment and/or vice versa. Large-scale metagenome analyses of the global marine environment have shown low abundance of *Klebsiella* in open oceans (Sunagawa et al., 2015), and the absence of Kp in samples of fish, seawater and sediments collected from open waters are in accordance with the previous study. Our study indicates that Kp may be largely present in the marine environments influenced by anthropogenic activities. Kp may follow numerous transmission routes to the marine environment, including sewage pollution, animal faeces, marine mammals and run-off from land, especially during periods with heavy rainfall (Jang et al., 2010; Roe

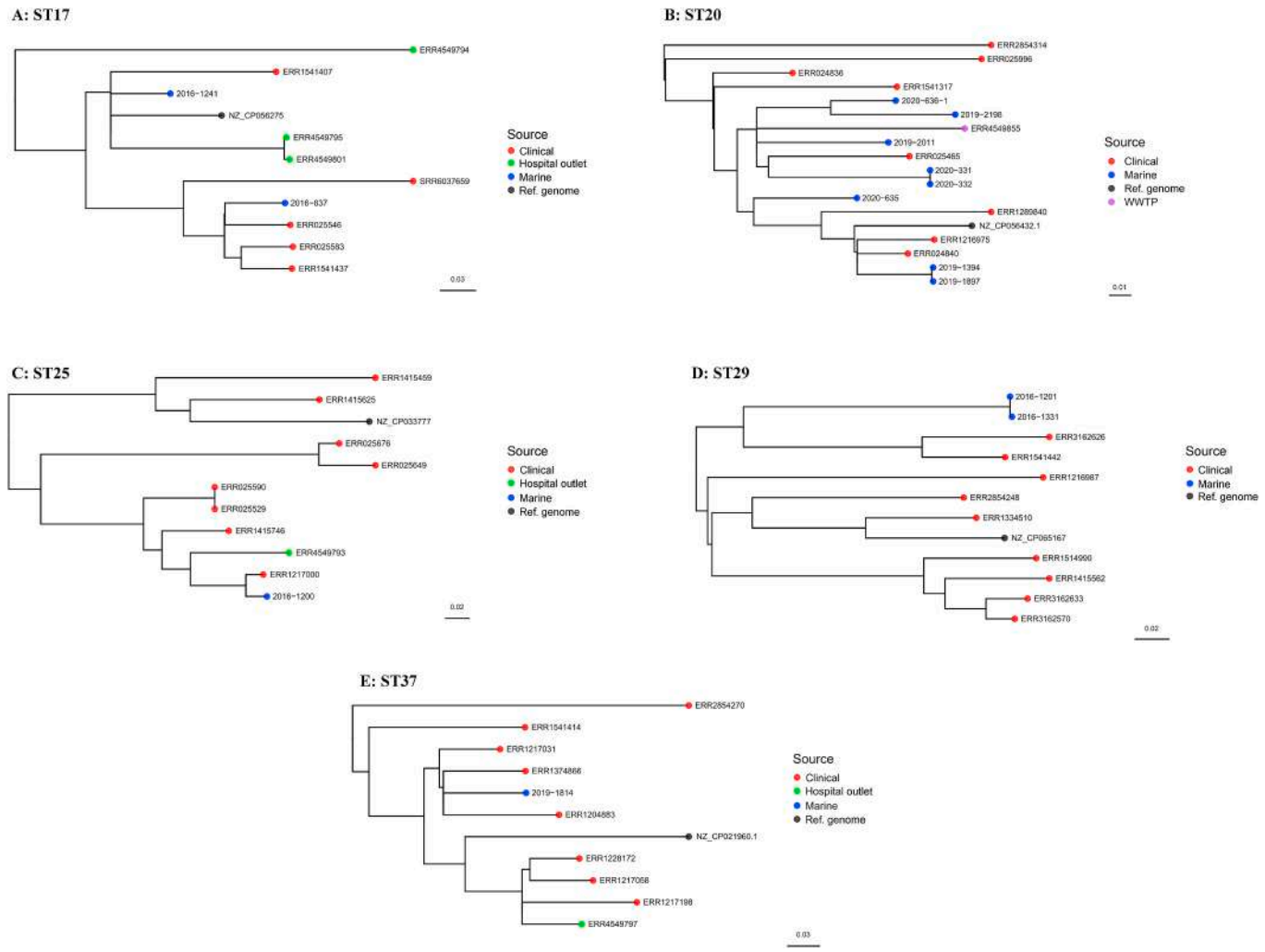

Fig. 2. Comparison of globally disseminated *Klebsiella pneumoniae* sequence types (STs) isolated from the marine environment and isolates of the same STs of human origin. A: ST17, B: ST20, C: ST25, D: ST29, E: ST37.

et al., 2015). This may explain the high genetic diversity of Kp observed in our study.

Our study included an ST specific comparison of a small set of marine isolates belonging to globally disseminated STs with human/clinical isolates. ST17, ST20 and ST37 isolates from the marine environment differed from isolates of human origin by 149–588 SNPs, indicating that isolates belonging to these STs recovered from the marine environment are not clonally related to the human/clinical genomes included in the study. Kp isolate 2016-1200 isolated from *M. edulis* collected from a production location in the middle of Norway carried multiple ARGs as well as genes encoding the yersiniabactin siderophore associated with human infection. We found a close genetic relationship between this isolate, belonging to ST25, and a clinical isolate from Germany causing BSI (24 SNPs). Further comparison revealed that the two isolates shared the same ARGs, HMRGs, virulence genes and plasmid replicons. Kp ST25 is associated with infections in both humans and animals (Bidewell et al., 2018; Wyres et al., 2020a, 2020b; Struve et al., 2015). The presence of MDR Kp with acquired virulence genes in bivalves reared for human consumption is especially worrisome, both with regards to transmission of pathogenic bacteria to the human population and the spread of ARGs and virulence genes in the food-production chain.

Overall, the frequency of acquired ARGs was low in Kp and other members of the KpSC isolated from the marine environment. Five of the antibiotic resistant Kp isolates also carried genes encoding resistance to heavy metals (*pco, sil, mer* and/or *ars*). This was also seen in the single *K. quasipneumoniae* subsp. *quasipneumoniae* isolate with acquired ARGs. These isolates also carried IncFIB(K) or IncFIB(K)(pCAV1099-114) plasmid replicons. Recently, we reported the co-occurrence of $bla_{CTX\text{-}M\text{-}3}$, $bla_{TEM\text{-}1}$, *pco, sil* and *ars* genes on an IncFIB(K)/IncFII(pKP91) plasmid in Kp from bivalves (Håkonsholm et al., 2020). HMRGs were common in our collection of Kp isolated from marine sources. This has also been reported in Kp from cattle suffering from mastitis, where *pco, sil* and *ars* genes were commonly found. The same study also showed lower frequencies of HMRGs in strains of human and environmental origin (Zheng et al., 2021). Heavy metals, especially copper, is commonly used in anti-fouling agents in aquaculture, and is also present in fish feed (Burridge et al., 2010; Grefsrud et al., 2021). Further, heavy metals are used in fertilisers in agriculture (Seiler and Berendonk, 2012), and may thus be introduced to the marine environment through run-off from land. Low concentrations of heavy metals are sufficient to select for and maintain the presence of antibiotic resistant bacteria in the environment (Gullberg et al., 2014). Thus, Kp isolates carrying heavy metal resistance genes may persist in metal contaminated marine environments and potentially contribute to dissemination of clinically important antibiotic resistance genes and related plasmids in the environment.

Yersiniabactin is one of the major virulence factors in Kp associated with human infections (Holt et al., 2015). In our study, *ybt* was identified in ten isolates, suggesting that Kp with pathogenic potential are present in bivalves produced for human consumption. Additionally, two isolates had capsule loci encoding capsule types associated with hvKp (K2 and

K57) (Russo and Marr, 2019). These findings suggest that potentially pathogenic Kp strains are present in the marine environment.

The presence of Kp in food and its association with human colonisation and infection is not well understood (Wareth and Neubauer, 2021). Since several studies on Kp in food have focused on retail food or food from markets (Hartantyo et al., 2020; Aguilar-Bultet et al., 2020), it is difficult to know where in the food-production chain the contamination has occurred (Huynh et al., 2020). Although no Kp were recovered from bivalves cleared for market, our study shows that bivalves from commercial production locations and coastal waters can carry Kp. Furthermore, we show close genetic relatedness between isolates from the marine environment and clinical isolates associated with human infections and MDR in bivalves produced for human consumption. Our study therefore supports the notion that consumption of raw or undercooked bivalves potentially may represent a risk of GI colonisation by Kp.

5. Conclusions

Our study reveals high genetic diversity among Kp isolated from seawater and bivalve molluscs collected from the Norwegian marine environment, including globally disseminated STs associated with MDR and hypervirulence. Along with ARGs, HMRGs were widespread in Kp from the marine environment, suggesting potential for co-selection of antibiotic resistance. Further, we show that Kp carrying clinically relevant ARGs and virulence genes genetically related to clinical isolates were present in bivalves, indicating potential for seafood borne transmission of Kp to humans. Our study thus indicates that the marine environment, especially the coastal environment, is a potential source of Kp, and further illustrates the need for environmental monitoring of pathogens and antimicrobial resistance.

Authorship contribution statement

Fredrik Håkonsholm., Nachiket P. Marathe, Bjørn Tore Lunestad, Iren H. Löhr and Cecilie S. Svanevik contributed to the design and conception of the study. Fredrik Håkonsholm performed the experiments, bioinformatic analyses were done by Fredrik Håkonsholm and Marit A.K. Hetland. Fredrik Håkonsholm prepared the first draft of the manuscript, all authors reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data availability

The raw reads, genome assemblies and annotations are available under BioProject PRJNA591480. BioSample accession number and GenBank accession number for the individual genomes included in the study are presented in Table S1.

Declaration of competing interest

The authors declare 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.2022.113967.

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Internal exposure of Flemish teenagers to environmental pollutants: Results of the Flemish Environment and Health Study 2016–2020 (FLEHS IV)

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ABSTRACT

The Flemish Environment and Health Study (FLEHS) collects information on internal exposure to a broad range of environmental chemicals in the general population in Flanders, the Northern region of Belgium. The aim is to establish biomonitoring exposure distributions for the general population in support of public health and environmental policy, environmental risk assessment and risk management decisions. In 2017–2018, urine and blood samples were collected from 428 teenagers by a stratified clustered two stage randomized design. Samples were analyzed for a broad range of biomarkers related to exposure to chlorinated and newer pesticides, brominated and organophosphate flame retardants (BFR/OPFR), polychlorinated biphenyls (PCBs), bisphenols, phthalates and alternative plasticizers, per-and polyfluoroalkyl substances (PFAS), polycyclic aromatic hydrocarbons (PAHs), benzene, metals and trace elements. The geometric mean levels and percentiles of the distribution were estimated for each biomarker, for the whole study population and following stratification for sex, the household educational attainment and the residence area's urbanicity.

Geometric means of biomarkers of lead, dichlorodiphenyltrichloroethane (DDT), PCBs, PAHs, regulated phthalates and bisphenol A (BPA) were lower than in the previous FLEHS cycles.

Most biomarker levels were below health-based guidance values (HB-GVs). However, HB-GVs of urinary arsenic, blood lead, blood cadmium, sum of serum perfluorooctane sulfonate (PFOS) and perfluoro-1-hexanesulfonate (PFHxS) and the urinary pyrethroid metabolite (3-PBA) were exceeded in respectively 25%, 12%, 39.5%, 10% and 22% of the teenagers. These results suggest that the levels of exposure in the Flemish population to some environmental chemicals might be of concern.

At the same time, we noticed that biomarkers for BPA substitutes, metabolites of OPFRs, an expanded list of PFAS, glyphosate and its metabolite could be measured in substantial proportions of participants. Interpretation of these levels in a health-risk context remains uncertain as HB-GVs are lacking.

Household educational attainment and residential urbanicity were significant exposure determinants for many biomarkers and could influence specific biomarker levels up to 70% as shown by multiple regression analysis.

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The research consortium also took care of the broader external communication of results with participants, policy makers, professional groups and civil society organizations. Our study demonstrated that teenagers are exposed to a wide range of chemicals, it demonstrates the success of public policies to reduce exposure but also points to concern and further priorities and needs for follow up.

Abbreviations

1-OH PYR	1- Hydroxypyrene
2,4-D	2,4-Dichlorophenoxyacetic acid
3-PBA	3-Phenoxybenzoic acid
AML	Algemeen Medisch Laboratorium
AMPA	Aminomethylphosphonic acid
ANOVA	Analysis of Variance
As	Arsenic
BBOEHEP	2-Hydroxyethyl bis(2-butoxyethyl) phosphate
BBzP	Benzyl butyl phthalate
B-Cd	Blood cadmium
BDCIPP	Bis(1,3-dichloro-2-propyl) phosphate
BE	Biomonitoring Equivalent
BFR	Brominated flame retardants
BMI	Body Mass Index
BPA	Bisphenol A
B-Pb	Blood lead
BPF	Bisphenol F
BPS	Bisphenol S
CAS	Chemical Abstracts Service
Cd	Cadmium
CHOL	Cholesterol
CI	Confidence Interval
Cr	Creatinine
cx-MEPP	Mono-(2-ethyl-5-carboxypentyl) phthalate
DDE	Dichloro-diphenyl-dichloroethylene
DDT	Dichloro-diphenyl-trichloroethane
DEHA	Di-(2-ethylhexyl) adipate
DEHP	Bis(2-ethylhexyl) phthalate
DEHTP	Di-(2-ethylhexyl) terephthalate
DEP	Diethyl phthalate
DiBP	Diisobutyl phthalate
DINCH	Di-(iso-nonyl)-cyclohexane-1,2-dicarboxylate
DnBP	Dibutyl phthalate
ECHA	European Chemicals Agency
EFSA	European Food Safety Agency
EHPHP	2-Ethylhexyl phenyl phosphate
eNO	Exhaled nitrogen oxide
ETS	Environmental Tobacco Smoke
EU	European Union
FAOSTAT	Food and Agriculture Organization of the United Nations
FLEHS	Flemish Environment and Health Study
FLU	Fluorene
GDPR	General Data Protection Regulation
GerES	German Environmental Survey
γ-HCH	Gamma-hexachlorocyclohexane
GLY	Glyphosate
GM	Geometric Mean
HB-GVs	Health-based guidance values
HBM	Human biomonitoring
HBM I	HBM-I value
HBM II	HBM-II value
HCB	Hexachlorobenzene
HCH	Hexachlorohexane
IARC	International Agency for Research on Cancer
ISCED	International Standard Classification of Education
LOD	Limit of Detection
LOQ	Limit of Quantification
MEHP	Mono-(2-ethylhexyl) phthalate
MEP	Monoethyl phthalate
MiBP	Mono-isobutyl phthalate
Mn	Manganese
MnBP	Mono-n-butyl phthalate
n	Sample size
NAPH	Naphthalene
NES	Neurobehavioral Evaluation System
NUTS	Nomenclature of Territorial Units for Statistics
OH	Hydroxy
OPFR	Organophosphate flame retardants
OXC	Oxychlordane
P50	50th Percentile
P90	90th Percentile
P95	95th percentile
PAHs	Polycyclic aromatic hydrocarbons
Pb	Lead
PBDEs	Polybrominated diphenylethers
PCBs	Polychlorinated biphenyls
PFAS	Per-and polyfluoroalkyl substances
PFDA	Perfluoro-n-decanoic acid
PFHxS	Perfluoro-1-hexanesulfonate
PFNA	Perfluorononan-1-oic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
PHE	Phenanthrene
PIH	Provincial Institute of Hygiene of Antwerp
RQ	Risk Quotients
S	Serum
SD	Standard deviation
SES	Socio-economic status
SG	Specific Gravity
β-HCH	Beta-hexachlorocyclohexane
t,t'-MA	t,t'-Muconic acid
TCPγ	3,5,6-Trichloro-2-pyridinol
TG	Triglycerides
TI	Thallium
TN	Trans-nonachlor
TRA	Toxic relevant arsenic
U	Urinary
US-CDC	US-Centers for Disease Control and Prevention

1. Introduction

Environmental health concerns and a decree of Preventive Health Care voted in 2003 in Flanders, the Northern region of Belgium, led to the mandatory set up of a network for surveillance of human exposure and/or effects of exposure to physical and chemical factors in the population, intending to take measures to protect public health. As a follow-up, every five years since 2002, a cross-sectional cohort was established to assess the complex relationship between environmental pollution and

health. The Flemish Environment and Health Study (FLEHS) recruits a sample of a selected age group that is representative of sex and geographical area. Targets are set to include participants from different socio-economic statuses and the degree of urbanization of their residential neighborhood. FLEHS periodically measures concentrations of environmental chemicals and/or their metabolites in blood and urine of the study participants and relates it to their personal environment and life style (Schoeters et al., 2012). These exposure biomarkers, measured in easily accessible body fluids or tissues, aggregate chemical uptake from different sources and exposure routes and reflect the internal dose of a chemical (Angerer et al., 2007). In the fourth FLEHS campaign, FLEHS IV (2016–2020), 80 exposure biomarkers, belonging to eight different chemical classes, were measured including regulated compounds and substitutes that replace regulated and banned compounds.

Teenagers of 14 and 15 years old were included in FLEHS IV. They are considered sentinels of their local living environment and have been included in each of the FLEHS cycles. Teenagers may be particularly vulnerable to environmental exposures. The biology of adolescence is distinctive and provides opportunities for unique actions of toxicants both in terms of disruption of function and disruption of maturation (Golub, 2000). Getting adolescents involved in a human biomonitoring (HBM) study raises awareness of lifestyle and environment and how this may connect to exposure to hazardous chemicals and their health.

Exposure biomarkers were selected for chemical substances known to be toxic to humans (in particular carcinogenic or endocrine disruptor effects) and present in the environment of the Flemish population. We included biomarkers for chemicals that are associated with the residential neighborhood's spatial planning (metals, combustion-related compounds, pesticides), dietary habits (metals, persistent organic compounds, pesticides, plasticizers, per-and polyfluoroalkyl substances) and housing (combustion-related compounds, flame retardants, plasticizers). We also included biomarkers that were measured in previous HBM cycles, allowing to evaluate changes over time. For some of these substances, regulation at the European Union (EU) level and the member states exists. The efficiency of the regulation to decrease human exposure can be checked by HBM. As regulated substances are often replaced by other substances for which information on exposure and health effects is lacking, we also included biomarkers for chemicals of emerging concern.

This manuscript provides an overview of the internal exposure distributions of biomarkers in Flemish teenagers and puts them in a health risk context if health-based guidance values (HB-GVs) are available (Wilhelm, 2014). HB-GVs are considered as biomonitoring concentrations that are linked by physiology based pharmacokinetic modelling to chemical-specific intake limits. They correspond to binding effect levels derived from experimental animal studies or directly from human data based on a relationship between internal concentrations and health effects (Apel et al., 2020a). We compared the recent measurements in FLEHS IV with the levels measured in the previous cycles (Schoeters et al., 2017). Furthermore, we tested whether internal exposure to environmental chemicals was different depending on the urbanicity of the residence area and on the household educational attainment, as a proxy marker for socio-economic status (SES). Next to that, insight is given in the way these results were reported back to the participants and to the community they belong to.

2. Materials and methods

2.1. Study design and sampling

Four hundred twenty-eight teenagers aged 14 and 15 years old, took part in the FLEHS IV study. The sampling was organized between September 2017 and June 2018 according to a stratified clustered two stage sampling design. Within the age group, the study sample was representative of geographical location and sex. The first stratification corresponded with the Flemish provinces which are considered as level 2 according to the nomenclature of territorial units for statistics (NUTS) in the EU. The number of participants per province was proportional to the number of inhabitants per province. Primary sampling units were schools that were randomly selected in each province. Distances between schools had to be at least 20 km and one school in the highest quartile of socially deprived attendants was included in each province to ensure participation of all socio-economic categories. Teenagers and one of the parents had to give their signed informed consent. Further inclusion criteria were: living in Flanders for at least five years and teenagers and parents mastered enough Dutch to fill out extensive questionnaires. Exclusion criteria were: more than one questionnaire not filled out, blood and urine sample missing, being held back in school for more than one year, attending a boarding school. Data of one subject were not considered in the analysis due to pregnancy. The FLEHS IV study protocol was approved in June 2017 by the Antwerp University Hospital Ethics committee (Belgian registration number B300201732753). Included as well was a statement on the report-back of the individual exposure results to the parents and teenager, and if preferred to their general practitioner. The medical practitioner of the Provincial Institute of Hygiene (PIH) also intercepted individual questions of respondents on their results afterwards.

Before clinical examination, teenagers filled out extensive questionnaires on health status and life style patterns such as the use of cosmetics, tobacco and alcohol, dietary habits, time spent in traffic, and their opinion on their environmental risk perception, attitudes and eco-behavior. Additionally, a short questionnaire was filled out at the day of sampling on recent exposure (i.e. within the last three days), e.g. smoking, medication, alcohol and food consumption. Parents filled out a questionnaire on the home environment, the housing conditions, pregnancy of the data subject, SES (e.g. educational level of the parents, household income). Teenagers and parents together filled out the food questionnaire. The participants chose to use electronic or paper questionnaires; the latter was preferred by 60% of parents and 40% of teenagers. The main characteristics of the study participants are presented in Table 1 and compared to information of the general Flemish population of the same age group to check representativity of the FLEHS IV study participants.

Clinical examinations were organized at the school location, they took about 1 h and were performed by trained nurses. The teenagers donated a urine sample of minimally 46 mL, a hair sample of 3 cm that was taken close to the scalp and a 35 mL blood sample. Urine samples were collected in clean metal-free polyethylene containers; they were kept at 4 °C and processed within 24 h. Samples were divided into aliquots with glass vials as recipients for measurement of the biomarkers for plastic compounds, polypropylene tubes for measurement of the biomarkers for benzene, PAHs and arsenic species. Metal-free polyethylene tubes were used for measurement of the other metal biomarkers. Until analysis, all samples were kept at −20 °C, except the vials for measurement of biomarkers of benzene and PAHs that were kept at −80 °C. The blood samples were immediately processed: 2 aliquots of whole blood and serum were kept at 4 °C and stored at −20 °C or −80 °C within 12 h in a central lab. When field work was finished, all samples, together with field work blanks and control samples, were shipped on dry ice to the analytical laboratories until sample work-up.

Clinical examinations included measuring body height and body weight while teenagers were not wearing shoes but were fully clothed. Abdominal circumference provided information on obesity risk. Blood pressure was measured according to the European Society of Hypertension guidelines using an automated blood pressure instrument. Exhaled nitrogen oxide (eNO) was measured as an indicator for airway inflammation. A Neurobehavioral Evaluation System computerized battery of tests (NES) was used to assess sustained attention, short-term memory and manual motor speed. Sustained attention was assessed by continuous performance and Stroop test, the short-term memory by the digit span forward and backward and the motor speed by a finger tapping task (Kicinski et al., 2016). Collection, storage, transfer, and use of

Table 1
Main characteristics of teenagers (14–15 years) selected in the FLEHS IV study in comparison with the Flemish population at age 15. Sample size and % of the study population.

	FLEHS IV	Flemish reference population 15 years
Sex		Statbel 2018 [1]
Boys	199 (46.5%)	34502 (51.3%)
Girls	229 (53.5%)	32795 (48.7%)
Age (years)		
≤14.5	117 (27.3%)	
14.5–15.5	277 (64.7%)	
>15.5	34 (7.9%)	
Educational level		Number of students in the third year of secondary education in schools of the Flemish region, 2017–18[2]
Vocational education	79 (18.5%)	20.6%
Technical education	133 (31.1%)	27.9%
General education	216 (50.5%)	49.6%
Art education	0 (0%)	1.9%
Household educational attainment (ISCED)		Statbel 2018 [3]
Primary	26 (6.2%)	19%
Secundary	140 (33.4%)	40%
Tertiary	254 (60.4%)	41%
Country of birth		Flemish region, 2016 [4]
Non-EU	43 (10.1%)	11.3%
EU	36 (8.4%)	9.2%
Belgium	348 (81.5%)	79.5%
Urbanicity (Eurostat)		Eurostat 2016
Rural areas	59 (13.8%)	9%
Towns and suburbs	311 (72.7%)	72%
Cities	58 (13.6%)	19%
Province of residence		Statbel, 2018 [1]
Antwerp	117 (27.3%)	28.2%
East-Flanders	91 (21.3%)	23.0%
West-Flanders	82 (19.2%)	18.2%
Limburg	62 (14.5%)	13.3%
Flemish-Brabant	76 (17.8%)	17.4%
Season		
Autumn	100 (23%)	
Winter	138 (32%)	
Spring	190 (44%)	
Summer	0 (0%)	
Smoking behavior		Student survey, 2016–2017 [5]
Never or once	409 (95.8%)	93.1%
Seldom	8 (1.9%)	4.1%
Daily	10 (2.3%)	2.7%
Exposure to environmental tobacco smoke		
Yes	161 (38.2%)	
No	261 (61.9%)	
Alcohol use		Student survey 2016–2017 [5]
Never or very seldom	365 (85.7%)	68.2%
< weekly	52 (12.2%)	23.4%
Weekly	9 (2.1%)	8.4%
Body Mass Index boys [6]		Food consumption survey 2014 [7]
Underweight	17 (8.5%)	10.0%
Normal weight	152 (76.4%)	77.6%
Overweight, obese	30 (15.1%)	12.5%
Body mass Index girls [6]		Food consumption survey 2014 [7]
Underweight	18 (7.9%)	10.7%
Normal weight	156 (68.1%)	69.0%
Overweight, obese	55 (24.0%)	20.4%

[1] https://Statbel.fgov.be/.
[2] http://www.ond.vlaanderen.be/.
[3] https://Statbel.fgov.be/; Highest educational level of Flemish adults between 25 and 64 years (n = 3 445 221) with BSO: Vocational Secondary Education, TSO: Technical Secondary Education, ASO: General Secondary Education, KSO: Artistic Secondary Education.
[4] Flemish Regional Indicators (VRIND) 2017; https://www.vlaanderen.be/publicaties/vrind-2017-vlaamse-regionale-indicatoren.
[5] Flemish Expertise Centre Alcohol and drugs: questioning of students 13-16 years- (2016–2017), https://www.vad.be/.
[6] Flemish growth curves of 2004, https://www.vub.be/groeicurven/.
[7] Sciensano 30/08/2019, information from Flemish students (10–17 years); International Obesity Task Force Criteria used as cut off points.

data were carried out according to the European General Data Protection Regulation (GDPR, Regulation (EU) 2016/679). The database was registered at the Belgian Committee for Privacy Protection (VT005081316). All data were pseudonymized prior to further analysis. According to the communication strategy and its agreed guiding principles, the participants received their interpretable personal results, positioned against the median and 90th percentile of the total study group and compared to the thresholds (HB-GVs). They also received a summary of the aggregated study results; background information on the chemical substances and suggestions for health promotional measures. The generic information is also made available on the website https://www.milieu-en-gezondheid.be/, including short video presentations. It is the consortium's broad interdisciplinary configuration - including toxicologists, biologists, medical scientists, statisticians and social scientists - that ensures the combination of these different components in the study design.

2.2. Selection and analysis of exposure biomarkers

Arsenic (As), cadmium (Cd), lead (Pb) and thallium (Tl) were included as metals. Biomarkers for these compounds have been measured in previous cycles.

PAHs and benzene were selected as combustion-related compounds. We have biomonitoring data from previous FLEHS studies for the pyrene metabolite 1- hydroxypyrene (1-OH PYR) and for t,t'-muconic acid (t,t'-MA) as benzene metabolite. In the present study, we included additional metabolites for some more volatile PAHs: naphthalene (NAPH), phenanthrene (PHE) and fluorene (FLU).

Plastic compounds included bisphenols and metabolites of phthalates that were measured in the urine samples.

To assess exposure to pesticides, biomarkers for pyrethroids, chlorpyrifos, the phenoxyherbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and glyphosate (GLY) and its metabolite aminomethylphosphonic acid

(AMPA) were measured for the first time in Flemish teenagers. Legacy pesticides, such as beta-and gamma-hexachlorocyclohexane (β-HCH and γ-HCH), p,p'-dichloro-diphenyl-trichloroethane (DDT) and its metabolite p,p'-dichloro-diphenyl-trichloroethane (DDE), the chlordane-related compounds oxychlordane (OXC) and trans-nonachlor (TN) and the fungicide hexachlorobenzene (HCB) were measured in serum samples in FLEHS IV and in previous studies.

Flame retardants measured in serum included the polybrominated diphenylethers (PBDEs), while metabolites of organophosphate flame retardants (OPFRs), that are replacing the PBDEs, were measured in urine. Other persistent substances that were measured are marker PCBs (PCB138, PCB153 and PCB180) and PFAS. Marker PCBs were also measured in teenagers in all three previous FLEHS studies. We measured for the first time in serum of teenagers, recruited from the general population, twelve different long-chain perfluoroalkyl substances, including perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorononan-1-oic acid (PFNA) and the slightly shorter perfluorohexane-1-sulphonic acid (PFHXS) (Supplemental Material S 1).

Table 2 lists the targeted parent compounds, the biomarkers that were selected, the analytical methods and the references to publications that report on the treatment of samples, analytical methods and quality assurance protocols. Table 2 also lists the Limit of Detection (LOD) or Limit of Quantification (LOQ) as provided by the analytical laboratories. Information on linearity, reproducibility, repeatability, accuracy, precision and quality assurance procedures of each of the analytical methods was collected. Triglycerides (TG) and cholesterol (CHOL) were routinely measured in blood samples. The following formula calculated total blood lipid concentration- TL = 1.33*TG + 1.12*CHOL*148 (g/L) (Covaci and Voorspoels, 2005) and was used to standardize lipid-soluble serum biomarkers. Specific gravity (SG) and creatinine (Cr) were determined in urine by refractometry and the Jaffe method at the General Medical Laboratory (Algemeen Medisch Laboratorium -AML, Antwerp, Belgium). Urinary biomarker concentrations were normalized for SG using the following formula: $biomarker_{SG} = C_{biomarker} * (1.024\text{-}1)/(SG\text{-}1)$ with $biomarker_{SG}$ as the normalized biomarker concentration, $C_{biomarker}$ as the measured biomarker concentration per liter urine and SG as the specific gravity of the urine sample (Pearson et al., 2009). Urinary dilution was checked based on criteria set for unsubstituted urine samples (Barbanel et al., 2002). All urine samples had SG above 1.001 and Cr above 5 μg/dL.

2.3. Statistical analysis

Detection frequencies were calculated for each chemical biomarker. Statistical analyses were carried out if biomarker values were above LOQ in at least 60% of the samples. Values below the LOQ were imputed with a random value (between 0 and the LOQ), drawn from the estimation of the lognormal distribution of all values by fitting a truncated lognormal distribution using only values above the LOQ (Lubin et al., 2004). The median, 90th and 95th percentile (P90, P95) and the geometric mean (GM) with 95% confidence interval (95% CI) were calculated. The CI for the percentiles are distribution-free using order statistics (Hahn et al., 2016). PCBs are reported as the sum of the marker PCBs (PCB138, PCB153, PCB180).

The proportion of participants with values above HB-GVs was calculated for biomarkers for which HB-GVs were available. We selected HB-GVs derived by international organizations such as the European Chemicals Agency (ECHA) or the European Food Safety Agency (EFSA), or published in peer-reviewed literature with preference to more recently derived values (Table 3). In addition, risk quotients (RQ) were calculated as the ratio of the biomarker concentration, at the GM or P95, to the chemical-specific HB-GV: RQ = [biomarker]/HB-GV).

Several biomarkers have been measured in the same age group in previous FLEHS studies, starting from 2002. Comparisons in time until FLEHS III have been published earlier (Schoeters et al., 2017). To allow comparison with previous FLEHS cycles, biomarkers were treated in the same way as in previous cycles. Values below LOD/LOQ were imputed by half LOD/LOQ. To check analytical comparability, biobank samples from previous FLEHS cycles were included in the present analytical runs. Multiple regression models were established based on the pooled dataset of the different cycles to evaluate the influence of time on chemical biomarker concentrations. To account for differences in composition of the sampled study participants between the successive cycles, all models were adjusted for sex, smoking, age and body mass index (BMI). Additionally, urinary biomarkers were adjusted for Cr and lipid soluble serum biomarkers were adjusted for blood lipids. All these variables were categorized (Table 1). Analysis of variance (ANOVA) was used to test for differences among biomarker-specific GMs of different FLEHS studies. If differences were statistically significant ($p < 0.05$), a two sided t -test was used to assess the difference of the GM of the FLEHS IV HBM data with those of previous studies. This allows complementing the time trend analysis (Schoeters et al., 2017) with the new data of the FLEHS IV study.

We also tested whether neighborhood urbanicity and SES of the household influenced the exposure biomarkers. Urinary biomarkers were normalized to SG, lipid soluble serum biomarkers were normalized to serum lipids. Participants' home addresses were geocoded. The Eurostat definition of urbanicity was used to classify the degree of urbanization of the residence area. The Eurostat definition is based on a combination of geographical contiguity and population density, applied to 1 km^2 population grid cells (Eurostat, 2018). Eurostat defines neighborhoods as 1) cities: densely populated areas where 50% or more of the population lives in urban centers with a population density of at least 1500 inhabitants per km^2 and at least 50 000 inhabitants, 2) towns/suburbs: intermediate density areas where 50% or more of the population lives in urban clusters with a population density of at least 300 inhabitants per km^2 and a minimum population of 5000 inhabitants, 3) rural areas: sparsely populated areas outside of city centers and urban clusters. The variable could be calculated for all participants.

The highest household educational attainment was taken as a proxy for the SES of the household. The information was available for 420 out of 428 participants. Individuals with missing values were not included in the analysis. Three categories were considered based on to the Belgian education system: primary (no educational attainment, primary school, lower secondary school), secondary (higher secondary school) and tertiary (higher education attainment). These levels correspond to the codes 0–2, 3–4 and 5–8 of the International Standard Classification of Education (ISCED). To test the influence of neighborhood urbanicity and SES of the household on the exposure biomarkers, multiple regression models were built. The exposure markers were used as dependent variables. They were log-transformed to achieve normal distributions. A first model was built that included the variables of interest, either urbanicity or educational attainment, as well as variables that may influence the exposure biomarkers but are known not to be associated with the variables of interest such as sex, age, sampling season and additionally serum ferritin for metals, SG for urinary markers, blood lipids for lipid-soluble serum markers. Serum ferritin, SG and blood lipid concentrations were used as continuous variables and other variables were categorized. We assessed whether the variable of interest was significant in these models. If significant ($p < 0.05$) or borderline significant ($p < 0.10$), the estimated biomarker levels were compared with the reference categories which were the primary educational level for the models with the household educational attainment as the variable of interest and with cities for the degree of urbanicity as a variable of interest. Further models included variables that may influence the exposure biomarkers and may be linked to the variable of interest such as smoking behavior, BMI, being breastfed. Boys and girls were classified as underweight, average weight, overweight or obese according to the sex- and age-specific 2004 Belgian growth curves (Roelants et al., 2009). Finally, we tested whether differences in biomarker levels between girls and boys remained significant in a multivariate model after adjustment for the above-mentioned variables, household educational attainment

Table 2
Biomarker levels measured in FLEHS IV teenagers normalized to specific gravity (urinary markers) and serum lipids (lipid-soluble serum biomarkers).

Parent compound (CAS nr)	Biomarker	Analytical Method	Unit	LOD/ LOQ	Sample size	% above LOD/ LOQ	GM (95% CI)	P50	P90	P95 (95%CI)
Metals and trace elements in urine										
Anorganic arsenic7440-38-2	Arsenobetaïne	UPLC-MS/MS(De Craemer et al., 2017)	µg/ L	LOD = 0.1	194	83.5	1.44 (0.96;2.15)	2.17	30.1	46.8 (34.8;78.3)
	Arsene(III)			LOD = 0.1	194	70.6	0.18 (0.14;0.23)	0.32	0.88	1.19 (0.98;1.80)
	Arsene(V)			LOD = 0.1	194	26.3	NC	<LOD	0.28	0.36 (0.29;0.58)
	Mono methyl arsenate (MMA)			LOD = 0.1	194	83.0	0.35 (0.28;0.45)	0.60	1.29	1.66 (1.44;2.16)
	Dimethyl arsenate (DMA)			LOD = 0.1	194	100	3.59 (3.29;3.91)	3.28	8.07	11.62 (9.66;14.58)
	Toxic relevant arsenic (TRA)(sum As III, As V, DMA, MMA)			NA	194		4.62 (4.26;5.02)	4.23	10.30	14.16 (11.48;16.52)
Cadmium7440-43-9	Cadmium (U-Cd)	HR-ICP-MS (Schroijen et al., 2008)		LOD = 0.010	415	100	0.300 (0.287;0.313)	0.287	0.494	0.630 (0.545;0.850)
Thallium 7440-28-0	Thallium (U-Tl)			LOD = 0.002	415	100	0.355 (0.345;0.365)	0.359	0.502	0.562 (0.539;0.582)
Metals and trace elements in blood										
Cadmium7440-43-9	Cadmium (B-Cd)	HR-ICP-MS (Schroijen et al., 2008)	µg/ L	LOD = 0.007	419	100	0.19 (0.18;0.20)	0.18	0.29	0.38 (0.34;0.51)
Thallium 7440-28-0	Thallium (B-Tl)		ng/ L	LOD = 0.651	419	100	27.3 (26.7;27.9)	26.8	35.6	38.9 (37.3;42.0)
Lead7439-92-1	Lead (B-Pb)		µg/ L	LOD = 0.048	419	100	7.7 (7.4;8.0)	7.7	12.8	14.3 (13.6;17.5)
Manganese7439-96-5	Manganese (B-Mn)		µg/ L	LOD = 0.120	419	100	9.4 (9.1;9.6)	9.3	13.7	15.6 (14.6;16.2)
Copper7440-50-8	Copper (B-Cu)		µg/ L	LOD = 0.551	419	100	816 (801;831)	792	1024	1252 (1093;1378)
Zinc7440-66-6	Zinc (B-Zn)		mg/ L	LOD = 0.008	419	100	5.29 (5.21;5.37)	5.35	6.53	6.88 (6.72;7.08)
Polycyclic aromatic hydrocarbons and benzene in urine										
Pyrene129-00-0	1-Hydroxypyrene (1-OH PYR)	UPLC-MS/MS(Verheyen et al., 2021)		LOQ = 0.015	412	97.6	0.067 (0.063;0.071)	0.066	0.134	0.164 (0.142;0.218)
Naphthalene91-20-3	2-Hydroxynaphthtalene (2-OH NAPH)			LOQ = 0.150	413	100	4.07 (3.73;4.43)	3.75	12.39	19.4 (15.1;24.5)
Fluorene86-73-7	sum of 2- and 3-Hydroxy-fluorene (2-OH FLU + 3-OH FLU))			LOQ = 0.030	413	99.5	0.208 (0.197;.0.220)	0.192	0.403	0.555 (0.466;0.689)
Phenanthrene85-01-8	2-Hydroxyphenantrene (2-OH PHE)			LOQ = 0.015	414	97.6	0.074 (0.070;0.078)	0.072	0.147	0.178 (0.162;0.205)
	3-Hydroxyphenantrene (3-OH PHE)			LOQ = 0.014	414	98.8	0.073 (0.069;0.077)	0.072	0.133	0.168 (0.142;0.219)
	sum of 1- and 9-Hydroxy-phenantrene (1-OH PHE + 9-0H PHE)			LOQ = 0.031	414	98.1	0.111 (0.104;0.118)	0.105	0.240	0.323 (0.266;0.546)
	4-Hydroxyphenantrene (4-OH PHE)			LOQ = 0.014	414	7.00	NC	<LOQ	<LOQ	0.018 (<LOQ;0.030)
Benzene71-43-2	T,t'-muco nic acid (t,t'-MA)	HPLC-UV (Schoeters et al., 2017)		LOD = 2	415	99.3	92.0 (83.8;101.0)	101	251	422 (300;600)
Bisphenols in urine										
80-05-7	Bisphenol A (BPA)	GC-MS/MS (Gys et al., 2020)	µg/ L	LOQ = 0.3 µg/L	414	85.7	1.07 (0.98;1.18)	1.15	3.10	5.13 (3.71;5.85)
620-92-8	Bisphenol F (BPF)			LOQ =	414	96.6	0.17 (0.15;0.19)	0.15	0.73	1.12 (0.86;1.63)

(continued on next page)

Table 2 (*continued*)

Parent compound (CAS nr)	Biomarker	Analytical Method	Unit	LOD/ LOQ	Sample size	% above LOD/ LOQ	GM (95% CI)	P50	P90	P95 (95%CI)
80-09-1	Bisphenol S (BPS)			0.02 µg/L LOQ = 0.04 µg/L	414	83.3	0.13 (0.11;0.14)	0.14	0.46	0.83 (0.68;1.13)
77-40-7	Bisphenol B (BPB)			LOQ = 0.02 µg/L	414	57.0	NC	0.03	0.08	0.11 (0.10;0.13)
843-55-0	Bisphenol Z (BPZ)			LOQ = 0.03 µg/L	414	37.0	NC	<LOQ	0.08	0.18 (0.11;0.31)
1478-61-1	Bisphenol AF (BPAF)			LOQ = 0.02 µg/L	414	12.0	NC	<LOQ	0.02	0.03 (0.03;0.04)
Phthalates in urine										
Diethyl phthalate84-66-2	Monoethyl phthalate (MEP)	LC-MS/MS (Bastiaensen et al., 2021a)	µg/L	LOQ = 0.5	407	100	37.9 (33.3;43.2)	27.2	244.9	569 (346;1332)
Di-n-butyl phthalate84-74-2	Mono-n-butyl phthalate (MnBP)			LOQ = 0.5	407	100	19.7 (18.5;21.0)	19.2	44.8	56.8 (52.2;78.3)
Di-isobutyl phthalate84-69-5	Mono-isobutyl phthalate (MiBP)			LOQ = 0.5	407	100	25.5 (23.5;27.7)	22.5	70.9	120 (83.5;184)
Butylbenzyl phthalate85-68-7	Mono-benzyl phthalate (MBzP)			LOQ = 0.2	407	98.3	3.0 (2.7;3.4)	2.4	15.9	36.0 (27.8;43.5)
Di-2-ethylhexyl phthalate117-81-7	Mono-(2-ethylhexyl) phthalate (MEHP)			LOQ = 0.5	407	83.5	12.5 (11.7;13.4)	12.2	31.9	41.0 (35.9;53.3)
	Mono-(2-ethyl-5-oxohexyl) phthalate (5-oxo-MEHP)			LOQ = 0.2	407	99.5	4.2 (3.9;4.6)	4.1	11.0	15.1 (12.7;19.3)
	Mono-(2ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP)			LOQ = 0.2	407	99.7	6.7 (6.2;7.2)	6.6	17.5	21.5 (19.5;29.6)
	Mono-(2-ethyl-5-carboxypentyl) phthalate (5-c x-MEPP)			LOQ = 0.5	407	100	16.4 (15.7;17.1)	16.2	28.7	34.2 (31.7;41.4)
Pesticides in urine										
Pyrethroids8003-34-7	3-Phenoxybenzoic acid (3-PBA)	HPLC-TQMS (Andersen et al., 2021)	µg/L	LOD = 0.03	415	99.5	0.953 (0.870;1.044)	0.871	2.77	4.14 (3.09;5.75)
Chloropyrifos 2921-88-2	3,5,6-trichloro-2-pyridinol (TCPγ)			LOD = 0.3	415	98.5	4.25 (3.98;4.54)	4.45	10.1	12.3 (10.7;13.6)
Phenoxyherbicide94-75-7	2,4-dichlorophenoxyaceticacid (2,4-D)			LOD = 0.03	415	96.1	0.259 (0.238;0.282)	0.274	0.733	1.003 (0.830;1.135)
Glyphosate1071-83-6	Glyphosate (Gly)	GC-MS/MS (Lemke et al., 2021)	µg/L	LOQ = 0.1	415	41.4	NC	<LOQ	0.280	0.391 (0.332;0.468)
A-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid1066-51-9	Aminomethylphosphonic acid (AMPA)		µg/L	LOQ = 0.1	415	55.9	NC	0.11	0.284	0.371 (0.323;0.448)
Chlorinated pesticides in serum										
Hexachloro Benzene 118-74-1	Hexachlorobenzene (HCB)	GC-ECNI/MS (Covaci and Voorspoels, 2005)	ng/g lipid	LOQ = 2 ng/L	415	100	7.5 (7.2;7.8)	7.6	12.0	14.1 (12.7;15.2)
Dichlorodi Phenyltri Chloroethane 50-29-3	DDT			LOQ = 4 ng/L	415	74.5	1.8 (1.6;2.1)	2.4	8.1	16 (10.9;22.1)
	DDE			LOQ = 4 ng/L	415	100	40.9 (37.9;44.2)	34.4	123	193 (149;247)
Chlordane39765-80-5	Oxychlordane (OXC)			LOQ = 2 ng/L	415	95.4	1.21 (1.14;1.28)	1.21	2.39	3.38 (2.73;3.79)
	Trans-nonachlor (TN)			LOQ = 2 ng/L	415	85.3	0.77 (0.72;0.83)	0.80	1.71	2.37 (2.08;2.62)
Lindane58-89-9	Beta-hexachlorocyclohexane (β-HCH)			LOQ = 2 ng/L	415	95.9	1.13 (1.06;1.20)	1.12	2.15	3.01 (2.39;4.78)

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Table 2 (*continued*)

Parent compound (CAS nr)	Biomarker	Analytical Method	Unit	LOD/ LOQ	Sample size	% above LOD/ LOQ	GM (95% CI)	P50	P90	P95 (95%CI)
	Gamma-hexachlorocyclohexane (γ-HCH)			LOQ = 2 ng/L	415	16.1	NC	<LOQ	<LOQ	0.80 (0.70;0.90)
Polychlorinated biphenyls in serum										
Polychlorinated biphenyls	Marker PCBs (sum of PCB 138, 153, 180)	GC-ECNI/MS (Covaci and Voorspoels, 2005)	ng/g lipid	LOQ = 2 ng/L	415	NA	21.6 (20.4;22.9)	20.8	47.8	57.1 (51.5;72.8)
Brominated Flame retardants in serum										
41318-75-6	BDE 28	GC-ECNI/MS (Covaci and Voorspoels, 2005)	ng/g lipid	LOQ = 1 ng/L	415	0.48	NC	<LOQ	<LOQ	<LOQ
5436-43-1	BDE 47			LOQ = 1 ng/L	415	53.6	NC	<LOQ	0.80	1.20 (1.00;1.60)
60348-60-9	BDE 99			LOQ = 1 ng/L	415	39.7	NC	<LOQ	0.80	1.00 (0.90;1.20)
32534-81-9	BDE 100			LOQ = 1 ng/L	415	0.72	NC	<LOQ	<LOQ	<LOQ
488710-22-1	BDE 153			LOQ = 2 ng/L	415	21.4	NC	<LOQ	0.70	0.90 (0.80;1.40)
207122-15-4	BDE 154			LOQ = 2 ng/L	415	37.5	NC	<LOQ	0.70	0.90 (0.80;0.90)
207122-16-5	BDE 183			LOQ = 2 ng/L	415	0.00	NC	<LOQ	<LOQ	<LOQ
Organophosphate flame retardants and plasticizers (PFRs) in urine										
Triphenylphosphate115-86-6	4-Hydroxyphenyl phenyl phosphate (4-OH-DPHP)	LC-MS/MS (Bastiaensen et al., 2018)	μg/L	LOQ = 0.5 μg/L	413	13.5	NC	<LOQ	0.72	1.05 (0.83;1.47)
	Diphenyl phosphate (DPHP)			LOQ = 0.1 μg/L	413	99.0	1.36 (1.27;1.46)	1.33	3.19	3.97 (3.49;4.47)
	Hydroxyphenyl diphenyl phosphate (OH-TPHP)			LOQ = 0.01 μg/L	413	5.69	NC	<LOQ	<LOQ	0.01 (<LOQ;0.01)
2-ethylhexyl diphenyl phosphate1241-94-7	2-Ethylhexyl phenyl phosphate (EHPHP)			LOQ = 0.05 μg/L	413	99.3	4.18 (3.89;4.50)	4.13	10.6	12.7 (11.6;15.7)
	2-Ethyl-5-hydroxyhexyl diphenyl phosphate (5-OH-EHDPHP)			LOQ = 0.01 μg/L	413	98.3	0.09 (0.08;0.10)	0.09	0.27	0.34 (0.30;0.41)
Tris(2-chloroisopropyl phosphate13674-84-5	1-Hydroxy-2-propyl bis(1-chloro-2-propyl) phosphate (BCIPHIPP)			LOQ = 0.04 μg/L	413	95.2	0.70 (0.61;0.80)	0.69	3.59	6.88 (4.78;14.34)
	Bis(1-chloro-2-propyl) phosphate (BCIPP)			LOQ = 1 μg/L	413	8.53	NC	<LOQ	<LOQ	2.93 (1.27;4.38)
Trischloroethylphosphate115-96-8	Tris(chloroethyl) phosphate(TCEP)			LOQ = 0.04 μg/L	413	28.7	NC	<LOQ	0.09	0.12 (0.10;0.15)
Tris(2-butoxyethyl) phosphate78-51-3	Bis(2-butoxyethyl) phosphate (BBOEP)			LOQ = 0.05 μg/L	413	10.2	NC	<LOQ	0.05	0.10 (0.08;0.13)
	2-Hydroxyethyl bis(2-butoxyethyl) phosphate (BBOEHEP)			LOQ = 0.005 μg/L	413	95.6	0.040 (0.037;0.045)	0.037	0.129	0.245 (0.147;0.320)
	Bis(2-butoxyethyl) 3′-hydroxy-2-butoxyethyl phosphate (3-OH-TBOEP)			LOQ =	413	5.81	NC	<LOQ	<LOQ	0.01 (<LOQ;0.02)

(*continued on next page*)

Table 2 (*continued*)

Parent compound (CAS nr)	Biomarker	Analytical Method	Unit	LOD/ LOQ	Sample size	% above LOD/ LOQ	GM (95% CI)	P50	P90	P95 (95%CI)
Tris(1.3-dichloro-2-propyl) phosphate13674-87-8	Bis(1,3-dichloro-2-propyl) phosphate(BDClPP)			0.01 µg/L LOQ = 0.05 µg/L	413	80.4	0.29 (0.25;0.33)	0.33	1.52	2.93 (1.77;4.03)
Tri-n-butyl phosphate126-73-8	Di-n-butyl phosphate (DNBP)			LOQ = 0.15 µg/L	413	17.1	NC	<LOQ	0.22	0.35 (0.23;0.48)
Per- and polyfluoroalkyl substances in serum										
335-67-1	Perfluorooctanoic acid (PFOA)	UPLC-MS/MS (Supplemental Materials S1)	µg/L	LOQ = 0.2 µg/L	410	100	1.03 (1.00;1.07)	1.00	1.60	1.80 (1.70;2.00)
1763-23-1	Perfluorooctane sulfonate (PFOS)			LOQ = 0.2 µg/L	410	100	2.16 (2.02;2.30)	2.10	4.95	7.30 (6.10;8.00)
355-46-4	perfluoro-1-hexanesulfonate (PFHxS)			LOQ = 0.2 µg/L	410	96.6	0.48 (0.46;0.51)	0.48	0.97	1.30 (1.20;1.60)
375-95-1	Perfluorononanoic acid (PFNA)			LOQ = 0.2 µg/L	410	82.2	0.31 (0.30;0.32)	0.31	0.59	0.70 (0.63;0.86)
375-73-5	perfluoro-1-butanesulfonate (PFBS)			LOQ = 0.2 µg/L	410	0	NC	<LOQ	<LOQ	<LOQ
2706-90-3	perfluoro-n-pentanoic acid (PFPeA)			LOQ = 0.2 µg/L	410	0	NC	<LOQ	<LOQ	<LOQ
307-24-4	perfluoro-n-hexanoic acid (PFHxA)			LOQ = 0.2 µg/L	410	5.37	NC	<LOQ	<LOQ	<LOQ
375-85-9	Perfluoroheptanoic acid (PFHpA)			LOQ = 0.2 µg/L	410	1.22	NC	<LOQ	<LOQ	<LOQ
335-76-2	Perfluorodecanoic acid (PFDA)			LOQ = 0.2 µg/L	410	42.2	NC	<LOQ	0.38	0.49 (0.42;0.63)
2058-94-8	perfluoro-n-undecanoic acid (PFU(n)DA)			LOQ = 0.2 µg/L	410	7.56	NC	<LOQ	<LOQ	<LOQ
335-76-2	Perfluorodeconaoic acid (PFDoODA)			LOQ = 0.2 µg/L	410	1.22	NC	<LOQ	<LOQ	<LOQ
60270-55-5	perfluoro-heptanesulfonate (PFHpS)			LOQ = 0.2 µg/L	410	2.44	NC	<LOQ	<LOQ	<LOQ

GM: geometric mean, LOD: limit of detection, LOQ: limit of quantification, NC: geometric mean was not calculated because less than 60% of the samples could be quantified. UPLC-MS/MS: ultra-high-performance liquid chromatography with tandem mass spectrometry, HPLC-UV: high-performance liquid chromatography with ultra-violet spectroscopy, HR-ICP-MS: High Resolution Inductively Coupled Plasma Mass Spectrometry, HPLC-TQMS: high-performance liquid chromatography/triple quadrupole mass spectrometry, GC-MS/MS: gas chromatography with high-resolution mass spectrometry, LC-MS/MS: liquid chromatography with high-resolution mass spectrometry.

and neighborhood urbanicity.

3. Results and discussion

3.1. Study population

Forty-seven schools were contacted and 43% (n = 20) of them responded positively. Overall, 34% of the invited teenagers agreed to participate. The sociodemographic, lifestyle and residential characteristics of the study participants are summarised in Table 1 and compared with the characteristics of the general Flemish population for this age group. Our study population consisted of 428 teenagers. Its composition reflected the general population of the same age group with slightly more girls compared to boys, but distribution between the sexes is close to being equal. There were relatively more inhabitants of rural areas. The household educational attainment was higher in our study population as seen in previous FLEHS studies (Morrens et al., 2017) but representation improved over time thanks to measures that lowered thresholds to participate. The distribution over school types of the participants accorded well with that of Flanders in general. A minority of 8.1% of teenagers and/or a parent was born in another EU country, 11.1% was born outside the EU. Only 2.3% were daily smokers, which is a decrease compared to previous FLEHS studies and in line with the general Flemish trends. About one-third of the participants reported being exposed to environmental tobacco smoke. Fewer participants consumed alcohol weekly than in the general population and compared to previous FLEHS studies. The distribution of BMI in categories was following data from the general population with a mean BMI of 21.0 kg/m^2 and a standard deviation (SD) of 3.7 kg/m^2. Because recruitment was carried out in collaboration with the schools, no samples were

Table 3
Environmental chemicals, their respective biomarkers and exposure health-based guidance values applied in the FLEHS IV study.

Environmental chemical	Biomarker	Type of Health based guidance value (HB-GV)	Corresponding toxic endpoint	Unit	Health based guidance value	% participants exceeding HB-GV
Metals and trace elements						
Arsenic	U-TRA	BE	Hyperpigmentation and vascular effects	µg/L	6.4	25
Cadmium	U- Cd	HBM-GV	Kidney functioning	µg/g Cr	0.2	39.5
Thallium	U- Tl	HBM I	Fatigue, Sleeplessness	µg/L	5	0
Lead	B-Pb	EFSA	Developmental neurotoxicity	µg/L	12	12
		ECHA	Other effects		45	0
Pesticides						
Pyrethroïds	U-PBA	BE	Neurotoxicity	µg/L	1.7	22
					87	0
Chlorpyrifos	U-TCPγ	BGV	Cholinesterase inhibition	µg/L	520	0
2,4-dichloro Phenoxyaceticacid	U-2,4D	BE	Kidney toxicity	µg/L	10500	0
Polychlorinated biphenyls and halogenated pesticides						
PCBs	S- PCB	HBM I	Liver toxicity	ng/L	3500	0
		HBM II			7000	0
HCB	S-HCB	BE	Liver toxicity	ng/g vet	25	0.5
DDT	S-DDT + DDE + DDD	BE	Liver toxicity	ng/g vet	5000	0
Bisphenols and phthalates						
Bisphenol A	U-BPA	HBM-GV	Kidney weight	µg/L	230	0
DEP	U-MEP		Growth retardation	µg/L	18000	0
DnBP	U-MnBP	HBM-GV	Male reprotox	µg/L	190	0.5
DiBP	U-MiBP	HBM-GV		µg/L	230	1.9
BBzP	U-MBzP	HBM-GV		µg/L	3000	0
DEHP	U-5OH-MEHP + 5oxo-MEHP	HBM-GV		µg/L	500	0
Per- en polyfluorinated substances						
PFOA + PFNA	S-PFOA + S-PFNA	EFSA	Vaccination response	ng/mL	2	4
PFOS + PFHxS	S-PFOS + S-PFHxS	EFSA	Vaccination response	ng/mL	4.9	10

Health-based guidance values: HB-GV derived by HBM4EU for U-Cd (Lamkarkach et al., 2021), U-BPA (Apel et al., 2020b), U-MnBP, U-MiBP, U-MBzP, U-5OH-MEHP+5oxo-M (Lange et al., 2021b), BE value for U-TRA (Hays et al., 2010), BE for 3-PBA (Aylward et al., 2018), U-2,4D (Aylward and Hays, 2015), BE for S-HCB lower value corresponds to an external limit value of 0.05 µg/kg.day set by Health Canada (Aylward et al., 2010), BE for S-DDT + DDE corresponds to an external limit value of 0.5 µg/kg-day (Kirman et al., 2011), BGV for TCPγ (Arnold et al., 2015), BE for U-MEP (Aylward et al., 2009), B-Pb limit values derived from EFSA opinion 2013 ("Scientific Opinion on Lead in Food | European Food Safety Authority,")and RAC 2019 (European Chemical Agency (ECHA), 2018), limit values for S-PFAS derived from EFSA (Schrenk et al., 2020), HBM I for U-Tl from German HBM (Schulz et al., 2011), HBM I and HBM II for sum marker PCBs (PCB138, PCB153, PCB180) from German HBM (Apel et al., 2017).

collected during the summer season.

3.1.1. *Biomonitoring results*

For each chemical, Table 2 presents summary statistics from the FLEHS IV HBM program for a relevant biomarker (parent chemical,

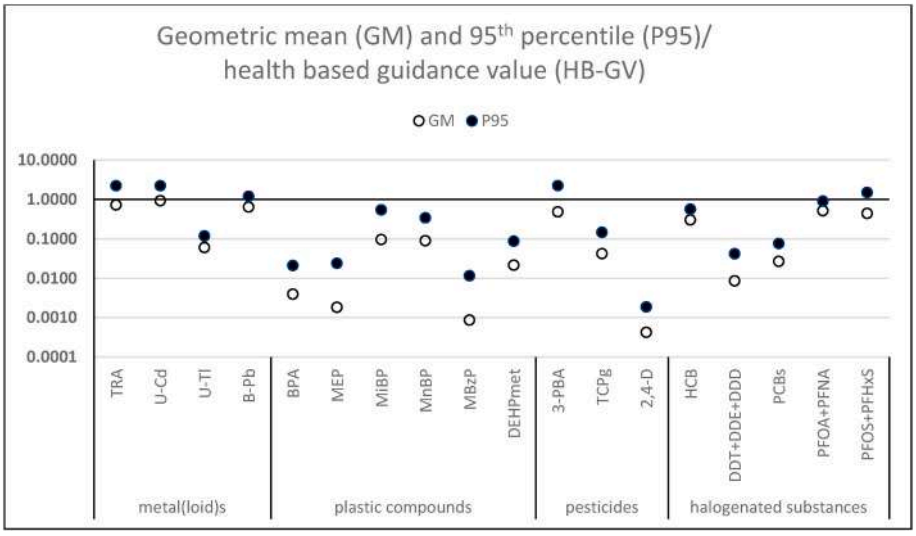

Fig. 1. Chemical specific risk coefficients based on comparisons of internal exposures with corresponding health based guidance values.

metabolite or sum of metabolites). Urinary markers were normalized to SG to adjust for urinary dilution. Urinary biomarker data normalized to Cr are presented in Supplemental Materials S2 and the volume based biomarker data (blood and urine) in Supplemental Materials S3.

HBM-GVs are listed in Table 3 and the proportion of the study participants that exceeded these guidance values is indicated. Sometimes, different HB-GVs for the same biomarker are listed reflecting the differences in opinions of risk assessors and risk assessment committees in expressing exposure-related health concerns. The HB-GVs that are considered refer only to non-cancer endpoints. Fig. 1 shows the risk coefficient (RQ) as the GM and P95 divided by the most sensitive HB-GV for the chemical-specific biomarker.

Fig. 2 displays changes in biomarker levels over time with the GM biomarker concentrations expressed as percentages of the concentrations at the first measuring point. Several biomarkers have been measured in the same age group in previous FLEHS studies, starting from 2002.

3.2. Metals and trace elements

Cd, Pb, As and Tl were included as metals. B-Pb and B-Cd were measured in all previous FLEHS cycles, toxicologically relevant arsenic (TRA) was included in FLEHS II and FLEHS III, U-Tl in FLEHS II. Levels of B-Pb decreased compared to the previous FLEHS studies (Fig. 2A). Success may relate to continued efforts to implement measures such as changing waterpipes in old houses. Mean biomarker levels of B-Cd and TRA remained stable, while U-Tl increased slightly. In some industrial and historically contaminated Flemish areas, elevated concentrations of cadmium and lead in soil still exist. Thallium is an emerging metal used increasingly for electronic and wireless applications and may be emitted near smelters, power plants and cement factories. It may enter different environmental compartments including the food chain (Aprea et al., 2020). Levels of naturally occurring As in groundwater raise concern for home grown food consumption and the use of well water as drinking or showering water (Monteiro De Oliveira et al., 2021).

Between 12 and 39.5% of the participants exceeded the HB-GVs for exposure to TRA, U-Cd, B-Pb (Table 3) and adverse health outcomes cannot be excluded. The GM of U-Cd, B-Pb and U-TRA are close to the HB-GVs (Fig. 1). Cd and As are classified by IARC as known human carcinogens (Group I) and Pb as a possible human carcinogen (Group 2B)(International Agency for Research on Cancer, 2019). No exceedance was observed for U-Tl, there are few population studies, but health effects at low Tl levels are reported, especially there is a concern for neurotoxicity (Campanella et al., 2019).

B-Tl and B-Cd were higher in teenagers from lower educated households but significance disappeared after adjustment for smoking. The positive association of TRA with the family's educational attainment was borderline significant ($p < 0.10$). A similar association with high SES has been described in the HBM program of the US-Centers for Disease Control and Prevention (US-CDC) and was mediated by more fish and shellfish consumption (Tyrrell et al., 2013). B-Pb and blood manganese(B-Mn) were borderline ($p < 0.10$) associated with residence area, with B-Pb higher in residents from rural areas and B-Mn higher in residents from towns and suburbs (Fig. 4). Underlying causes are not clear but may relate to living in older houses with leaded waterpipes.

U-Cd, U-TRA and B-Cu were higher in girls, B-Pb and B-TI were higher in boys after adjustment for household educational attainment, urbanization characteristics, sampling season, age of the participants.

FLEHS IV GM of B-Cd and B-Pb are higher than reported by US-CDC and Health Canada for sampling in the same period (>2015) in a comparable age group (12–19 years old), while the GM TRA levels of FLEHS IV are similar (US-CDC) (Health Canada, 2019). Comparison with HBM programs in Europe (VITO, 2021), showed that GM of TRA is lower than reported for 7 year-old children from Northeast Italy (Bocca et al., 2020), P50 B-Cd and B-Pb of FLEHS IV are lower than those measured in adults and in children sampled in the Czech HBM program of 2015–2016 (Černá et al., 2017), P50 B-Pb of FLEHS IV and GerES V teenagers (14–17 years) were similar but P50 B-Cd was higher in FLEHS IV teenagers (Vogel et al., 2021) (Supplemental materials S4).

3.3. Combustion-related compounds

Benzene and PAHs are considered relevant for spatial planning as they relate to traffic exhaust, industrial emissions and heating of buildings. PAHs are also a relevant group of compounds in the indoor environment, as they are produced during cooking and heating (IARC Working Group on the Evaluation of Carcinogenic Risks, 2010). We have biomonitoring data from previous FLEHS cycles for 1-OH PYR as pyrene metabolite and for t,t'-MA as benzene metabolite in urine. We included additional metabolites for some smaller volatile PAHs in the current study: NAPH, PHE and FLU. 2-OH NAPH is the most abundant biomarker of the low molecular weight PAHs (Table 2).

GM levels of 1-OH PYR have decreased for the first time in the successive FLEHS studies, while levels of t,t'-MA increased (Fig. 2B). This coincides with a decreasing trend in air emissions of PAHs in Flanders that was reported in 2014 for the first time since 2000, while the benzene concentrations in the air decreased until 2014, but showed a slight increase thereafter (Vlaamse Milieumaatschappij, 2017).

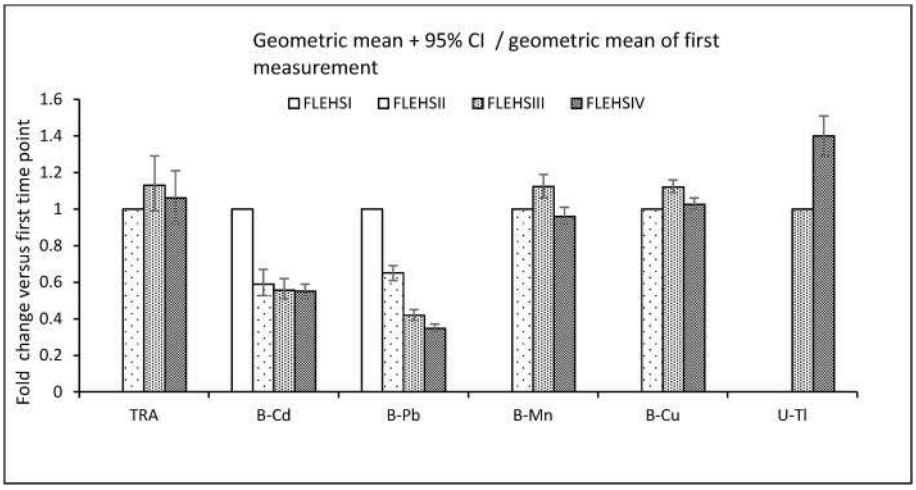

Fig. 2A. Changes over time of urinary biomarkers (U) of trace elements adjusted for sex, smoking behavior, age, creatinine; blood biomarkers (B) adjusted for sex, age and smoking behavior.

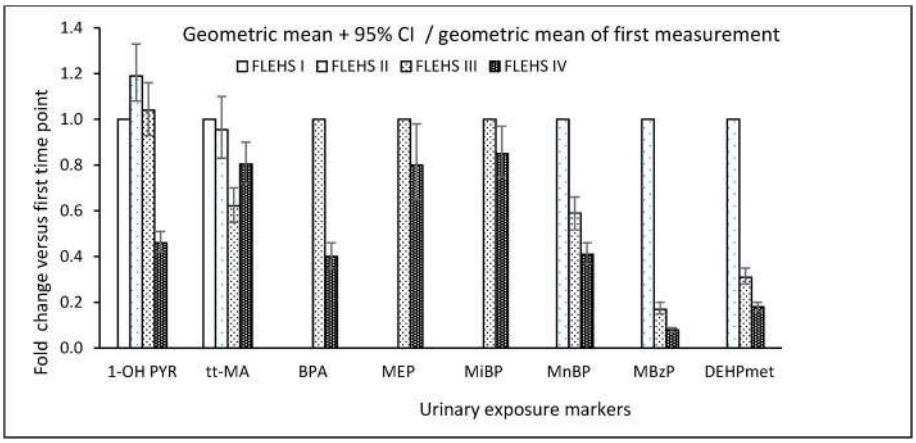

Fig. 2B. Changes over time of urinary biomarkers for combustion products and plastic compounds adjusted for sex, smoking behavior, age, body mass index (BMI) and creatinine.

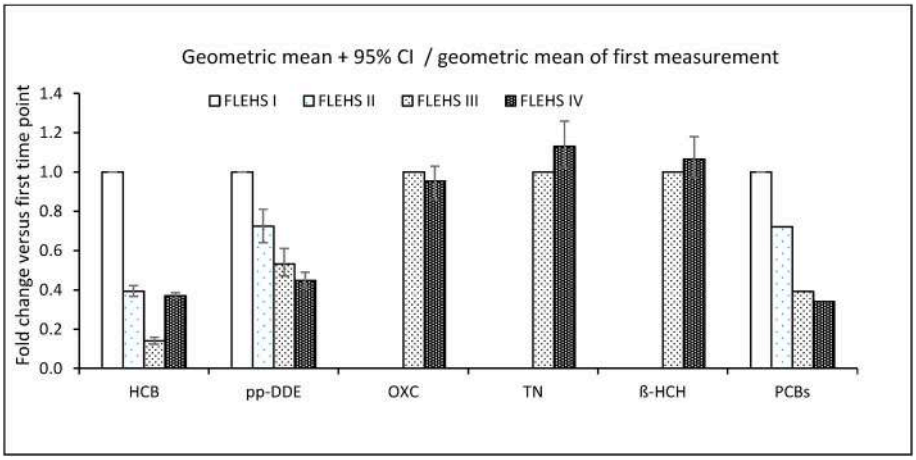

Fig. 2C. Changes over time of serum biomarkers of halogenated substances adjusted for sex, smoking behavior, age, BMI and serum lipids.

No HB-GVs are available, but both substances are classified as known human carcinogens by IARC (Group I)(International Agency for Research on Cancer, 2019). Based on annual air concentrations, extra cancer risks were estimated for benzene between 1/156 000 and 1/587 000 and for benzo-a-pyrene between 1/50 000 and 1/210 000 depending on the place of residence and if levels would remain the same in the future (Vlaamse Milieumaatschappij, 2020). FLEHS IV study results indicate that adverse effects on the adolescent endocrine and immune system cannot be excluded(Verheyen et al., 2021).

Several of the PAH metabolites (1-OH PYR, sum of 2-OH FLU and 3-OH FLU, 2-OH PHE) and also t,t'-MA were elevated in teenagers from lower-educated households (Fig. 3). We also observed higher levels of 1-

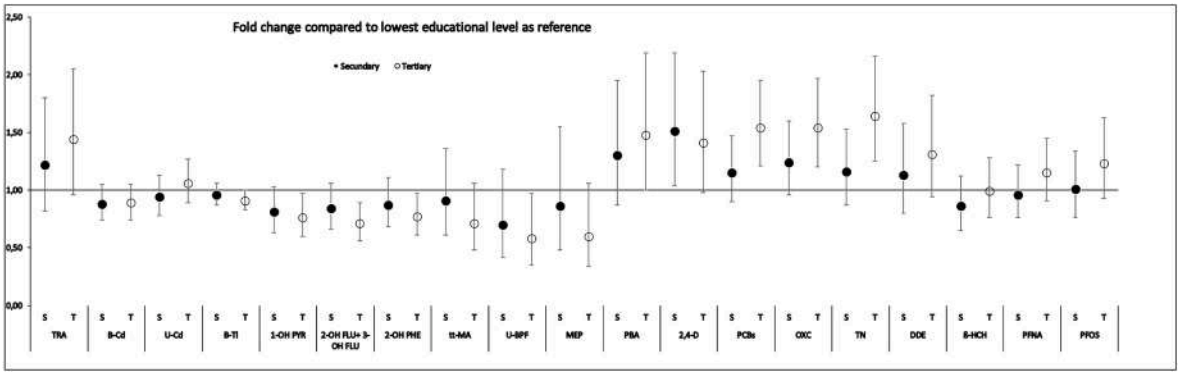

Fig. 3. Biomarkers that show significant ($p < 0.10$) associations with highest educational level attainment of the household: primary (no educational attainment, primary school, lower secondary school), secondary (higher secondary school) and tertiary (higher education attainment). These levels correspond to the codes 0–2, 3–4 and 5–8 of the International Standard Classification of Education (ISCED). The primary educational level is used as reference category. Fold change and 95% CI are presented. $P < 0.05$ for U-Cd, B-Tl, 2-OH NAPH, sum of 2-OH FLU and 3-OH FLU, 2-OH PHE, tt'-MA, MEP, BPF, PCBs, OXC, TN, HCB, PFNA and PFOS and borderline significant ($p < 0.10$) for TRA, 1-OH PYR, the pesticide markers 3-PBA, 2,4-D, DDE, β-HCH.

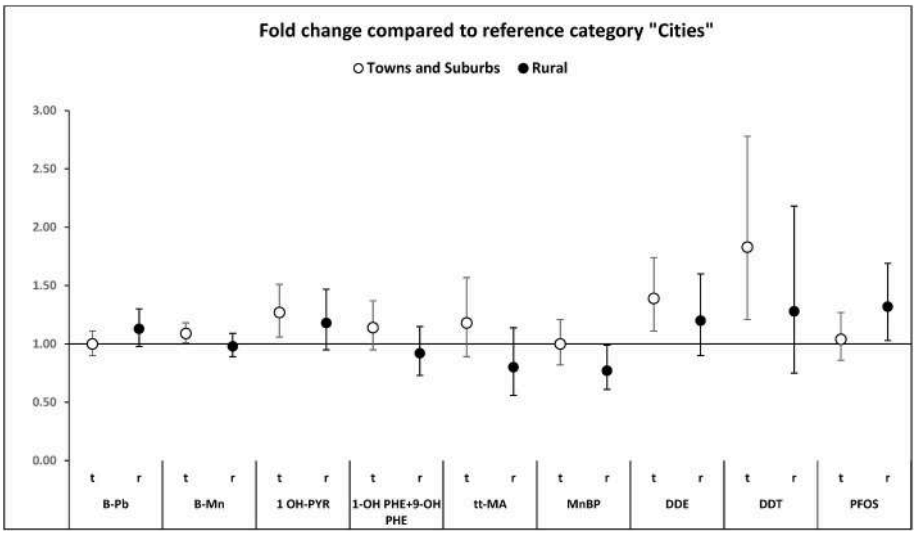

Fig. 4. Biomarkers levels that are associated ($p < 0.10$) with the neighborhood urbanicity of the participants. The neighborhoods are categorized according to the EUROSTAT definitions and "cities "are used as the reference category. Fold change and 95%CI are presented.

OH PYR, the sum of 1 and 9-OH PHE and t,t'-MA in residents from towns and suburbs. Fig. 4 displays the fold change of these biomarkers (GM and 95% CI) with cities as the reference category. Significance ($p < 0.05$) remained after adjustment for smoking. Exposure to environmental tobacco smoke (ETS) at home, living close to industry and heavy traffic have been suggested as exposure determinants for PAHs (Iamiceli et al., 2020; Sochacka-Tatara et al., 2018). Levels of 2-OH NAPH were higher in girls than in boys.

We compared with large representative international studies from similar age groups and sampling periods (Supplemental material S2). The urinary GM/P50 levels in this study were below the ones reported by the HBM programs of Health Canada with sampling in 12–19 years old teenagers in 2014–2015 (Health Canada, 2017) and were similar or lower than reported by GerES V for 14–17 years old teenagers with sampling in 2014–2017 (Murawski et al., 2020).

3.4. Plastic compounds

Bisphenols have been used to produce polycarbonate plastics and epoxyresins and are present in various consumer products including toys, food packaging materials, food cans (Vandenberg et al., 2007). Phthalates are added to soften plastics and are also used in a lot of consumer products. People may be exposed by dermal contact, by food and by inhalation as these compounds settle on house dust (Luis et al., 2021). BPA, Diethyl phthalate (DEP), Dibutyl phthalate (DnBP), Diisobutyl phthalate (DiBP), Benzyl butyl phthalate (BBzP) and Bis (2-ethylhexyl) phthalate (DEHP) are regulated under REACH at the EU level (ECHA, 2018). As reported earlier (Gys et al., 2021), three of the six measured bisphenols could be quantified in more than 60% of teenagers. Levels of BPA were higher than those of PBF and BPS. Only BPA was measured in FLEHS III, the GM in the FLEHS III study was almost double compared to the FLEHS IV study (Fig. 2B). No significant health risk for BPA is expected as the recently derived HB-GVs are not exceeded (Apel et al., 2020a), no HB-GVs are published for the other bisphenols. Only BPF was higher in teenagers from lower educated households but not the other bisphenols. Also a German study reported higher BPA levels in children from families with lower SES status which was a composite variable based on parent's income, education, and profession (Tschersich et al., 2021).

The levels of BPA are in the same range as those found in other studies from a comparable time frame. The P95 BPA and BPF levels (volume based) are somewhat lower than the HBM reference values for children (6–17 years old) measured in the French Esteban study (2014–2016) (Fillol et al., 2021), while BPS levels were roughly ten times lower. P50 BPA levels (volume based) found in German teenagers (14–17 years old) (GerESV 2014–2017) are higher (Tschersich et al., 2021).

MEP showed the highest GM concentration followed by MiBP, MnBP and cx-MEPP. Significant decreases in GM levels of regulated phthalates (DEHP, DiBP, DnBP and DBzP) were observed compared to the levels measured in the previous FLEHS III study (Fig. 2B). Many consumer products are labelled and consumers nowadays often have the choice to buy BPA-free or phthalate-free products. The recently derived HB-GVs of DnBP and DiBP were exceeded in respectively 0.5 and 1.9% of the participants (Table 3), while no exceedances were measured for DEHP metabolites, demonstrating the efficacy of regulation(Lange et al., 2021a).

The results confirmed earlier observations that MEP, which is mainly used in personal care products, showed higher levels in teenagers from lower educated households (Fig. 3) (Den Hond et al., 2015). MnBP levels were lower in residents from rural areas compared to cities. SES status and degree of urbanization should be considered as surrogate markers for specific behaviors as discussed extensively before (Bastiaensen et al., 2021a). After adjustment for household educational attainment, urbanization characteristics, sampling season, age of the participants BPA, MEP, MnBP and MEHP levels were higher in girls than in boys. This may relate to differences in exposures or metabolism. The demand for plastics and plasticizers remains and a comprehensive study revealed that metabolites of substitute plasticizers such as di-(iso-nonyl)-cyclohexane-1,2-dicarboxylate (DINCH), di-(2-ethylhexyl) terephthalate (DEHTP) and di-(2-ethylhexyl) adipate (DEHA) could be quantified in urine samples of Flemish teenagers (Bastiaensen et al., 2021a). The toxicological database for these substitutes is not yet complete and their impact on human health is not fully understood.

We reported earlier (Bastiaensen et al., 2021a) that concentrations of phthalate metabolites measured in this study were generally consistent with studies of adolescents from Canada (Health Canada, 2019) and Germany (Schwedler et al., 2020). Our P95 volume based levels for MiBP, MEP, MBzP, MnBP and the DEHP metabolites were below the French reference values for 6–17 years old children (Fillol et al., 2021) (Supplement S4).

3.5. Pesticides

Biomarkers for pyrethroids, chlorpyrifos, the phenoxyherbicide 2,4-D and glyphosate were measured for the first time in spot urine samples

of a representative sample of Flemish teenagers. No time trends for internal exposures in the Flemish region are yet available. These short-lived pesticides are used professionally in agricultural areas but also privately in homes and gardens. Residues present in commercially bought food and locally grown food may also expose the general population. According to FAOSTAT, pesticide use per area of cropland in Belgium (6.96 kg/ha) is about four times above the European average (1.66 kg/ha) (FAO, 2019).

Pyrethroids are synthetic pesticides that are increasingly used as insecticides in public and private places indoors and outdoors and replace some of the banned halogenated pesticides. The potential for human exposure is high, both from the intake of residues in food items and by dermal and inhalation exposures via direct contact and from dust (Morgan, 2012). They are regulated at the EU level in food and as plant protection products (Andersen H.R., 2021). We measured 3-phenoxybenzoic acid (3-PBA) which is a common metabolite of several widely used pyrethroids (Cypermethrin, Deltamethrin, Permethrin, lambda-Cyhalothrin, D-Phenothrin and D-Phenothrin Fluvalinate-tau). 3-PBA could be quantified in nearly all urine samples.

Chlorpyrifos was one of the most used organophosphate insecticides, but in 2017 its use was limited to certain applications in Flanders. At EU level it is no longer approved since 2019 (Andersen H.R., 2021). The chlorpyrifos metabolite, TCPγ, could still be detected in all samples from FLEHS IV teenagers. It is anticipated that levels will decrease due to the recent regulations.

GLY is worldwide the most used herbicide. The use of GLY and its degradation product AMPA by non-professionals has been restricted in Flanders since 2018, but it is still primarily used by farmers. GLY and the AMPA metabolite could be measured in respectively 41.45 and 55.9% of the FLEHS IV teenagers (LOQ 0.1 μg/L). Flemish adolescents with GLY below LOQ had on average more diluted urine samples (SG of 1.024703) versus samples with GLY above LOQ (SG of 1.020276). This underestimates the fraction of exposed participants. The average dilution factor, calculated by the formula (1.024-1)/(SG-1), was 1.22 fold higher for samples below LOQ than for samples above LOQ. If the same dilution factor would be applied, 45 samples out of 172 with values above LOQ would be no longer quantifiable.

Although results of spot urine samples of short-lived biomarkers should be interpreted with caution (Bastiaensen et al., 2021c), 22% of the study participants exceeded the most sensitive HB-GV for 3-PBA (Table 3). The most sensitive HB-GV assumes that all parent compounds have the same toxic potency as the known most toxic compound. Neurotoxic and endocrine effects are health concerns associated with pyrethroids and chlorpyrifos (Koureas et al., 2012; Saillenfait et al., 2015), while 2,4-D is classified as possible human carcinogen (Group 2B) (International Agency for Research on Cancer, 2019). IARC has classified GLY as probably carcinogenic (Group 2A) (International Agency for Research on Cancer, 2019), while the EU considers its aquatic toxicity as the basis for regulation (Kalofiri et al., 2021).

Levels of 2,4-D and 3-PBA were higher in the teenagers from higher educated households (Fig. 3). Higher levels of pyrethroid metabolites in relation with higher household educational attainment were also observed in an Italian study (Bravo et al., 2019) but not in other studies (Fernández et al., 2020a; Norén et al., 2020; Rodzaj et al., 2021).

The P50 of the pyrethroid biomarkers 3-PBA of FLEHS IV teenagers is higher than reported for adolescents from Sweden (Norén et al., 2020), 12-19 year-old teenagers from Canada (Health Canada, 2019), Polish urban-dwelling young adults (Rodzaj et al., 2021), children in Trieste (Bravo et al., 2019) but lower than those of children in the Spanish Valencian region (Fernández et al., 2020) and comparable to children from the Belgian Walloon region (Pirard et al., 2020). The P50 chlorpyrifos metabolite TCPγ, is higher in FLEHS IV teenagers than reported in Italian, Spanish and Swedish studies (Supplemental materials S4). The P95 of 2,4-D is higher in Flemish teenagers than in Swedish adolescents (Norén et al., 2020), but lower than in Spanish children (Fernández et al., 2020). The detection frequency of GLY and AMPA with quantification limit of 0.1 μg/L, was comparable to German children with GLY and AMPA quantified in respectively 52% and 46% of (GerESV 2014–2017) samples (Lemke et al., 2021) using the same analytical method and LOQ. A recent study of Slovenia reported, depending on the sampling season, between 22 and 27% detects for GLY and between 50 and 56% for AMPA in children and teenagers between 7 and 15 years old, LOQ was also 0.1 μg/L (Lemke et al., 2021; Stajnko et al., 2020).

Metabolites of DDT, lindane (β-HCH), the chlordane related compounds and HCB were detected in the majority of serum samples of FLEHS IV teenagers. Chlorinated pesticides are legacy chemicals as their production and use is eliminated or restricted under the Stockholm convention; however these chemicals are still present in the environment and the food chain. Food is the primary exposure source for the general population (Keswani et al., 2021). OXC, TN and HCHs were measured in teenagers of FLEHS III with sampling in 2013. DDT, DDE and HCB were measured in all the previous FLEHS studies with sampling in 2003–2004 (FLEHS I), 2007–2008 (FLEHS II), 2013 (FLEHS III). Only levels of DDE are gradually decreasing over the different FLEHS studies, but decreases in concentrations could not be observed for the other halogenated pesticides (Fig. 2 C).

HB-GVs relate to the endocrine disrupting properties associated with these compounds (Gheidarloo et al., 2020). GM and P95 did not exceed HB-GVs. Only a few individuals exceeded the HB-GV of HCB (Table 3). However, significant exposure-effect associations have been observed with HCB and DDT metabolites in earlier FLEHS studies (Croes et al., 2015). The carcinogenic properties of these compounds remain of concern as IARC classified DDT as probably carcinogenic (Group 2A), HCB, chlordane and HCH isomers as possibly carcinogenic (Group 2B) (International Agency for Research on Cancer, 2019).

The household educational attainment explained a part of the variability of OXC, TN, HCB ($p < 0.05$) and of DDE, β-HCH ($p < 0.10$) as shown by multiple regression models adjusted for age, sex, sampling season and blood lipids. Significance for DDE and β-HCH disappeared after correction for BMI and being breastfed. Mean biomarker levels of OXC, TN, HCB and DDE were highest at the highest educational attainment (Fig. 3). Previous studies have observed similar associations for the halogenated compounds (Bandow et al., 2020; Morrens et al., 2012) and these were associated with higher intake of egg products, meat and fish (Arrebola et al., 2018). DDE and DDT biomarker levels were significantly higher in teenagers from towns and suburban areas than from cities (Fig. 4). Higher consumption of locally produced eggs and food have been associated with a higher burden of halogenated substances in previous FLEHS cycles (Colles et al., 2021; Den Hond et al., 2009).

Compared to girls, boys had higher biomarker levels of HCB, DDE, β-HCH, OXC and TN after adjustment for household educational attainment, urbanization characteristics, sampling season, age of the participants.

Recent HBM data from the German children and adolescent study (GerESV 2014–2017) revealed similar GM of HCB, β-HCH was twice as high in Germany, while DDE was 56% higher in FLEHS IV participants (Bandow et al., 2020).

3.6. PCBs and flame retardants

Marker PCBs were quantifiable in serum of all teenagers of FLEHS IV and were measured in the same age group in the three previous FLEHS studies. GM PCB levels decreased in FLEHS IV compared to the previous FLEHS III study (Fig. 2C). None of the BFR congeners could be quantified in more than 60% of the participants (only 4 mL serum samples were available for the analysis of the halogenated chemicals). We noticed that the detection frequency of PBDE153 decreased from 61,4% in FLEHS II to 38% in FLEHS III to 21.3% in FLEHS IV applying the same LOQ. HB-GVs of PCBs, based on liver toxicity, were not exceeded in the study participants (Table 3). IARC classified PCBs in group I (International Agency for Research on Cancer, 2019).

PCBs and BFRs are another class of legacy chemicals that are regulated under the Stockholm convention. PCBs have cooling properties and were produced for specific applications, while PBDEs were used as flame retardants. These chemicals are banned, but remain circulating in the environment and the general population is exposed mainly to food (Lebelo et al., 2021).

As observed for some other biomarkers of chlorinated chemicals, teenagers of higher educated households had higher PCB burdens (Fig. 3). Levels of PCBs were higher in boys than in girls after adjustment for household educational attainment, urbanization characteristics, sampling season, age of the participants.

Compared with German children and teenagers (GerES 2014–2017) (Bandow et al., 2020), PCBs in Flemish teenagers were somewhat lower if GM are compared (Supplemental Materials S4).

BFRs are being replaced by alternatives such as OPFRs. These chemicals are used as flame retardants but also as plasticizers in a wide variety of consumer products such as furniture, textiles, decoration and building materials, paints, floor polish, resins and polyvinyl chloride plastics (Chokwe et al., 2020). Six biomarkers of five OPFRs were found in practically all participants. The GM and P95 were highest for 2-ethylhexyl phenyl phosphate (EHPHP), a biomarker for 2-ethylhexyl diphenyl phosphate. No HB-GVs were available, as information on the toxicology of these compounds is still limited. Some of these chemicals are suspected developmental and carcinogenic toxicants (Alzualde et al., 2018; Wei et al., 2020). No significant associations with household educational attainment or urbanicity have been observed in this reference population. A more extended study that included 582 participants has identified higher levels of BBOEHEP and BDCIPP in teenagers from higher educated households (Bastiaensen et al., 2021b).

3.7. Per- and polyfluoroalkyl substances

PFOS and PFOA were quantified in all serum samples, demonstrating ubiquitous exposure. PFOS showed the highest concentrations followed by PFOA, while levels of PFNA and PFHxS were lower. Perfluoro-n-decanoic acid (PFDA) could be quantified in 42.2% of the participants, while the concentrations of the other PFAS were mostly below the LOQ of 0.2 μg/L. The PFAS are a large group of compounds with interesting properties: water, dirt and oil repellent. They are applied in a diversity of consumer products, but food may also be an exposure source for humans. Their persistency in the environment, accumulation and long half-life-in humans are a growing concern (Fromme et al., 2009). Our HBM-data may be compared with internal concentrations that correspond with the recently by EFSA proposed intake limits based on a reduced vaccination response as critical effect (Schrenk et al., 2020). Respectively 10 and 4% of our participants exceeded these values for the sum of PFOS and PFHxS (4.9 ng/mL), and for the sum of PFOA and PFNA (2 ng/mL) (Table 3). Various health effects including developmental effects, liver toxicity, immune effects have been associated with these persistent compounds and their endocrine properties (Rappazzo et al., 2017). IARC classified PFOA in group 2B (International Agency for Research on Cancer, 2019). Regulation in the EU has restricted the production and use of PFOS and PFOA in a variety of applications (ECHA, 2021), while recent risk assessment made by EFSA proposed to regulate the sum of four substances: PFOA, PFOS, PFHXS, PFNA (Schrenk et al., 2020). Although decreasing levels for PFOS and PFOA have been described earlier in Flemish newborns (Colles et al., 2020), our new study demonstrates that concerns for adverse health effects cannot be excluded. Moreover, we have measured only a very small fraction of the fluorinated compounds that enter the market and many replacements of the restricted compounds have not been measured in human matrices yet (Kaiser et al., 2021).

Teenagers from higher educated households showed higher levels of PFOS and PFNA (Fig. 3). This confirms results of higher PFAS levels associated with higher socio-economic position described in German children and adolescents (Duffek et al., 2020), in children and pregnant mothers from European urban birth cohorts (Montazeri et al., 2019) and in relation with higher income as found in a meta-analysis including five HBM studies (Buekers et al., 2018). Differences in breastfeeding and dietary patterns have been suggested as underlying reasons (Buekers et al., 2018; Duffek et al., 2020). We observed higher levels of PFOS in residents from rural areas compared to those from cities (Fig. 4). Earlier studies in Flanders have shown that higher consumption of locally grown foods occurs more in rural areas and is associated with higher internal exposure levels of several persistent compounds such as PFOS and PFOA (Colles et al., 2020). Levels of PFOA and PFHXs were higher in boys than in girls.

The GM serum levels of PFOS, PFOA, PFHxS and PFNA showed similar values compared to recently published values from French and Canadian children (Fillol et al., 2021; Health Canada, 2019) and from German teenagers (Duffek et al., 2020) (Supplemental Materials S4).

3.8. Strengths and weaknesses

Our aim was to recruit a population sample of 14- and 15-year old teenagers that was representative for geographical location and sex and included participants from different socio-economic strata and residential areas with varying degrees of urbanization. We compared our population sample with available population characteristics of Flemish teenagers. We found some overrepresentation of teenagers from rural areas but a good match for most other characteristics including SES. Accordingly, no extra weighing factors were introduced as we consider our population exposure estimates to represent 14–15 year' old Flemish teenagers. We could not sample in the summer months as schools (our primary sampling units) were closed. Dietary habits and life style may change with season. Season of sampling was a significant covariate for several biomarkers. Not sampling equally in all seasons may have somewhat biased representativity of the biomarkers. To assess impact of educational attainment of the household and degree of urbanization of the residence area, the statistical models were adjusted for season of sampling.

We have compared our exposure distributions with other general population studies that sampled teenagers of approximately the same age as exposures may differ according to age (Supplemental Materials S4). Also, the sampling years should be comparable as chemical production, use patterns and regulations change over time. Only a few countries carried out HBM studies of a representative sample of the general population after 2015 and most studies reported HBM data from a more extended age group. Interpretations are hampered by differences in study and sampling design, laboratory analysis, reported LOQs and data handling. Further harmonization of study design and assessment of the comparability of biomarker analysis such as currently ongoing in Human Biomonitoring for Europe (HBM4EU) will benefit comparability of HBM data in Europe (Ganzleben et al., 2017).

Improved analytical techniques allowed quantifying a wide diversity of chemical compounds in the same individual. The presence of a chemical itself in serum or urine does not imply adverse health risks. Comparison of the biomarker data with corresponding HB-GVs was possible for only 18 of the 80 biomarkers. HB-GVs change regularly as more information becomes available; different organizations may come to different conclusions and handle different HB-GVs. HB-GVs are available for non-cancer endpoints. Some of the chemicals are classified by IARC as known (PAHs, benzene, Cd, As, PCBs), probable (GLY, DDT) or possible (Pb, 2,4-D, HCB, HCH, chlordanes, PFOA) human carcinogens and levels should be as low as reasonably achievable to avoid extra cancer risks.

Enhanced risks also assume chronic exposure at the same level throughout life.

Certainly for short-lived chemicals, such as pyrethroids and phthalates, the results of spot urine measurements may vary within an individual even within a day. More frequent sampling per individual is needed to interpret exposure data at the individual level. To assess

exposure determinants of these short-lived chemicals large sample sizes are needed. With more than 400 individuals (except for As biomarkers) sampled within a narrow age range, sample size was sufficient to assess the impact of important covariates such as the urbanicity of the residence area and the household educational attainment.

The present manuscript describes exposures and risks for individual chemicals. However, we acknowledge that increasingly complex chemical mixtures are detected in particular samples which will need new methods and approaches to quantify their risks.

4. Conclusions

The study emphasizes the success of chemicals' regulation: all recently restricted phthalates (DEHP, DiBP, DnBP, DBzP) and BPA biomarkers were lower than in the previous study (FLEHS III). Internal exposure to cadmium, lead decreased. The sum of PCBs was reduced to one third in 15 years. However, this was not seen for the chlordanes, with even significant increases of TN. Substitutes of regulated chemicals (PFAS, flame retardants and plasticizers) popped up in many samples. Little is known about their toxicity, warranting further follow up of the exposure levels in the population and toxicity information to prevent future health risks.

Adverse health risks at the population level are still expected for some traditional pollutants such as cadmium, lead, arsenic, polycyclic aromatic hydrocarbons, benzene and perfluoroalkyl substances, emphasizing the need for further measures and control of sources. Health risks cannot be excluded as more than 5% of the study participants exceeded HB-GVs of TRA, urinary Cd (U-Cd), blood Pb (B-Pb), pyrethroids and PFAS. The GM of U-Cd was close to the guidance values. GM and P95 of HCB, DiBP, DnBP and P95 of TCPγ and urinary Tl (U-Tl) were within one order of magnitude of the HB-GVs. A few individuals exceeded the guidance values of HCB, DnBP and DiBP.

We have specifically looked into the relationship between urbanization and internal exposure as Flanders is densely populated and urbanization is expected to further increase in the future. Household educational attainment and residential urbanicity influenced biomarker levels in both directions. Markers of several PAH metabolites, MeP and BPF were higher in teenagers from lower educated households, while biomarkers of halogenated substances were often higher in teenagers from higher educated households. PFOS was higher in residents of rural areas, while residents from towns and suburbs showed the highest levels of benzene and PAH metabolites but also of DDE and DDT. Underlying causes may relate to differences in environment, lifestyle and food habits and need further research to reduce internal exposure efficiently.

The Information on exposure will support prioritization and evaluation of policies and will inform on personal choices that people can make themselves.

Ethics

The study protocol was approved in June 2017 by the Antwerp University Hospital Ethical committee (Belgian Registry Number B300201732753).

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.113972.

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Maternal exposure to traffic-related ambient particles and risk of gestational diabetes mellitus with isolated fasting hyperglycaemia: A retrospective cohort study in Beijing, China

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ABSTRACT

Background: Ambient particles have been associated with gestational diabetes mellitus (GDM), however, no study has evaluated the effects of traffic-related ambient particles on the risks of GDM subgroups classified by oral glucose tolerance test (OGTT) values.

Methods: A retrospective analysis was conducted among 24,001 pregnant women who underwent regular prenatal care and received OGTT at Haidian Maternal and Child Health Hospital in Beijing, China, 2014–2017. A total of 3,168 (13.2%) pregnant women were diagnosed with GDM, including 1,206 with isolated fasting hyperglycaemia (GDM-IFH). At a fixed-location monitoring station, routinely monitored ambient particles included fine particulate matter ($PM_{2.5}$), black carbon (BC) and particles in size ranges of 5–560 nm ($PNC_{5\text{-}560}$). Contributions of $PNC_{5\text{-}560}$ sources were apportioned by positive matrix factorization model. Logistic regression model was applied to estimate odds ratio (OR) of ambient particles on GDM risk.

Results: Among the 24,001 pregnancy women recruited in this study, 3,168 (13.2%) were diagnosed with GDM, including 1,206 with isolated fasting hyperglycaemia (GDM-IFH) and 1,295 with isolated post-load hyperglycaemia (GDM-IPH). We observed increased GDM-IFH risk with per interquartile range increase in first-trimester exposures to $PM_{2.5}$ (OR = 1.94; 95% Confidence Intervals: 1.23–3.07), BC (OR = 2.14; 1.73–2.66) and $PNC_{5\text{-}560}$ (OR = 2.46; 1.90–3.19). $PNC_{5\text{-}560}$ originated from diesel and gasoline vehicle emissions were found in associations with increases in GDM-IFH risk, but not in GDM-IPH risk.

Conclusion: Our findings suggest that exposure to traffic-related ambient particles may increase GDM risk by exerting adverse effects on fasting glucose levels during pregnancy, and support continuing efforts to reduce traffic emissions for protecting vulnerable population who are at greater risk of glucose metabolism disorder.

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1. Introduction

Gestational diabetes mellitus (GDM) has been traditionally defined as glucose intolerance with first detection during pregnancy (Chen et al., 2021). The International Diabetes Federation (IDF) has reported that 14.8% of Chinese pregnant women have GDM, with increasing prevalence (Gao et al., 2019). Studies have found that pregnant women with GDM tend to have higher risk of cardiovascular diseases and metabolic syndrome in the years post pregnancy (Chen et al., 2021; Lee et al., 2007), and their offspring are more likely to develop obesity, cardiometabolic condition and impaired intellectual abilities in later life (Hu et al., 2015). Besides traditional risk factors, such as genetic predisposition and body adiposity, emerging evidence has suggested that ambient air pollution is associated with increased GDM risk, but less is known about the potential pathophysiologic mechanisms (Zhang et al., 2020a; Zhu and Zhang, 2016).

It has been globally recognized that ambient particles, especially fine particulate matter ($PM_{2.5}$) can be an important risk factor for abnormal glucose metabolism through increased systemic inflammation, endothelial dysfunction and insulin resistance (Rao et al., 2015; Zhang et al., 2020a). However, smaller particles (e.g., ultrafine particles) may have greater toxicity than larger particles (e.g., $PM_{2.5}$) due to much greater active surface areas, which can adsorb more toxic components (Lanzinger et al., 2016; Zhang et al., 2020b). Ambient particulate matter is a complex mixture from various sources, and vehicle exhaust particles have been identified as the most components in ambient particles resulting from chemical and biological responses (Park et al., 2018). In metropolitan areas worldwide, traffic emissions have been found to be the major sources of ambient particles (Huang et al., 2018). Prior research has shown that maternal exposures to traffic-related air pollution, traffic density and residential proximity to major roads were associated with increased GDM risk (Malmqvist et al., 2013; Yorifuji et al., 2015).

Commonly, the diagnosis of GDM is established based on fasting and post-load glucose values from the oral glucose tolerance test (OGTT) results during the mid-gestation (Papachatzopoulou et al., 2020). Approximately half of the pregnancies complicated by GDM showed abnormal fasting glucose values, whereas the rest showed abnormal post-load glucose values (Sacks et al., 2012). Clinical studies found that fasting and post-load hyperglycaemia in normal subjects were characterized by different and specific beta-cell defects, which might represent differential deterioration processes of glucose metabolism (Kotzaeridi et al., 2021; Mengozzi et al., 2020). Previous studies reported that GDM complicated pregnancies with isolated fasting hyperglycaemia (GDM-IFH) were typically less responsive to exercise and diet therapy, and had higher risk for macrosomic newborns than those with isolated post-load hyperglycaemia (GDM-IPH) (Burt et al., 2012; Papachatzopoulou et al., 2020). In addition, several prior studies have assessed the potential associations between maternal exposure to $PM_{2.5}$ and OGTT values, including fasting and post-load glucose values, and suggested that the associations were especially pronounced in the elevation of the fasting glucose levels during pregnancy (Hu et al., 2021; Lin et al., 2020). However, no study examined the effects of maternal ambient particle exposures on the risks of differential GDM subgroups classified by OGTT values, which could be critical in stratifying effective and individualized intervention strategy against air pollution exposure.

In this study, the GDM complicated pregnancies were classified into GDM-IFH, GDM-IPH and GDM with combined hyperglycaemia (GDM-CH) based on the presence of fasting and post-load hyperglycaemia resulting from OGTT. Here we hypothesized that exposures to traffic-related ambient particles would increase blood glucose values and GDM risk. We explored the potential glycemic effects of ambient $PM_{2.5}$, black carbon (BC), and particles in size ranges of 5–560 nm ($PNC_{5\text{-}560}$) during preconception and early pregnancy on OGTT glucose levels, including fasting, 1-h and 2-h glucose levels, and the risks of GDM-IFH, GDM-IPH and GDM-CH. We further applied positive matrix factorization (PMF) models to assess the contributions of $PNC_{5\text{-}560}$ sources, and examined the glycemic effects of source-specific $PNC_{5\text{-}560}$ on OGTT glucose levels and GDM risks.

2. Material and methods

2.1. Study participants

Data for this retrospective cohort study was extracted from Haidian Maternal and Child Health Hospital in Beijing, China. We collected information on the identification number, maternal age at pregnancy, maternal ethnicity, maternal weight at various stages of pregnancy, residential address, presence or absence of a history of diabetes, gravidity, parity, last menstrual period (LMP), the year and season of conception, gestational age, fetal gender and fetal weight. Pregnant women were excluded if they were diagnosed with diabetes prior to gestation, had non-singleton deliveries, lived outside Beijing according to residential addresses, had living births with weight <500 or $>5{,}000$ g, with gestational age <28 or >44 weeks, or with missing values of fetal gender (Hu et al., 2015). Pregnant women undertook plasma glucose measurement in early pregnancy, if fasting plasma glucose values ≥ 7.0 mmol/L, they were diagnosed with overt diabetes in pregnancy and excluded from the analyses (Yang et al., 2014). The final analytic sample consisted of 24,001 mother-infant pairs. This research was approved by grand IRB-2020001-1 from the Institutional Research Review Board at the National Health Commission.

2.2. Outcome and covariates

All of the participants underwent an OGTT for GDM screening between 24 and 28 weeks of gestation. According to the International Association of Diabetes and Pregnancy Study Groups standard, GDM was diagnosed based on fasting blood glucose values ≥ 5.1 mmol/L, 1-h glucose values ≥ 10.0 mmol/L or 2-h glucose values ≥ 8.5 mmol/L. In addition, we categorized GDM into three subgroups: 1) GDM-IFH, fasting glucose values ≥ 5.1 mmol/L but post-load glucose values were in the normal range; 2) GDM-IPH, post-load glucose values ≥ 10.0 mmol/L at 1 h and/or ≥ 8.5 mmol/L at 2 h but fasting glucose values were in the normal range; 3) GDM-CH, fasting blood glucose values ≥ 5.1 mmol/L and post-load glucose values ≥ 10.0 mmol/L at 1 h and/or ≥ 8.5 mmol/L at 2 h (Kotzaeridi et al., 2021).

We adjusted for other risk factors of GDM: maternal age at pregnancy (<25, 25–29, 30–34, 35–39 or ≥ 40), maternal ethnicity (Han Chinese or other), gravidity (0 or ≥ 1), parity (0 or ≥ 1), the season of conception (spring: March, April, May; summer: June, July, August; fall: September, October, November; or winter: December, January, February), the year of conception (2014, 2015 or 2016) and fetal sex (girl or boy). We also adjusted for maternal weight at the 12th week of gestation and gestational weight gain (GWG) from 12th to 26th gestational weeks.

2.3. Air pollution measurements

We acquired information on air pollution from a fixed-location air pollution monitoring station located within 600 m of a major traffic road surrounding urban Beijing area (Feng et al., 2019; Liang et al., 2020). At this monitoring location, routinely monitored ambient particles included $PM_{2.5}$ (BAM-1020; Met One Instruments, Inc), BC (AE-33; Magee Scientific), and $PNC_{5\text{-}560}$ with a total of 32 size distribution segments (FMPS Model 3091; TSI). We further calculated daily average PNC in size ranges of 5–25 nm ($PNC_{5\text{-}25}$), 25–100 nm ($PNC_{25\text{-}100}$) and 100–560 nm ($PNC_{100\text{-}560}$), which reflected the nucleation mode, the Aitken mode and the accumulation mode, respectively (Han et al., 2016). The details of source apportionment for $PNC_{5\text{-}560}$ have been described in the supplementary material, and the detailed summaries of the resolved five factors were shown in Figs. S1 and S2. In addition, EC9800 series ambient gas analyzers (EcoTech Pty, Ltd, Australia) was

used to measure sulfur dioxide (SO_2), nitrogen dioxide (NO_2), carbon monoxide (CO) and ozone (O_3), and a Met One unit (Met One Instruments, Inc) was used to measure ambient temperature and relative humidity (RH). We averaged exposure data over three different exposure time-windows based on the LMP and gestational age: preconception (90 days prior to LMP), first trimester (LMP to the 12th gestational weeks) and second trimester (the 13th to the 26th gestational weeks).

2.4. Statistical analysis

We first calculated descriptive statistics for demographic characteristics and trimester-specific air pollution concentrations, and conducted Spearman correlations between respective exposure variables. We then performed logistic regression models to analyze the associations between maternal exposures to air pollutants and the risks of GDM-IFH, GDM-IPH and GDM-CH, and performed linear regression models for OGTT glucose levels, including fasting, 1-h and 2-h blood glucose levels (Hu et al., 2021; Ye et al., 2020). The basic models were built including maternal age, maternal ethnicity, gravidity, parity, the season and year of conception, fetal gender, maternal weight and GWG. The temperature and RH with natural splines function were then adjusted in the basic models based on minimizing Akaike's information criterion (AIC) (Wu et al., 2019), and the choice of degrees of freedom (DF) for temperature and RH in each exposure time-windows was shown in Table S1. Air pollutants including $PM_{2.5}$, BC, size-fractioned $PNC_{5\text{-}560}$, and gaseous pollutants were introduced separately into the basic models. For each identified source of $PNC_{5\text{-}560}$, we included both the source-related $PNC_{5\text{-}560}$ and the remaining $PNC_{5\text{-}560}$ in basic models (Samoli et al., 2016). Further, we conducted a stratified analysis to examine potential differences in the associations of ambient particles with GDM risks and OGTT glucose levels according to maternal age (<35 years old, and ≥35 years old).

We applied distributed lag models to characterize the exposure-lag-response associations of weekly-specific ambient particle exposures on GDM risks and OGTT glucose levels, and identify the critical timing of exposure (Gasparrini, 2014; Hu et al., 2021). The maximum lag was set at 39 weeks that covered preconception (13 weeks prior to LMP) and first two trimesters (LMP to the 26th gestational weeks). We also performed several sensitivity analyses to verify the reliability of main models: 1) we conducted two-pollutant models for ambient particles to examine the confounding effects of gaseous pollutants, except when the Spearman's correlation coefficients of pairwise pollutants were higher than 0.6 (Xu et al., 2019a); 2) we conducted a sensitivity analysis without adjusting for the season of conception; 3) we restricted residential address of participants to Haidian District; 4) we excluded fetal macrosomia, which has been commonly defined as birth weight greater than 4000 g (Shang et al., 2022).

All models were conducted by using R version 3.5.3, and $P < 0.05$ were considered statistical significance. For OGTT glucose levels, the Bonferroni correction was applied at $P < 0.017$ (0.05/3) to assess the significance of multiple testing (Xu et al., 2019b). The effect estimates were calculated as odds ratios (ORs) for GDM risk per interquartile range (IQR) increase in each air pollutant value, and percent changes for OGTT glucose levels.

3. Results

Among 24,001 pregnant women with singleton live births, 3,168 (13.2%) pregnant women were diagnosed with GDM (Table 1). Overall, the mean fasting, 1-h, and 2-h glucose values resulting from OGTT were 4.5 mmol/L, 7.4 mmol/L, and 6.3 mmol/L, respectively. The daily average air pollutant concentrations and the spearman correlation coefficient values for all environmental variables were described in Table S2 and Table S3, respectively. The period-specific air pollutant concentrations were similar among the three exposure periods, and the average values during the first trimester of $PM_{2.5}$, BC, $PNC_{5\text{-}25}$, $PNC_{25\text{-}100}$ and $PNC_{100\text{-}560}$ were 85.9 μg/m^3, 4.7 μg/m^3, 7,480 #/cm^3 for, 7,195 #/cm^3 for, and 5,951 #/cm^3, respectively (Table 2). The average contributions of major sources to $PNC_{5\text{-}560}$ were 40.9% from nucleation, 21.1% from gasoline vehicle emissions, 20.1% from diesel vehicle emissions and 9.8% from secondary aerosols.

Table 1
Characteristic of study population in Beijing, China, 2014–2017.

Characteristics (N = 24,001)	mean ± SD or N	(%)
Maternal age (years), N (%)		
<25	814	3.4
25-29	10,378	43.2
30-34	9,528	39.7
35-39	2,836	11.8
≥40	445	1.9
Ethnicity, N (%)		
Han Chinese	22,590	94.1
Other	1411	5.9
Gravidity (times), N (%)		
0	13,597	56.7
≥1	10,404	43.3
Parity (times), N (%)		
0	19,656	81.9
≥1	4,345	18.1
Season of conception, N (%) [a]		
Spring (March–May)	5,677	23.7
Summer (June–August)	6,519	27.2
Fall (September–November)	6,061	25.3
Winter (December–February)	5,744	23.8
Year of conception, N (%)		
2014	7,849	32.7
2015	8,110	33.8
2016	8,042	33.5
Maternal weight at the 12th week of gestation (kg), mean ± SD	58.7 ± 0.2	
GWG from 12th to 26th gestational weeks (kg), mean ± SD	5.7 ± 0.1	
Fetal sex, N (%)		
Girl	11583	48.3
Boy	12418	51.7
OGTT glucose levels (mmol/L), mean ± SD		
Fasting glucose levels	4.5 ± 0.5	
1-h glucose levels	7.4 ± 1.6	
2-h glucose levels	6.3 ± 1.2	

Abbreviation: GDM, gestational diabetes mellitus; GWG, gestational weight gain; SD, standard deviation; OGTT, oral glucose tolerance test.

As shown in Fig. 1, per IQR increase in maternal $PM_{2.5}$ exposure during the first trimester (OR = 1.56; 95%CIs: 1.19, 2.04) and second trimester (OR = 1.70; 95%CIs: 1.37, 2.10) was significantly associated with increased GDM risk. IQR increases in first-trimester exposures to BC, $PNC_{5\text{-}560}$, $PNC_{5\text{-}25}$, $PNC_{25\text{-}100}$ and $PNC_{100\text{-}560}$ were also associated with increased GDM risks of 1.41 (95%CIs: 1.23, 1.63), 1.43 (95%CIs: 1.22, 1.67), 1.08 (95%CIs: 0.97, 1.20), 1.61 (95%CIs: 1.35, 1.93) and 1.26 (95%CIs: 1.11, 1.44), respectively. The associations between ambient particles and risks of GDM subgroups were also shown in Fig. 1. IQR increases in first-trimester exposures to $PM_{2.5}$, BC, $PNC_{5\text{-}25}$, $PNC_{25\text{-}100}$ and $PNC_{100\text{-}560}$ were also associated with increased GDM-IFH risks of 1.94 (95%CIs: 1.23, 3.07), 2.14 (95%CIs: 1.73, 2.66), 1.36 (95%CIs: 1.15, 1.62), 2.94 (95%CIs: 2.19, 3.94) and 2.01 (95%CIs: 1.62, 2.49), respectively. The associations between ambient particle exposures and percent changes in OGTT glucose levels were shown in Fig. 2. Fasting glucose levels were also significantly associated with first-trimester exposures to $PM_{2.5}$, BC and size-fractioned $PNC_{5\text{-}560}$. No association of size-fractioned $PNC_{5\text{-}560}$ exposures with GDM-IPH risk, 1-h and 2-h blood glucose levels was observed.

Fig. 3 showed the associations of source-specific $PNC_{5\text{-}560}$ with GDM risk, GDM-IFH risk and fasting glucose levels. Per IQR increases in first-trimester exposures to gasoline and diesel vehicle emissions were also associated with increased GDM-IFH risks of 1.62 (95%CIs: 1.29, 2.05) and 2.39 (95%CIs: 1.77, 3.21), respectively. For each IQR increment in

Table 2
The air pollution levels during the preconception, first trimester and second trimester in pregnant women in Beijing, China, 2014–2017.

Variables	Exposure window	Mean ± SD	25th percentile	Median	75th percentile
Particles					
$PM_{2.5}$, μg/m^3	Preconception	81.8 ± 23.4	60.9	75.8	100.6
	1st trimester	85.9 ± 27.3	61.4	79.6	104.2
	2nd trimester	84.9 ± 25.1	65.0	76.4	100.0
BC, μg/m^3	Preconception	4.5 ± 2.5	3.1	4.0	5.3
	1st trimester	4.7 ± 2.8	3.2	4.3	5.8
	2nd trimester	4.4 ± 1.6	3.1	4.2	5.3
$PNC_{5\text{-}560}$, # cm^{-3}	Preconception	17,561 ± 4,779	13,086	17,153	20,920
	1st trimester	20,617 ± 7,722	14,212	19,933	23,805
	2nd trimester	19,569 ± 7,282	13,581	19,706	23,260
$PNC_{5\text{-}25}$, # cm^{-3}	Preconception	7,445 ± 1,252	6,516	7,474	8,232
	1st trimester	7,480 ± 1,267	6,494	7,320	8,436
	2nd trimester	7,379 ± 1,356	6,304	7,323	8,489
$PNC_{25\text{-}100}$, # cm^{-3}	Preconception	9,124 ± 3,390	6,050	8,749	11,813
	1st trimester	7,195 ± 3,315	4,457	6,026	10,171
	2nd trimester	7,000 ± 3,485	4,457	5,852	10,173
$PNC_{100\text{-}560}$, # cm^{-3}	Preconception	4,681 ± 2,840	1,971	2,708	3,276
	1st trimester	5,951 ± 7,186	2,331	3,301	4,369
	2nd trimester	5,200 ± 6,151	2,104	3,183	4,190
Gaseous pollutants					
NO_2, μg/m^3	Preconception	61.7 ± 9.5	55.9	60.5	67.1
	1st trimester	65.0 ± 9.6	57.8	63.3	72.0
	2nd trimester	63.0 ± 7.6	57.1	62.3	68.4
SO_2, μg/m^3	Preconception	20.5 ± 10.7	12.2	17.8	26.3
	1st trimester	19.7 ± 10.4	12.3	17.6	23.1
	2nd trimester	18.9 ± 9.2	14.1	17.3	21.8
CO, ppm	Preconception	1.3 ± 1.0	0.7	0.9	1.4
	1st trimester	1.1 ± 0.5	0.7	0.9	1.4
	2nd trimester	1.0 ± 0.3	0.7	0.9	1.3
O_3, μg/m^3	Preconception	108.6 ± 48.1	62.9	115.4	151.6
	1st trimester	99.2 ± 51.9	46.2	97.5	150.7
	2nd trimester	87.1 ± 46.3	44.1	74.8	122.9
Identified $PNC_{5\text{-}560}$ sources					
Nucleation, # cm^{-3}	Preconception	4,237 ± 1,844	2,109	5,266	5,778
	1st trimester	5,867 ± 941	5,155	5,726	6,650
	2nd trimester	5,787 ± 961	5,118	5,648	6,636
Gasoline vehicle emissions, # cm^{-3}	Preconception	3,810 ± 1,467	2,408	3,904	4,964
	1st trimester	3,317 ± 943	2,522	3,150	3,951
	2nd trimester	3,247 ± 1,020	2,446	3,192	4,001
Diesel vehicle emissions, # cm^{-3}	Preconception	3,662 ± 2,629	1,424	2,991	5,737
	1st trimester	3,083 ± 2522	969	2,193	4,689
	2nd trimester	3,138 ± 2553	1,013	2,351	4,505
Secondary aerosols, # cm^{-3}	Preconception	1,620 ± 1,047	608	1,579	2,490
	1st trimester	1,466 ± 897	528	1,575	2,201
	2nd trimester	1,440 ± 858	573	1,483	2,149

Abbreviations: $PM_{2.5}$, fine particulate matter; BC, black carbon; PNC_X, particle number concentration in the given size range (X nm); NO_2, nitrogen dioxide; SO_2, sulfur dioxide; CO, carbon monoxide; O_3, ozone; SD, standard deviation.

first-trimester exposures to gasoline and diesel vehicle emissions, fasting glucose levels increased by 1.5% (95%CIs: 1.0%, 2.0%) and 10.0% (95% CIs: 9.3%, 10.7%), respectively. Fig. 4 showed that the increased GDM risk was significantly associated with $PM_{2.5}$ exposure during the 1st to 13th weeks before LMP and 5th to 26th gestational weeks, with the strongest association in the 26th gestational week (OR = 1.09; 95%CIs: 1.06, 1.11). For GDM-IFH risk, there were also two significant exposure windows: the 1st to the 12th weeks before LMP and the 7th to the 26th gestational weeks, with corresponding peak associations in the 5th week before LMP (OR = 1.08; 95%CIs: 1.04, 1.11) and the 13th gestational week (OR = 1.12; 95%CIs: 1.09, 1.16), respectively. Weekly-specific $PM_{2.5}$ exposure during the preconception and first two trimesters were significantly associated with elevated fasting glucose levels, with the strongest association in the 16th gestational week (percent change = 0.7%; 95%CIs: 0.6, 0.7).

For gaseous pollutants, increased risks of GDM, GDM-IPH and GDM-CH were associated with increases in NO_2, SO_2 and CO exposures during the first two trimesters, while negative associations were observed for O_3 (Table S4). First-trimester exposures to NO_2, SO_2 and CO were associated with elevated OGTT glucose levels, but the effect estimates for fasting glucose levels were higher than 1-h and 2-h blood glucose levels (Table S5).

Sensitivity analyses showed that the effect estimates of $PM_{2.5}$, BC and size-fractioned $PNC_{5\text{-}560}$ remained robust with adjustment for gaseous pollutants in two-pollutant models (Tables S6–S12), without adjusting for the season of conception, after restricting pregnant women living in Haidian District, or after excluding macrosomia (Tables S13–S19). Corresponding effect estimates for ambient particles during the first two trimesters were comparable to those observed during the first or second trimester (Tables S20–S21). In the subgroup analysis, $PM_{2.5}$ exposure during the first and second trimesters, as well as BC, $PNC_{5\text{-}560}$, $PNC_{5\text{-}25}$, $PNC_{25\text{-}100}$ and $PNC_{100\text{-}560}$ exposures during the first trimester generally showed stronger associations with GDM-IFH risk among women who were ≥35 years old, and similar patterns were observed for the associations between ambient particles and fasting glucose levels (Table S22).

4. Discussion

This is the first study to provide evidence linking maternal exposure to traffic-related ambient particles to GDM subgroups classified by fasting and post-load glucose values from OGTT. In specific, elevated fasting glucose levels (percent changes: 2.1%–5.8%) and increased GDM-IFH risks (ORs: 1.23–2.24) were observed in associations with IQR increases in first-trimester exposures to traffic-related air pollution

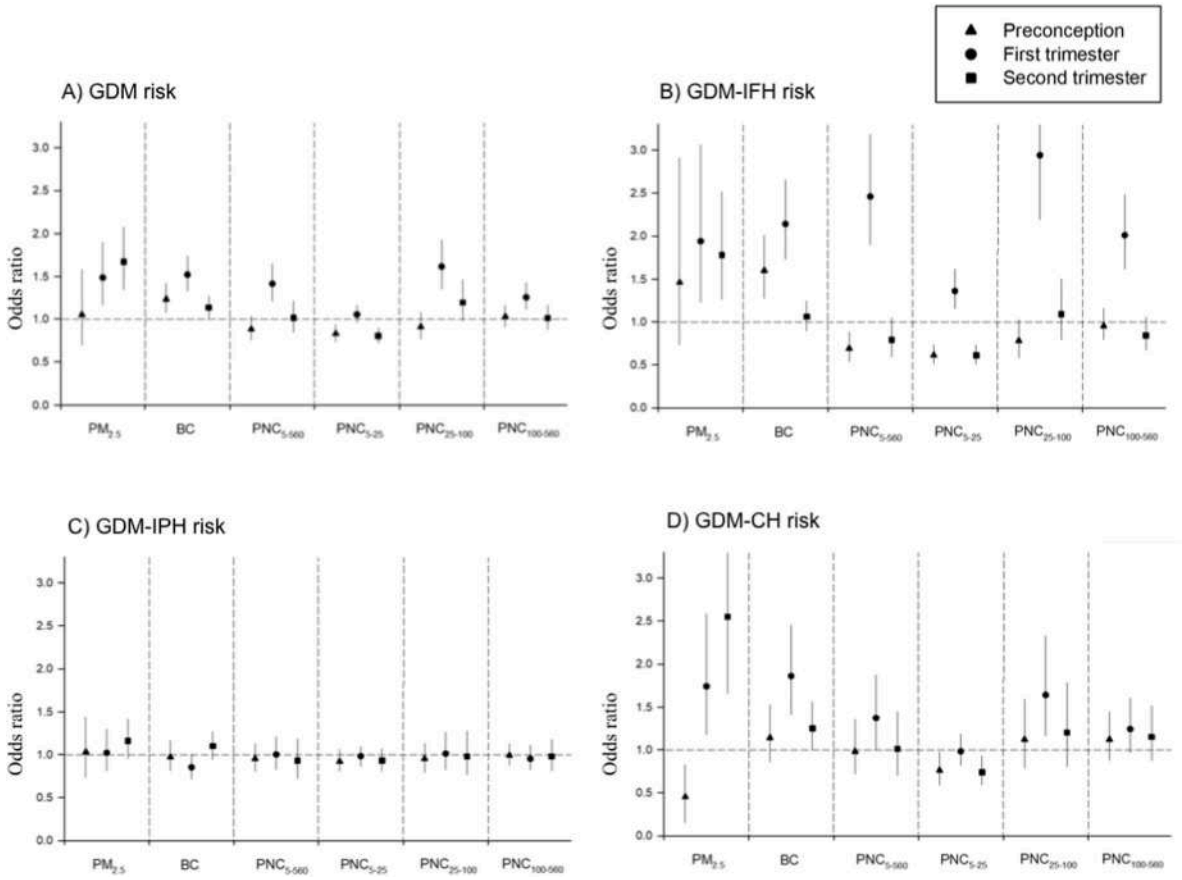

Fig. 1. Adjusted ORs and 95%CIs for GDM (A), GDM-IFH (B), GDM-IPH (C) and GDM-CH (D) risks associated with ambient particles (per IQR) during preconception and early pregnancy in Beijing, China, 2014–2017. Abbreviation: OR, odds ratios; CI, confidence interval; IQR, interquartile range; GDM, gestational diabetes mellitus; GDM-IFH, GDM with isolated fasting hyperglycaemia; GDM-IPH, GDM with isolated post-load hyperglycaemia; GDM-CH, GDM with combined hyperglycaemia; $PM_{2.5}$, fine particulate matter; BC, black carbon; PNC_X, particle number concentration in the given size range (X nm).

($PM_{2.5}$, BC and NO_2), as well as small-sized particles $PNC_{5\text{-}560}$ originated from traffic emissions. Our findings support the efforts to control traffic emissions in vulnerable population who have higher risk of glucose metabolism disorder.

We observed significantly associations between ambient $PM_{2.5}$ exposure and increased GDM risk in the preconception and first two trimesters of pregnancy, and the period from the 13th to the 26th gestational weeks might be a more susceptible exposure window. Recent meta-analyses have reported inconsistent associations between maternal $PM_{2.5}$ exposure and GDM risk during various exposure windows from preconception to the entire pregnancy (Tang et al., 2020; Zhang et al., 2020a). A case-control study reported that exposures to $PM_{2.5}$ in the 12 weeks prior to LMP, first trimester and second trimester were associated with significantly increased ORs of GDM at 1.10 (95% CIs: 1.03, 1.18), 1.09 (95%CIs: 1.02, 1.17) and 1.07 (95%CIs: 1.01, 1.14), respectively (Shen et al., 2017). Hu et al. reported that $PM_{2.5}$ exposures during the first and second trimesters were significantly associated with higher risks of GDM in Florida, USA, with ORs of 1.16 (95%CIs: 1.11, 1.21) and 1.15 (95%CIs: 1.10, 1.20) per 5 $\mu g/m^3$, respectively (Hu et al., 2015). A Chinese prospective cohort study estimated the associations of maternal weekly exposure to $PM_{2.5}$ on GDM risk, and reported that the 22nd week of gestation might be the most susceptible exposure window (Chen et al., 2021). In this study, we for the first time explored the effect of maternal $PM_{2.5}$ exposure on increased risk of GDM classified by OGTT results, and showed significant associations with increased risks of GDM-IFH and GDM-CH, but not of GDM-IPH. Consistent with study hypothesis, our results suggest that ambient particle exposures may increase GDM risk by exerting adverse effects on fasting blood glucose concentrations during pregnancy.

The evidence on the relationship between ambient air pollutants and OGTT glucose levels has been limited. Lin reported that per 10 $\mu g/m^3$ increase in $PM_{2.5}$ and PM_{10} exposures during the first two trimesters were associated with 0.07 mmol/L to 0.29 mmol/L increment in fasting glucose levels in Foshan, China (Lin et al., 2020). Another Chinese study reported that per IQR increment of $PM_{2.5}$ (15.97 $\mu g/m^3$) and BC (0.89 $\mu g/m^3$) during the second trimester increased 0.13 mmol/L (95%CIs: 0.07, 0.17) and 0.11 mmol/L (95%CIs: 0.08, 0.14) in fasting blood glucose levels, respectively, but did not identify association with 1-h or 2-h blood glucose levels (Hu et al., 2015). In this analysis, we found positive associations between maternal $PM_{2.5}$ exposure and elevated OGTT glucose levels, and the association was stronger in the elevation of fasting glucose levels during pregnancy. Different potential mechanisms may partially explain our finding of the differential associations in relation to ambient particle exposures between fasting and post-load glucose levels. Fasting hyperglycaemia has shown to be more closely related with hepatic insulin sensitivity and consequent hepatic glucose production, while post-load hyperglycaemia is more closely related to muscle insulin resistance (Abdul-Ghani et al., 2006; Hu et al., 2021). Therefore, hepatic insulin resistance might play important roles in ambient particles-induced glucose metabolism disorder during pregnancy (Kotzaeridi et al., 2021).

Research has also found that smaller ambient particles can adsorb more organic components, which were highly correlated with oxidative stress biomarkers (Araujo et al., 2008). Therefore, smaller particles could be more potent than $PM_{2.5}$ toward inducing a cascade of effects related to oxidative stress in epithelial cells and macrophages (Li et al.,

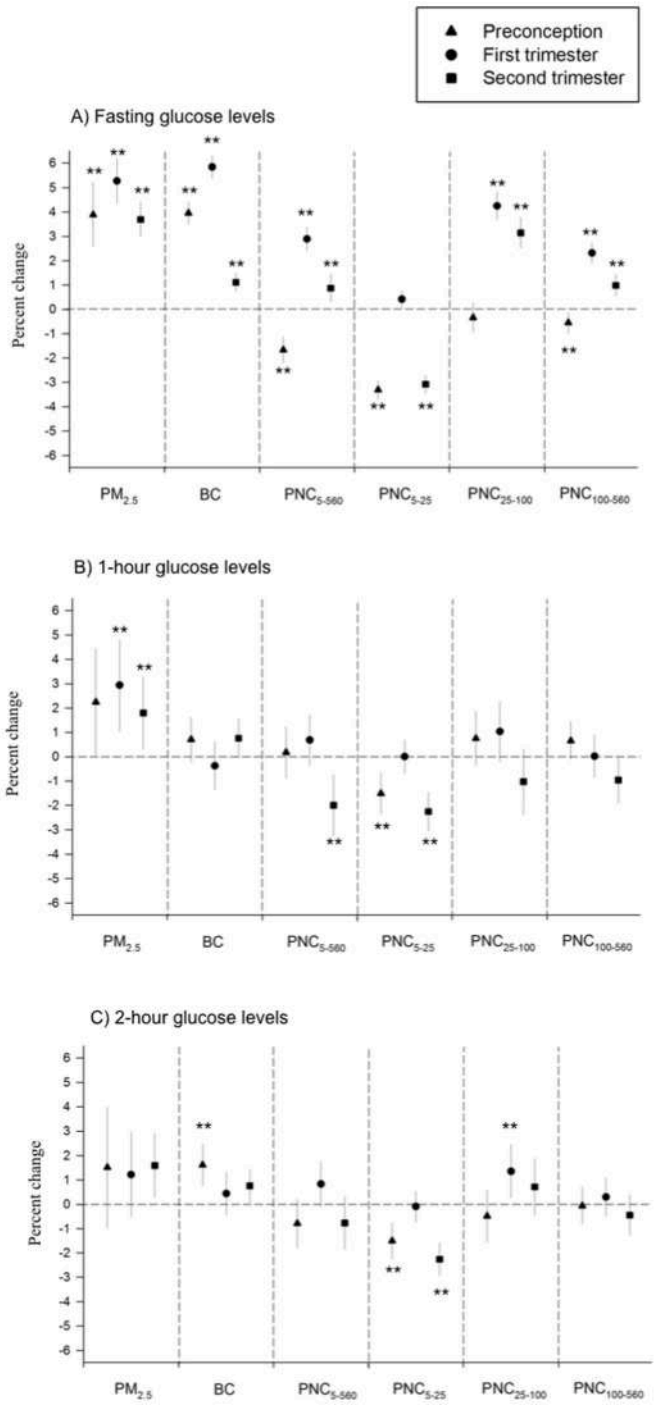

Fig. 2. Adjusted percent changes and 95%CIs in fasting (A), 1-h (B) and 2-h (C) glucose levels associated with ambient particles (per IQR) during preconception and early pregnancy in Beijing, China, 2014–2017. Bonferroni corrections with significance (P < 0.017) are indicated by double asterisks. Abbreviation: CI, confidence interval; IQR, interquartile range; $PM_{2.5}$, fine particulate matter; BC, black carbon; PNC_X, particle number concentration in the given size range (X nm).

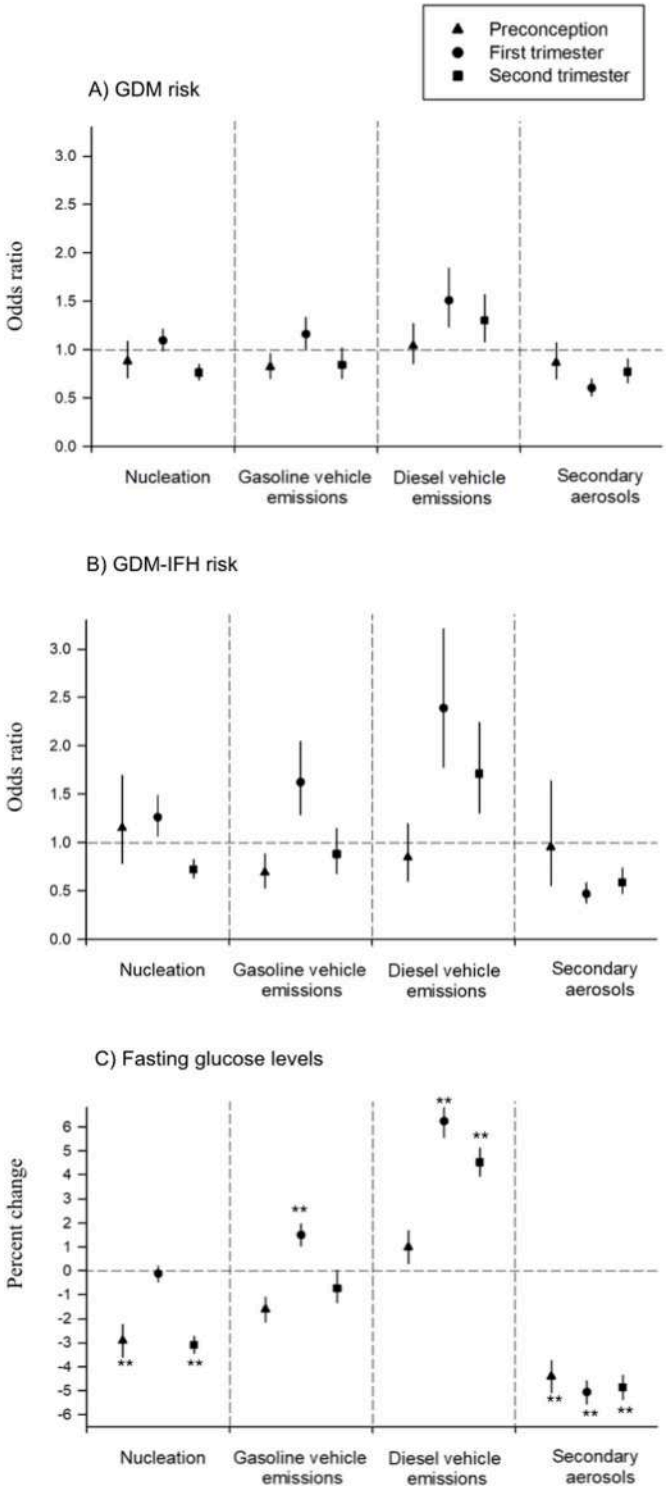

Fig. 3. Adjusted effect estimates and 95%CIs for GDM and GDM-IFH risks (A-B, odds ratios) and fasting glucose levels (C, percent changes) associated with major sources of $PNC_{5\text{-}560}$ (per IQR) during preconception and early pregnancy in Beijing, China, 2014–2017. Bonferroni corrections with significance (P < 0.017) are indicated by double asterisks for fasting glucose levels. Abbreviation: GDM, gestational diabetes mellitus; GDM-IFH, GDM with isolated fasting hyperglycaemia; CI, confidence interval; IQR, interquartile range; PNC_X, particle number concentration in the given size range (X nm).

2003). Very few studies have reported association between maternal exposure to smaller particles and GDM risk. Bai et al. found that each IQR change in exposure to UFPs was associated with increased risk of diabetes among general population (Hazard ratios = 1.06; 95%CIs = 1.05, 1.08) in Toronto, Canada (Bai et al., 2018). Chen et al. observed that each IQR increment (6.0×10^3 #/cm^3) in UFPs was associated with 14.0% (95%CIs: 6.4%, 20.9%), 6.6% (95%CIs: 0.4%, 12.4%), and 8.5%

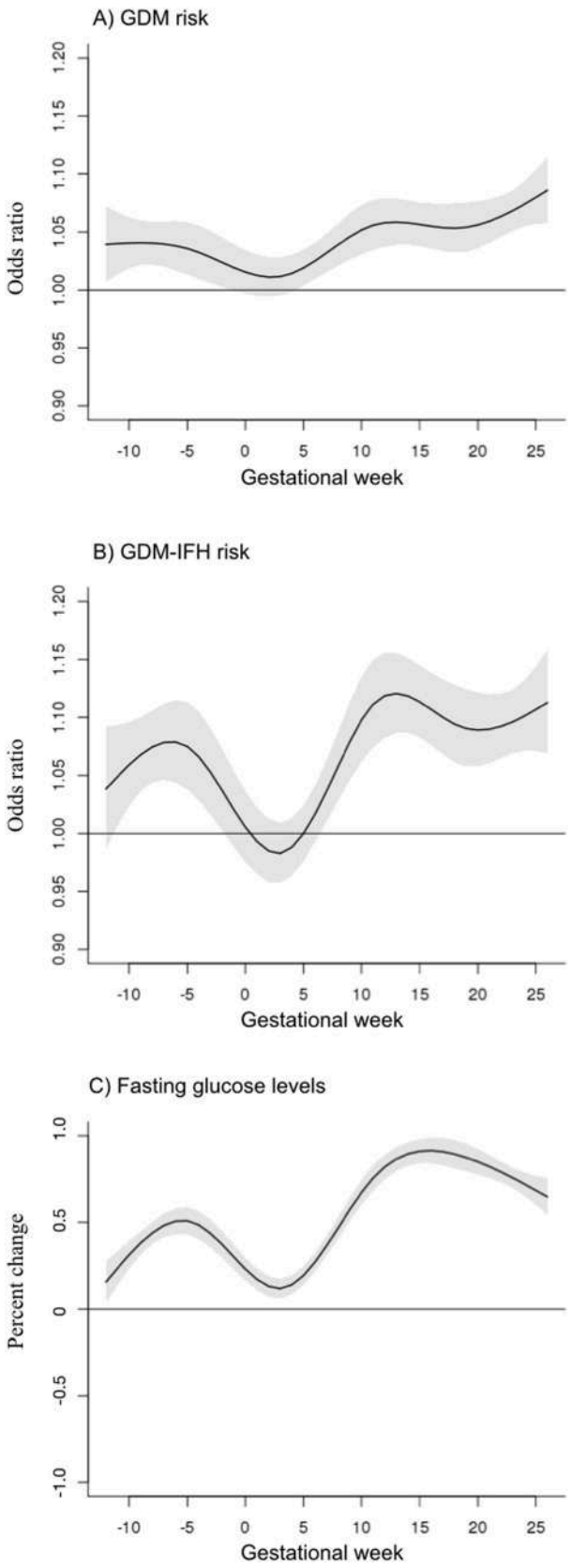

Fig. 4. Adjusted effect estimates and 95%CIs for GDM and GDM-IFH risks (A-B, odds ratios) and fasting glucose levels (C, percent changes) associated with weekly-specific $PM_{2.5}$ exposure (per IQR) in Beijing, China, 2014–2017. Abbreviation: GDM, gestational diabetes mellitus; GDM-IFH, GDM with isolated fasting hyperglycaemia; CI, confidence interval; IQR, interquartile range; PNC_X, particle number concentration in the given size range (X nm).

(95%CIs: 2.2%, 14.5%) decreases in adiponectin, leptin and resistin levels, which were the major mediators of insulin resistance and diabetes (Chen et al., 2020). Consistently, we found positive associations of first-trimester exposures to size-fractioned $PNC_{5\text{-}560}$ on the increased GDM-IFH risk and elevated fasting glucose levels.

In this study, traffic emissions, including gasoline and diesel vehicle emissions, have been identified as the major source of $PNC_{5\text{-}560}$ in associations with the increased GDM-IFH and GDM-CH risks in this study. Prior studies have reported that maternal exposure to traffic emissions was associated with increased GDM risk (Malmqvist et al., 2013; Yorifuji et al., 2015). Malmqvist et al. categorized the traffic density into three levels, including low (no road within 200 m), medium (road with $\leq$10 cars/min) and high (road with >10 cars/min), and reported that the risks of GDM versus non-GDM were also increased in pregnant women living in a neighborhood with the high (vs. low) level of traffic density (OR = 1.23; 95%CIs: 1.01, 1. 51) (Malmqvist et al., 2013). We also found significant increases in the risks of GDM-IFH and GDM-CH in association with exposures to BC, NO_2 and CO, which were all widely recognized surrogates of traffic emissions. Consistently, Zheng et al. reported higher GDM risk in association with increased BC exposure during the first two trimesters (OR = 1.09; 95%CIs: 1.08, 1.10) in Florida, USA (Zheng et al., 2020), and Choe et al. reported increased GDM risk in association with increased NO_2 exposure during the first trimester (OR = 1.05; 95%CIs: 1.01, 1.09) in New York City, USA (Choe et al., 2019). Our study identifying $PNC_{5\text{-}560}$ originated from traffic emissions in association with the increased GDM risk can provide a practicable guidance to reduce exposure in pregnant women that have higher risks of glucose metabolism disorder.

Our study had some strengths. Firstly, we classified the GDM complicated pregnancies into OGTT-IFH, OGTT-IPH and OGTT-CH based on the fasting and\ post-load glucose values from OGTT, and explored the differential impacts of ambient particle exposures on fasting and post-load hyperglycaemia during pregnancy, which has been rarely evaluated. Secondly, the continuous daily measurement of particles in size fractions of 5–560 nm over years in the metropolitan Beijing area provided essential information to apportion the sources of ambient particles in much smaller sizes by using PMF models. The estimated effects of ambient particle sizes between 5 and 560 nm and their sources can largely enhance the understanding of the increased GDM risk from ambient particles as a mixture. Concomitantly, we should note several limitations in this study. Firstly, pregnant women generally underwent OGTT in late second trimester to screen for GDM and thus we were only able to assume that the test occurred between 24 and 28 weeks of gestation by following IADPSG standards and recommendations, which might introduce potential misclassification on exposure timing estimates. Secondly, the estimated exposure of each participant was based on a single fixed-site monitoring station and without considering time-activity models and residential mobility during pregnancy, which might also introduce exposure misclassification that can lead underestimate of the effects toward null. Thirdly, pre-pregnancy body mass index of each participant was unavailable, we were only able to adjusts for maternal weight and GWG during the pregnancy. Fourthly, information on socioeconomic status (e.g., education, marital status and income) was also not available, limiting the ability to exclude the possibility of residual confounding by other unmeasured factors.

5. Conclusion

In this retrospective cohort study, we found that maternal exposures to traffic-related ambient particles were associated with elevated fasting glucose levels and increased GDM-IFH risk. Our findings enhance the current understanding of air pollution effects on glucose metabolism disorder during pregnancy, and highlight the public health importance of traffic emission control priority to minimize adverse effects in pregnant women.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2022.113973.

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Pollen allergy: Developing multi-sectorial strategies for its prevention and control in lower and middle-income countries

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ABSTRACT

Pollen allergy is considered a major public health problem that causes morbidity and subsequently affects a patient's quality of life. Pollen due to their large size cannot enter the thoracic regions of the respiratory tract but can affect the nasopharyngeal mucous membrane. At the same time, the submicronic-pollen particles can act as respirable particles reaching deeper into the upper airways leading to exacerbation of asthma, chronic obstructive pulmonary disease (COPD) and other allergic reactions. Based on the existing literature, expanding evidence shows that climate change and air pollutants could affect the pollen number, morphology, season, allergen content, and distribution pattern. Hence, this will influence the prevalence and occurrence of allergies linked to pollen exposure. Being a part of biogenic pollutants, pollen allergens are not expected to diminish in the foreseeable future. Therefore, it is imperative that steps need to be strengthened to improve and optimize preventive/adaptive strategies. This paper aims to review the major causes of widespread allergy, identify the major gaps, and suggest key preventive/adaptive measures to address the onset and exacerbation of pollen-related allergic diseases with a major focus on lower and middle-income countries. The study also discusses how-to implement the prevention and control measures at the individual, health care communities and organizations, Local Governments, National/International Governments levels to decrease the risk of illnesses associated with pollen allergy.

1. Introduction

Green spaces are considered critical components in providing ecosystem services by minimizing pollution, air temperature, noise, and soil erosion. Despite their undeniable benefits for humans, they could be associated with many concerns if not appropriately managed (Sousa-Silva et al., 2020). The most severe challenge related to the plants is the human allergic responses to pollen grains released during pollination.

Over the past few decades, the prevalence of pollen allergy has increased. This trend is projected to rise due to urbanization, air pollution, and climate change, particularly in urban areas (Pawankar et al., 2013; Aerts et al., 2021). The noticeable negative impact of pollen has been seen in the sensitive population. Pollen cannot enter the thoracic regions of the respiratory system due to their large size (10–80 μm), but they can impact the nasal and nasopharyngeal mucosal membranes. Also, submicronic-pollen particles can act as respirable allergens, which can reach deeper into the upper airways leading to various respiratory diseases. Exposure to allergic pollen from specific trees, grasses and weeds leads to a wide range of health effects. Though, a non-linear relationship has been described between different airborne pollen species with certain respiratory illnesses (Jones et al., 2021).

Pollen are also considered the primary cause of allergic rhinitis (AR). The prevalence of allergic rhinitis has increased in developed countries and affects about 10%–30% of adults and 20%–25% of children worldwide, posing severe repercussions on the quality of life (Hellings et al., 2017; Meng et al., 2020). However, several studies have found that pollen are also associated with the increasing problem of wheezing, atopic dermatitis (eczema) and exacerbation of asthma and chronic obstructive pulmonary diseases (COPD) in susceptible individuals (Cecchi et al., 2010; Singh and Mathur, 2021).

An extensive epidemiological study was conducted by ISAAC (International Study of Asthma and Allergies in Childhood) to determine the prevalence of asthma, allergic rhinitis, and atopic eczema in children and adolescents. The prevalence of current wheeze was found to be 7%

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in Indian children aged 6–7 years and 13–14 years (Krishna et al., 2020). In contrast, the overall prevalence of nasal symptoms and rhino-conjunctivitis is 12.5% and 3.3.% among Indian children aged 6–7 years, respectively, and 18.6% and 5.6% among Indian children 13–14 years respectively (Chandrika, 2017; Kondraju and Sudhakar, 2019). However, around 20–30% of the population suffers from allergic rhinitis in Europe and the USA (Wang et al., 2021). Numerous other studies were also conducted to determine the prevalence of allergic rhinitis, atopic dermatitis (AD), eczema and wheezing in children and adolescents (as shown in Table 1).

Several international organizations, namely, American College of Allergy, Asthma, and Immunology (ACAAI), American Society for Microbiology (ASM), European Aerobiology Society (EAS), Indian Aerobiology Society (IAS), Nordic Aerobiology Society (NAF), The Palynological Society (AASP), etc. are actively working in the field of aerobiology to address the various issues related to pollen allergy (https://sites.google.com/site/aerobiologyinternational/links). Apart from this, the Global Initiative for Asthma (GINA), European Academy of Allergy and Clinical Immunology (EAACI), American Academy of Allergy, Asthma & Immunology (AAAAI), Allergic Rhinitis and its Impact on Asthma (ARAI) are some of the leading organizations that have proposed guidelines and recommendations covering the risk factors, development of disease, proper diagnosis, healthcare pathway and treatment involved with allergic rhinitis, allergic diseases, seasonal allergic asthma and immunology (Agache et al., 2018; Boulet et al., 2019; Bousquet et al., 2008; Cingi et al., 2017; Horak et al., 2016). As majority of these guidelines and recommendations are not directly focusing on pollen allergy, therefore, to address these gaps, there is a need for appropriate policies and framework related to pollen allergy covering various aspects (types of allergic species, their allergenic potential, peak pollen seasons) for the prevention and control measures at individual and public level to decrease the risk of illnesses associated with pollen allergy.

In order to alleviate the effects of climate change and air pollution, the establishment and growth of green spaces and urban forests are encouraged to improve the quality of life. However, an increase in the green spaces may also enhance allergic pollen levels. Therefore, knowing pollen will always persist in the environment, developing and strengthening pollen allergy prevention and treatment strategies are needed. The core purpose of the current review is to critically examine the implementation gaps and barriers to address the issue of pollen allergy and to prevent pollen allergy to avoid new exacerbations and their potential strategic interventions. Not many diverse studies are available that highlight the unmet needs and barriers to preventing and controlling pollen allergies. Therefore, this study examined the scientific literature to understand or assess the gaps and barriers and recommended the key measures required in order to prevent and control pollen allergies with a major focus on lower and middle-income countries.

Table 1
Prevalence (%) of allergic rhinitis (AR), atopic dermatitis (AD), eczema and wheezing.

Diseases	Prevalence (%)	Study Area	Year	References
Allergic rhinitis	12.9–22.5	Denmark	1990–1998	Linneberg et al. (2000)
	23	Europe	2001	Bauchau and Durham (2004)
	32.2	United Kingdom	2001	Shamssain (2007)
	28.5	Pakistan	2007	Hasnain et al. (2009)
	11.1	China	2005	Zhang et al. (2009)
	23.1	Turkey	2008–2009	Cingi et al. (2010)
	28	Sweden	2007	Eriksson et al. (2012)
	5.8–13.7	Japan	2006	Honda et al. (2013)
	14.9	Beijing	2012	Zhang et al. (2013)
	19.9	United States	2001–2013	Hill et al. (2016)
	17.6	China	2011	Wang et al. (2016)
	28.3	Tehran	2013–2016	Shoormasti et al. (2018)
	24.4	India	2001–2003	Singh et al. (2018)
	22.2	Japan	2013–2017	Nakamura et al. (2019)
	17.1	Korea	2008–2017	Ha et al. (2020)
Atopic Dermatitis	17.8	Stockholm	1992–1994	Kihlström et al. (2002)
	3.9	Tehran	2013–2016	Shoormasti et al. (2018)
	21.2	Japan	2013–2017	Nakamura et al. (2019)
	3.9	Korea	2008–2017	Ha et al. (2020)
	4.65	French	2016	Richard et al. (2021)
Eczema	24.2	United Kingdom	2001	Shamssain (2007)
	21.8	Pakistan	2007	Hasnain et al. (2009)
	8.7–13.5	Japan	2005–2006	Honda et al. (2013)
	6.7	United States	2001–2013	Hill et al. (2016)
	3.7	India	2001–2003	Singh et al. (2018)
Wheezing	16.1	Bangladesh	2001	Zaman et al. (2007)
	11.7	Pakistan	2007	Hasnain et al. (2009)
	18.3	Thailand	2002	Bunnag et al. (2009)

2. Methodology

This comprehensive literature review explores and discusses the implementation gaps and measures to pollen allergy prevention and control from simple, basic, to more complex levels. The inclusion criteria for screening the relevant studies were: 1) recent publication in an international peer-reviewed journal available on PubMed, Google Scholar, Web of Science, Scopus, Science Direct, etc. database (in or after the year 2000). The latter was justified as the knowledge and clinical studies have been substantially evolved over time and the earlier studies do not provide much relevant data for current policies; 2) emphasis on articles that review hypersensitivity to pollen, related mechanism of action, clinical manifestations of allergic rhinitis and asthma, diagnostic test, cases of sensitivity to different pollen species and subsequent management; 3) published in English 4) both in-vivo and in-vitro studies were also considered. The exclusion criteria include 1) thesis, dissertations, case report, conference proceedings, letters to the editor and editorials; 2) language except for English. The literature review is not meant to be exhaustive but rather, it is intended to identify allergy severity, incidences, and complexity for developing integrated management strategies. The keywords were beginning with “pollen”, “pollen allergy”, “allergic rhinitis”, followed by the search words and their combinations: “allergies”, “pollinosis”, “hay fever”, “eczema”, “asthma”, “COPD patients”. The search was performed for articles published in English using Boolean logic searching methods (AND/OR/NOT/()/“ ”). Many of these articles were outside inclusion criteria and duplicated, and those were excluded. Additionally, a manual search for relevant articles was also carried out in each article’s bibliography section.

3. From where did the pollen allergy start?

Pollen grains are male biological entities produced by higher plant

cells that are essential for sexual reproduction. Pollen themselves are immobile and dispersion is aided by agents such as water, insects, birds and wind. Hence, they are considered as aeroplanktons (floating in the air) surrounding human beings. The first physician, John Elliotson, identified pollen grain allergy in 1831, whereas John Bostock first reported 'Hay Fever' in 1819. The detailed experiment was conducted by Phoebus (1862) and Charles Blackley (1873) (Cresti and Linskens, 2000; Xie et al., 2019). In 1873, Charles Blackley, in his book "Catarrhus aestivus" revealed the connection between pollen and pollinosis based on skin and provocation tests which leads to confirmation in the field of etiology. In 1908, Doctor A. Carini from Sao Paulo published the first paper on pollinosis, where the existence of hay fever was questioned in Brazil (Taketomi et al., 2006). Since then, pollen has received attention as an allergic agent by most physicians, botanists and aerobiologists. Further studies also show significantly higher pollinosis incidences in atopic individuals responding to grass and tree pollen (Frei and Leuschner et al., 2000; Jutel et al., 2005; Kailaivasan and Davies, 2018).

Numerous time-series studies correlate pollen seasons with hospital emergency room (ER) visits due to asthma and chronic obstructive pulmonary disease (COPD) (Jariwala et al., 2011; Darrow et al., 2012; Simunovic et al., 2020). Patients also show ocular pruritus with coryza, sneezing, nasal pruritus, nasal obstruction, rhinorrhea, angioedema and conjunctival hyperemia in any combination (Taketomi et al., 2006; Greiner et al., 2011). Two major symptoms, i.e., conjunctival hyperemia and nasal pruritus, differentiate pollinosis (hay fever) from the common cold. Clinically, allergic rhinitis (pollinosis or hay fever) is characterized by four major symptoms, i.e., sneezing, rhinorrhea, nasal itching and nasal congestion. Pollen are considered primary potential triggers of asthma and COPD; even though their size is large, they affect the upper airways of mucosa when inhaled. Each pollen grain is a double-walled structure (except in some cases) that contains different metabolites like proteins, carbohydrates, hormones, organic acids, pigments, minerals, etc. The proteins present inside pollen grains can activate strong immunological responses in the sensitized peoples known as Type-I hypersensitivity reactions. Thus, releasing the allergen from the pollen grain is a prerequisite for evoking allergic responses in sensitized individuals (Taketomi et al., 2006). There are two prerequisite mechanisms required for allergenic particles to be expelled from pollen grains: (i) allergens readily diffuses coming in direct contact with mucosal surfaces such as conjunctiva and nose, (ii) humidity and rain leads to rapid hydration of pollen to expel cytoplasmic inhalable material, capable of evoking IgE antibody-mediated allergic reactions, reaches lower airways due to small size and induces asthma (D'Amato et al., 2007b; Reid and Gamble, 2009; Stone et al., 2021).

These mechanisms reflect the probability of inducing epidemics (Ravindra et al., 2021a), like thunderstorm-related asthma and their linkage with the rise in hospital admission within a few hours of storm passage (Villeneuve et al., 2005; Losappio et al., 2011; Thien et al., 2018). It is not easy to evaluate the weather pattern on the prevalence of airborne pollen distribution patterns. However, the global rise in temperature and severe weather events studies indicate that they contribute to the dissemination of pollen grains and may further affect the sensitive and vulnerable population.

4. Environmental factors affecting airborne pollen

Pollen allergy is often considered a trivial disease. However, it is a major respiratory illness that causes morbidity and subsequently affects a patient's quality of life (Mridula et al., 2011; Greiner et al., 2011; Muzalyova et al., 2019). Due to the ubiquitous nature of pollen grains, it is estimated that a high percentage of humans are sensitive to these kinds of allergens. In contrast to some countries where the prevalence of allergic rhinitis (hay fever) has been stabilized or decreased, morbidity statistics are still rising in others (Pawankar et al., 2013; Guilbert et al., 2016). For example, it is established by European Community Respiratory Health Survey that the prevalence of allergic rhinitis has increased from 4% to 32% in Europe (Pawankar et al., 2013). There are many human-driven factors responsible for the increase of pollen allergens in the atmosphere, such as:

(i) Climate change and increase in global surface temperature in the atmosphere have a significant impact on plant life-cycle events (photosynthesis and plant growth) and their physiological parameters (such as pollen production, morphology and pollen season) (Ziello et al., 2012; Damialis et al., 2019; Poole et al., 2019). Hence, the overall relationship between climatic factors, meteorological conditions and pollen concentrations can be complex and multi-dimensional. CO_2 is considered the sole supplier of carbon to plants, which affects photosynthesis, plant growth, and pollen production (Ziska and Caulfield, 2000; Levetin and Van de Water, 2008). An increase in temperature and CO_2 influences pollen concentration, season timelines, strength and duration, and possibly increased allergenic content, which does not remain consistent and is found to be variable between various pollen emitting species in the atmosphere (Wan et al., 2002; Damialis et al., 2007; Cecchi et al., 2010; Cuinica et al., 2014; Beggs, 2015; Ziska et al., 2019). Therefore, these changing events affect the pattern of pollen that could affect pollinosis and exacerbate other allergic diseases.

(ii) Another contributing aspect in the magnitude and concentration of pollen is the change in meteorological conditions (Gross et al., 2019) and geographic distribution (Wjst et al., 2005; Skjøth et al., 2013). The pollen concentration at a particular site reflects the distribution of pollen sources within a few kilometers of the site (Ravindra et al., 2021c). Changes in meteorological factors, especially wind speed and direction, will directly impact aerobiological pollen activities such as emission, dispersal, transmission, and deposition. This will affect the prevalence and occurrence of pollen-related allergies (Mandal et al., 2008b; Poole et al., 2019). Some evidence supports that sensitive populations respond to abrupt weather changes in the environment, for example, thunderstorm events (Losappio et al., 2011; D'Amato et al., 2016; Hew et al., 2019; Sabih et al., 2020). Numerous studies reported the long-distance transport of pollen from their release sites. Pollen grains disperse and travel from one location to another due to changing weather conditions, exposing humans to new and novel allergens (Yang et al., 2019).

(iii) Rapid unplanned urbanization with high vehicular emissions was reported to be linked with rising incidences of pollen-induced respiratory allergies (Ghiani et al., 2012). Consistent studies support that people living in urban areas are more likely to suffer pollen allergies than people in rural areas (Wan et al., 2002; D'Amato et al., 2007a; Sénéchal et al., 2015; Charalampopoulos et al., 2021). Further, air pollutants and pollen are known to have a synergistic effect on human health (Beck et al., 2013). Air pollutants can attach to the surface of pollen grains and can modify morphology (change in diameter, thinning in exine, exine rupture) of these antigen carrying agents and alter the allergenic potential (Motta et al., 2006; Suárez-Cervera et al., 2008; Azzazy, 2016; Oduber et al., 2019). Acute and chronic exposure to various pollutants such as nitrogen dioxide (NO_2), sulfur dioxide (SO_2), ozone and particulate matter intensifies airway responsiveness to pollen in atopic individuals (Zhao et al., 2016).

These observations show a clear association between changing temperature, meteorological parameters, air pollution and ambient pollen number/concentration. The relationship between the climatic factors and airborne pollen production could have a manifold multifactorial system, as shown in Fig. 1. However, determining anticipated changes that are contributed by increased pollen allergy will require an extensive network of research.

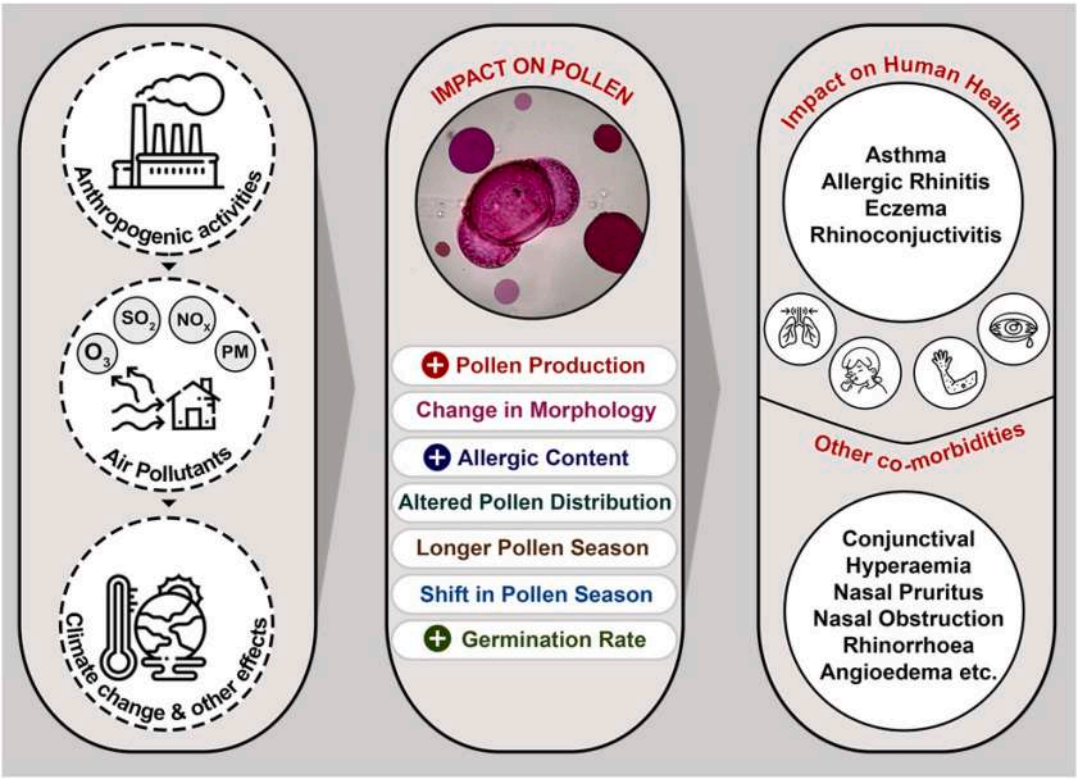

Fig. 1. Depicting impact of climate change on airborne pollen and human health.

5. What are the unmet needs and research gaps identified?

The prevalence of pollen allergy severity, incidences and complexity have dramatically increased over two decades (Damialis et al., 2007; Poole et al., 2019; Ziska et al., 2019; Simunovic et al., 2020). If not treated at early age, it can lead to "multi-system allergic diseases" in later life, which is life-threatening and may cause fatal reactions. There are various significant research gaps and challenges involved with increasing incidences of pollen allergies:

1. A high abundance of pollen has been associated with exacerbating various allergic responses and respiratory diseases (Singh and Mathur, 2021). The severity of allergic rhinitis due to the intricate interaction of genetic and environmental factors considerably increased within the last decade. Therefore, a growing requirement is to estimate accurate pollen concentration globally (D'Amato et al., 2015). Palynologists and botanists are always interested in measuring pollen concentration in the atmosphere. Various samplers have already been installed for the proper monitoring of airborne pollen allergens. Real-time measurements, which potentially offer immediate warning alerts to sensitized individuals, have been established at some places (Sofiev, 2019; Schaefer et al., 2021). However, a fully-reliable, operational, automated real-time aero-allergen monitoring program with global networking is necessary to collect and disseminate the obtained pollen data to develop long-time series data.
2. Several physicians, allergists and scientists have used aerobiology data in the medical community to properly diagnose and treat pollen allergic patients (Levetin and Van de Water, 2008). Most of the research has been done in Europe and the USA, providing better insights into pollen allergies. Hence, more research is needed in various lower- and middle-income countries, which are considered rich in floral diversity.
3. Studies from around the last three decades have shown that pollen allergens-based respiratory disease has increased in parallel to pollen production of some plant species (D'amato et al., 2001; Reid and Gamble, 2009; Osborne et al., 2017; Guilbert et al., 2018). In addition, changes over a period of time, potentially linked to climate change or globalization, can be used to explain and predict trends in the incidence of pollen allergies are missing. Therefore, the impacts of climate change on pollen and respiratory allergies caused need much more elucidation (Beggs, 2015).
4. In addition, there is a shortage of pollen allergy specialists/trainees who can provide the essential guidance and treatment, as well as lead the search for solutions to control allergies (Cecchi et al., 2010).
5. Among the ongoing studies related to plants and pollen allergens, research gaps have been found concerning the production and dissemination of allergens and specified proteins as well as their linkage with land use patterns, industrialization, environmental pollutants and extreme weather conditions (Ziska and Beggs, 2012).
6. Although various immunological and genetic studies also have been looking for different risk factors for allergic rhinitis and allergic asthma, focusing on identifying appropriate central genes to substantiate preventive measures (Papadopoulos et al., 2012). Meanwhile, some epidemiological studies have supported the famous "hygiene hypothesis" where early childhood exposure to microbes could protect against allergic diseases by providing the development of the immune system (Liu and Murphy, 2003; Mutius, 2007). To date, there is not sufficient evidence for the hygiene hypothesis, as complexities between the gene-environment relationships/interactions are apparent.
7. Pollen allergy is often considered a self-managed condition that enables atopic individuals to be at risk of missing health checkups and proper treatment (Medek et al., 2019). There is a lack of awareness among the people regarding pollen allergy, majorly in low-middle income countries. The symptoms of pollen allergy are somewhat similar to common flu and cold. Therefore, even if they have an allergy, itchy throat, or running nose, they try to treat it with home remedies. So, the dissemination of proper knowledge regarding

pollen allergy, allergen avoidance, their symptoms and management is needed to better address the pollen allergy ailment.

8. Longitudinal studies in children and adults, with and without treatments, are needed to better understand illness progression and treatment options.
9. Most European countries possess long-time pollen series data and forecasting models such as SILAM models, numerical forecasting and others (Sofiev et al., 2006; Muzalyova et al., 2021). But globally, data on airborne pollen concentration are limited; mainly, long-term time series are rarely available, especially for lower and middle-income countries. Major future challenges involve the collection of databases and applying these data streams into pollen forecasting systems using artificial intelligence and machine learning approaches. Above all, timely risk alerts personalized forecasts on allergy management are also required.

6. What are the recommendations to overcome the barriers to pollen allergy prevention and control?

Prophylaxis in pollen allergy is difficult, as humans work within the same environment and it is quite challenging to avoid airborne pollen exposure (Wang, 2005). But some basic preventive measures can be taken to reduce exposure, such as the public may be advised to stay indoors, keep windows and doors closed, avoid gardening or grass-cutting during peak pollen seasons when the quantity and propagation of airborne pollen are significant Spanish Aerobiology Network (REA, http://www.uco.es/investiga/grupos/rea/). Although not fully established but several ways to reduce allergic reactions have been suggested based on these three milestones as shown in Fig. 2 (Tanno et al., 2017).

The current work examined the scientific literature to understand the key measures and formulate recommendations (Fig. 3) to overcome the barriers for the prevention and control of pollen allergies as follows:

1) Adaptive actions required **at an individual level** to decrease the risk of illnesses associated with pollen allergy:
   - Keeping a regular check on pollen forecasts and minimizing exposures to the outdoor environment during high pollen loads
   - The use of HEPA (High-Efficiency Particulate Air) filters are recommended for the indoor environment to reduce exposure to pollen allergens (Sublett et al., 2010; Cipriani et al., 2017; Roubelat et al., 2020)
   - Use of already prescribed medications and seek medical help if necessary for sensitive people
   - Proper knowledge of allergen seasons will allow earlier administration of preventive medicines and improved management
2) Health Care Communities and Non- Governmental Organizations (NGO's):
   - Communicate and educate regarding the significance of limiting outdoor exposure on high pollutant days.
   - Regular training for patients and professionals
   - **Education and awareness:** Allergic rhinitis affects a substantial portion of the population who remain undiagnosed or inadequately treated. Therefore, educational programs must be conducted to deliver the importance of allergen avoidance and correct ways to prevent allergies.
   - **Defining pollen allergy:** Better understanding and adequately describing the **mechanism of pollen allergy** forms the fundamental basis for primary prevention. Lack of knowledge misleads to early prevention, which is primarily linked to delayed diagnosis. For this, numerous studies have been conducted in aerobiology, epidemiology, and phenology related to fundamental research. Still, the prophylaxis found has posed many doubts/queries, which remain unsolved/unanswered.
3) Local Governments and Agencies:
   - **Adequate knowledge of allergenic species:** Allergenic species are considered a major contributing factor to the increased incidence of allergic diseases. The intensity of allergy is directly linked to the species and taxa of airborne pollen, which depends on the threshold value of the individual species (Cariñanos and Casares-Porcel, 2011). Therefore, caution must be exercised before making green zones.
   - Keep a check and control on plant species to be planted
     1. Avoid botanical sexism (dioecious species) and plant perfect flowering plants, i.e., having male and female parts in the same flower (example: roses, tulips, lilies, orchids, apples, and cherries, etc.) so that pollination is reasonably contained.
     2. Planting monoecious plants (male and female flower grow on the same flower) offer an additional advantage over dioecious plants (distinct male and female plants), which require pollen to be spread widely (Eisenman et al., 2019).
     3. Choose plant species with low to medium pollen production. Non-allergenic or not highly allergic and entomophilous plant species over anemophilous plant species should be chosen judiciously to provide a healthy allergen-free atmosphere (Cariñanos and Casares-Porcel, 2011).
     4. Regulate and control exotic/invasive species
   - Complete knowledge of local flora distribution and guides should be available of allergenic pollen types, which are of utmost importance in managing pollen-related allergic diseases.
   - **Development of regional pollen calendar and its timely update:** Pollen calendars provide visual information about the numerous forms of airborne pollen types present throughout the year with their seasonality in a single image. They correspond as a preventive tool for sensitized individuals regarding minimizing exposure when the levels of aero-pollen are above thresholds (Mandal et al., 2008a; Elvira-Rendueles et al., 2019; Ravindra et al., 2021b). Public health officials can use calendar data to establish early warning systems, exposure guidelines and further advancements in therapy during high pollen loads. Moreover, providing access to the local pollen information will enhance the self-management of allergen avoidance and management. Therefore, pollen calendars are necessary to establish possible connections and contributions in the field of etiology to avoid allergic diseases such as pollinosis and allergic rhinitis.

Fig. 2. Graded response for pollen allergy prevention.

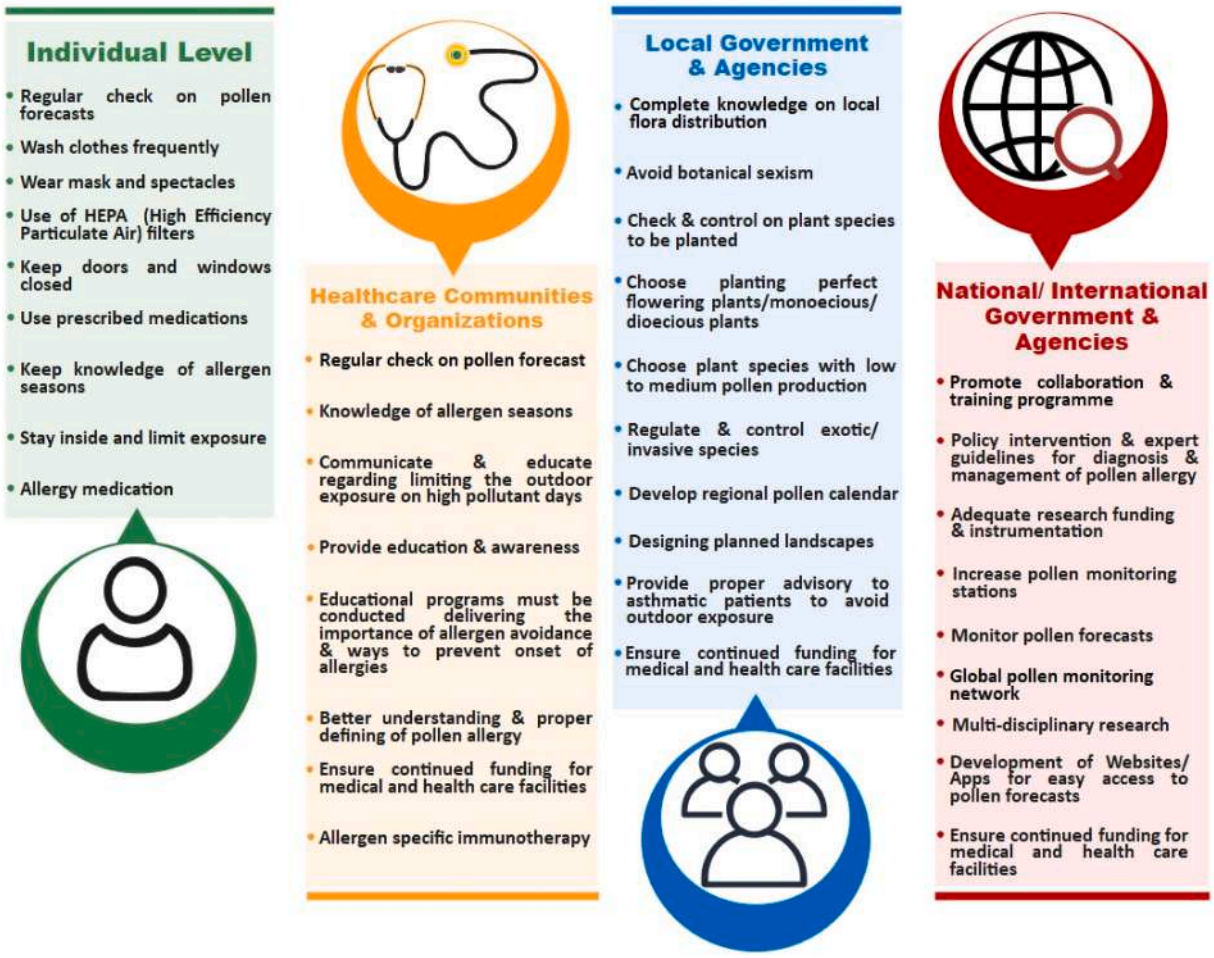

Fig. 3. Key measures and recommendations for preventing and controlling pollen allergy.

- **Designing planned landscapes and management** to produce a lesser amount of pollen allergens with context to local flora. Moreover, involving experts (Botanist, aerobiologists, epidemiologists, etc.) at the planning stage to design urban green spaces. Encourage the concept of a "third landscape" site promoting the selective colonization of various and transient species in order to reduce the existence of spontaneous low-diversity populations producing allergic pollen (Clement, 2007).
- To avoid outdoor exposure and overexertion during high pollen load days, proper advisory must be given to asthmatic patients.
- Co-morbidities due to pollen allergies must be addressed and proper medications should be made available during the peak pollen seasons.
- **Ensure continued funding for medical and health care facilities**-to provide access to high-quality allergen extracts for immunodiagnostics and ensure the safe delivery of allergen-specific immunotherapies.

4) National/International Governments and Agencies:

- **Collaboration and training programs:** Expand the research and collaboration to monitoring pollen trends, remote sensing, sensitization and development of asthma severity.
- **Proper Guidelines and Policy intervention:** It is crucial to set appropriate guidelines for the diagnosis and proper management of pollen allergy, mentioning the allergic species, its allergenic potential, peak pollen seasons with respect to geographic area. For example, the European Academy of Allergy and Clinical Immunology (EAACI) provided recommendations to improve the future scenario of asthma, allergy and other immunological diseases (Agache et al., 2018).

  Pollen also makes a significant fraction of the ambient air pollution. Therefore, more focus should be paid to develop climate change and air pollution mitigation strategies through policy formulation. Like in India, National Clean Air Programme (NCAP) and National Action Plan on Climate Change and Human Health (NAPCCHH), etc., could benefit from the inclusion of aerobiologists, environmental health specialists and specifically experts from plant biology and phenology to control bioaerosols. Not only this, but the improvement in air quality will help to reduce the mortality and morbidity due to air pollution. This will further result in better economic growth and human health. Fig. 4 depicts a proposed integrated framework to minimize pollen allergy as a case for India. Further, the Clean Air Initiative (CAI) by World Bank aims to advance a better understanding of pollution challenges and practical solutions on the Global Alliance on Health, Pollution and Climate Change, having focus only on the policy related to anthropogenic emissions. Thus, there is an urgent need to understand and incorporate the natural component, i.e., pollen, into the policies.
- **Multi-disciplinary research:** different sciences and disciplines pertaining to epidemiology, immunology, aerobiology, etc., are required to illustrate and explain factors owing to pollen allergy properly.
- **Research funding and instrumentation:** Designated laboratories to monitor and analyze daily samples. The database available will

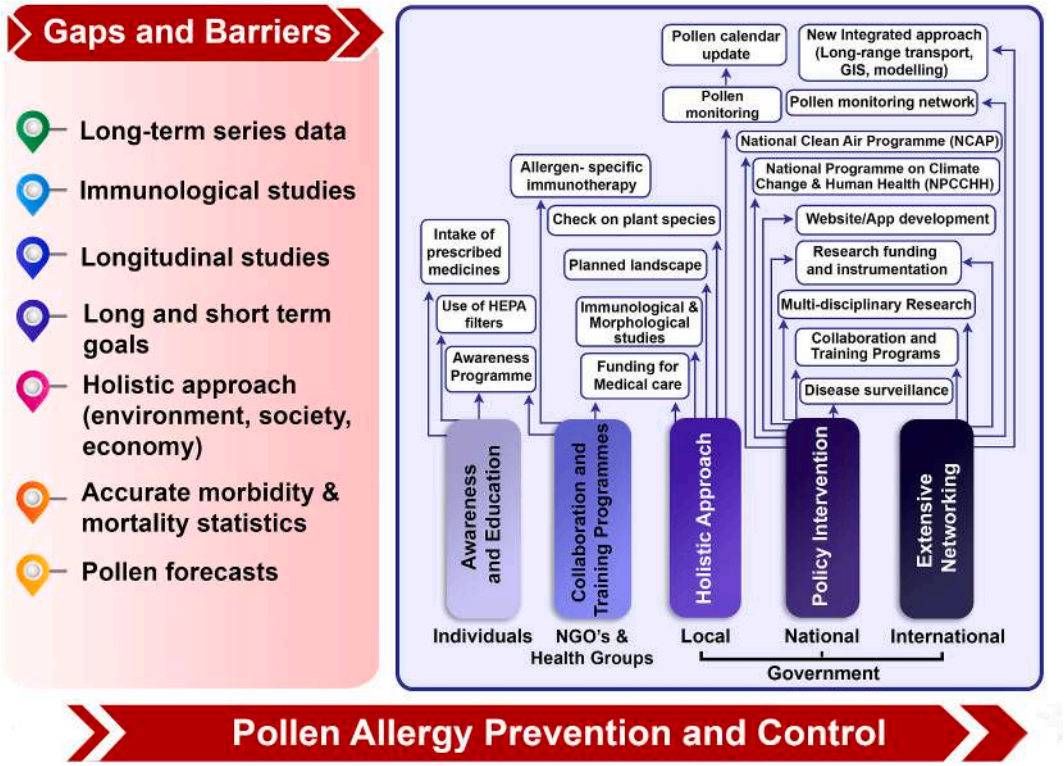

Fig. 4. Proposed integrated developmental framework for pollen allergy prevention as a case for India.

help to summarize the current and past allergen levels in the atmosphere in a very efficient manner.

- **Inadequate monitoring of pollen loads:** Sampling and aerobiological units for measurement of airborne pollen concentration are globally scarce, and most of them are established in high-income countries such as the United Kingdom, USA, Europe, etc.
- **Pollen Monitoring network:** in response to the increasing pollen allergy, a global pollen monitoring network is required to collect and disseminate the obtained pollen data. More monitoring stations could help to have larger pollen data pools. This will aid to understand the spatial and temporal trends of pollen concentration and will be fundamental for scenario analysis and policy formulation in the future.
- **Pollen Forecasting:** The aerobiology data is also valuable in creating pollen forecasts, development of medications and public awareness. Forecast for pollen levels will help to regulate and avoid allergies.
- **Development of Websites/Apps:** A well-designed website or mobile app is required featuring the pollen concentration and major allergenic species forecast for easy and prior dissemination of information for particular areas. A very few apps are available to deal with the specific arena, such as Pollen.com's Allergy Alert for the United States of America, My Pollen Forecast UK for the United Kingdom, etc.

Some measures have been recommended to prevent allergy, i.e., removing allergenic species from the environment, shown to be effective as a primary step but is ineffective when long-distance transport is considered. Another aspect of annual periodicity, when taken into account, is an essential feature in response to pollen allergy, as symptoms usually occur at the same time of the year in maximum cases (during pollination). But many have reported allergy symptoms before and after the pollen season start and ends. Thus, symptoms may occur seasonally or throughout the year. Therefore, pollen allergy may be indefinite and is masked by many other sensitization processes, which precludes or alters its clear clinical picture in some instances.

Therefore, to address the above issues, both short-term and long-term objectives are needed to reduce allergic diseases. Under short-term objectives, the main focus remains on emergency and unscheduled visits, a relation of the epidemic to emergency hospital admissions. In contrast, longer-term objectives involve new therapies, follow-ups with patients, extensive educational programs and courses to control and prevent allergies.

7. Conclusions

Pollen allergy is considered a trivial disease and has not attained much public health attention, specially in lower and middle-income countries. Various studies have already demonstrated that climatic change and air pollution may alter pollen production, morphology, allergenicity, season and their distribution; which may increase the prevalence of allergic diseases in humans. The prevalence of pollen-associated allergies has increased in the past three decades and is likely to rise in the future. Thus, there is an urgent need to develop a strategic, multi-professional, collaborative approach to tackle the burden of allergic rhinitis and co-morbidities. With every adaptation/preventive measure, attention needs to be given to the most vulnerable sub-populations: allergic asthma, allergic rhinitis, and eczema. However, special attention needs to be given in the education sector, training for patients and professionals, developing collaborated research programs, pollen forecasts, and ensuring continued funding for medical and health care facilities. Participation and association of healthcare communities and relevant regulatory authorities at the local, national and international levels may allow acting on pollen allergy prevention and control.

CRediT authorship contribution statement

Khaiwal Ravindra: Conceptualization, Methodology, Formal analysis, Visualization, Writing – review & editing. **Akshi Goyal:**

Methodology, Formal analysis, Writing – review & editing. **Suman Mor:** Supervision, Formal analysis, Writing – review & editing.

Declaration of competing interest

Authors confirm that there's no financial/personal interest or belief that could affect the objectivity. All authors read the paper and approved it.

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Proximity and density of unconventional natural gas wells and mental illness and substance use among pregnant individuals: An exploratory study in Canada

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ABSTRACT

Background: Hydraulic fracturing (fracking) is a method used to extract unconventional natural gas (UNG). Living near UNG operations has been associated with various health outcomes, but few have explored the association between UNG and mental health and substance use. Our objective was to evaluate the association between metrics of residential UNG well density/proximity and mental illness and substance use among pregnant individuals in Northeastern British Columbia, Canada.

Methods: Individuals who gave birth at the Fort St John hospital between December 30, 2006 and December 29, 2016 (n = 6278) were included in the study. Exposure was determined using inverse distance weighting (IDW) to calculate the density and proximity of UNG wells to the postal code centroid ofindividual's residential address at delivery. Four exposure metrics, categorized by quartiles, were calculated based on 50, 10, 5 and 2.5 km buffer zones around each postal code centroid. Logistic regression was used to separately evaluate associations between IDW quartiles of each metric and diagnosis of depression and anxiety prior to or during pregnancy, and self-reported substance use during pregnancy, controlling for relevant and available confounders.

Results: The second and third quartile (Q) of the 10 km IDW were associated with greater odds of depression (Q2: adjusted (aOR) 1.30, 95% (confidence interval) CI 1.03–1.64; Q3: aOR 1.35, 95% CI 1.07–1.70) compared to the first quartile, but not the fourth. Using the 5 km IDW, we observed a suggestive positive association with depression in the second and third quartile (aOR Q2: 1.21, 95% CI 0.96–1.53; aOR Q3: 1.24, 95% CI 0.98–1.57) compared to the first quartile. No statistically significant association was observed using the 2.5 km IDW exposure metric.

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Conclusion: We observed some evidence of greater odds of mental illness prior to or during pregnancy, and substance use during pregnancy in pregnant individuals living in postal codes with increased UNG well density/proximity, although associations were not observed in smaller buffer zones. This study adds to the growing literature on the adverse health outcomes surrounding living in proximity to UNG operations.

1. Introduction

Hydraulic fracturing is a shale gas extraction process using unconventional means of drilling to release trapped natural gas. Unconventional natural gas (UNG) production includes horizontal drilling at depths 1.5–3 km below the surface to create fissures at the shale layer and injecting millions of gallons of highly pressurized fracking fluid (water combined with chemical additives, sand, and/or silica) in existing wells to force natural gas up to the surface. Advocates of UNG production using fracking point to its local economic-boosting benefits, and lower energy costs compared to traditional oil and gas extraction (Fukui et al., 2017). However, UNG production can release fugitive chemical emissions leading to air pollution and water contamination from spills and well casing degradation (Wollin et al., 2020). The industrial process also leads to an influx of workers and diesel machinery, further adding to the air pollution and stress on rural community infrastructures (Adgate et al., 2014; Cunningham et al., 2020; Deziel et al., 2020).

Living near UNG operations has been adversely associated with health (Deziel et al., 2020), including birth outcomes (e.g., preterm birth, gestational weight, and congenital abnormalities) (Caron-Beaudoin et al., 2021a; Casey et al., 2016; Currie et al., 2017; Gonzalez et al., 2020; Hill, 2018; Janitz et al., 2019; Tran et al., 2021; Walker Whitworth et al., 2018; Whitworth et al., 2017; Willis et al., 2021), self-reported health symptoms (e.g., upper respiratory and dermal symptoms) (Rabinowitz et al., 2015; Tustin et al., 2017), asthma exacerbations (Rasmussen et al., 2016; Willis et al., 2020), cardiovascular health (Denham et al., 2021), hypertensive conditions during pregnancy (Willis et al., 2021) and mortality (Li et al., 2022). More recently, studies have explored the impact of nearby UNG sites on mental illness (Casey et al., 2018, 2019) given the well-documented heightened psychosocial stress experienced by community members (McDermott-Levy and Garcia, 2016; Perry, 2013; Sangaramoorthy et al., 2016; Soyer et al., 2020), and environmental contamination as a result of UNG processes and waste (Gonzalez et al., 2022; Li et al., 2020; Wollin et al., 2020). For example, a study with 4762 participants in Pennsylvania, U.S. reported that those living closer to more and larger wells had increased risk of depressive symptoms (Casey et al., 2018), and qualitative studies in Ohio and West Virginia documented feelings of anxiety and social stress among community residents impacted by UNG operations (Sangaramoorthy et al., 2016; Willow, 2014). A common concern flagged by community residents is the increase in alcohol and substance use with the insurgence of "boomtowns" and rapid population growth (Brasier et al., 2015). However, results pertaining to substance use in association with UNG have been mixed at best and this area requires further research. For example, Beleche and Cintina (2018) did not find a statistically significant association between UNG activity and drug abuse in Pennsylvania, while Mayer and Olson Hazboun (2019) reported a modest and statistically significant association between measures of county-level UNG production and binge and heavy drinking in the US (Beleche and Cintina, 2018; Mayer and Olson Hazboun, 2019).

Further, certain subpopulations living in proximity to UNG sites may be more vulnerable. Pregnancy can be a sensitive period for developing mental illness due to the stress surrounding the major life changes and immunological and hormonal changes (Valsamakis et al., 2019). In fact, approximately one fifth of pregnant individuals develop a perinatal mental illness (Andersson et al., 2006; Fawcett et al., 2019). A study in Pennsylvania examined the mediating role of maternal mental illness in the association between UNG well density and adverse birth outcomes (Casey et al., 2019). Although they did not observe a mediating effect, there was evidence of an association between living near UNG operations and perinatal anxiety and depression.

Canada is one of the largest producers of natural gas in the world, with 25% of the country's production taking place in Northeastern British Columbia (Adams et al., 2016a; Natural Resources Canada, 2019). In this region, more than 85% of the natural gas is produced using fracking (BCOGC, 2016), which represents more than 28,000 wells drilled since 1954 (Adams et al., 2016a; Hughes et al., 2015). The region sits on the Montney formation, an important source of natural gas (Adams et al., 2016b). Since the expansion of UNG, epidemiologic research on its human health effects has been playing "catch-up" with over 60% of peer-reviewed research published since 2014. While most studies investigated the impacts of proximity and density of UNG wells on physical health outcomes (e.g. birth outcomes, congenital malformations, cancer, respiratory and cardiovascular outcomes) (Bamber et al., 2019), very little is known about the potential impacts of UNG operations on mental health during pregnancy. This lack of research is even more evident in Canada, with only two studies published in the scientific literature that investigated the levels of exposure to contaminants associated with UNG during pregnancy (Caron-Beaudoin et al., 2018, 2019), and one study on the association between maternal proximity to UNG operations and birth outcomes (Caron-Beaudoin et al., 2021a). Our objective was to evaluate the association between metrics of residential UNG well density/proximity with mental illness prior to or during pregnancy, and substance use during pregnancy, in Northeastern British Columbia, Canada in an exploratory analysis.

2. Methods

2.1. Study population

Our retrospective birth cohort study included 6333 women who gave birth to singletons at the Fort St John hospital, the largest hospital in Northeastern British Columbia, Canada, from December 30, 2006 to December 29, 2016. To reduce inter-correlation, one pregnancy was randomly selected for women with multiple pregnancies during the study period, resulting in a total of 6278 pregnancies included in the analysis. Cohort data was obtained from Northern Health, the public health care provider for Northeastern British Columbia. This study was reviewed and approved by the Northern Health Research Review Committee and by the Université de Montréal Institutional Review Board (#18–001-CERES-D).

2.2. Estimation of exposure to UNG

The main exposure of interest was UNG wells density/proximity around each study participants' six-digit residential postal code centroid. More details on our exposure estimation strategy are published elsewhere (Caron-Beaudoin et al., 2021a). Briefly, to estimate the exposure of women to UNG wells during their pregnancy, we first geocoded pregnant individual's residential postal code coordinates using an online geocoder and visually verified them to be located near a population center. Approximately 80% of the postal codes were small (average 0.01 km^2) and located in a populated area, while the remaining 20% of postal codes were larger with an average area of 559 km^2 (Caron-Beaudoin et al., 2021a). For these larger postal codes, we identified the populated area by manually identifying the coordinates located in the centre of the main populated area using Google Maps.

Postal code coordinates were imported to QGIS (Desktop 3.0.1).

Next, data on UNG wells were obtained from the British Columbia Oil and Gas Commission. UNG wells in the Montney formation were identified using the spud date, i.e., the earliest known date ground was broken for well development. We included wells at all stages (including dormant and orphan wells) present at the time of pregnancy because of the lack of data distinguishing the difference phases in the database, and in order to also capture cases of fugitive emissions from all types of UNG wells (Kang et al., 2014; Schout et al., 2019).

We then generated a distance matrix between each individual's postal code centroid and each UNG well identified in British Columbia using QGIS. The inverse distance weighted (IDW) sum of UNG wells with a spud date prior to the delivery date for each of the study participants was calculated using the following formula: $IDW_x = \sum_{i=1}^{n} (\frac{1}{d_i})$, where "x" is the buffer distance, "n" is the total number of wells in the specified buffer, "i" is a given well in the specified buffer, and "d_i" is the exact distance (km) between that well and the postal code centroid. IDWs were calculated to represent the proximity and density of UNG wells within 10, 5, 2.5 km buffer around postal code centroids. All exposure metrics apply greater weight to closer wells, and although the larger buffers include a greater number of wells in their calculation, these wells are penalized by their distance. These buffers are consistent with previous studies (Caron-Beaudoin et al., 2021a; McKenzie et al., 2014; Stacy et al., 2015; Walker Whitworth et al., 2018; Whitworth et al., 2017). As an exploratory approach, we created a fourth IDW metric using a 50 km buffer to account for the highly dispersed population in rural Northeastern British Columbia (e.g., the region has a population density of 0.3 residents per square kilometer (Government of Canada, 2017)), because UNG operations could theoretically impact communities at larger distances via changes in community population demographics, traffic, and stress on local services. However, because capturing UNG well proximity and density within a buffer of this size may also be susceptible to confounding, we consider results pertaining to this metric as exploratory and they are presented in the Supplementary material. Each of the IDW metrics were categorized by quartiles, with each of the lowest quartiles treated as the reference group.

A map of Northeastern British Columbia including oil and gas wells is presented in Fig. 1.

2.3. Outcomes and covariables

Data regarding mental health and substance use was compiled by Northern Health. Maternal general depression, anxiety, bipolar disorder, and other mental illnesses as diagnosed by a healthcare provider were obtained from the British Columbia Perinatal Data Registry, which includes data abstracted from obstetrical and neonatal medical records from 99% of British Columbia births (Perinatal Services BC, n.d.). Bipolar disorder and other mental illnesses were subsequently dropped from analyses due to small numbers (n = 25 and 15, respectively). Of note, we could not distinguish the timing of diagnosis from these records and thus, our mental health outcomes represent prevalent mental illness among pregnant individuals that may have been diagnosed prior to pregnancy.

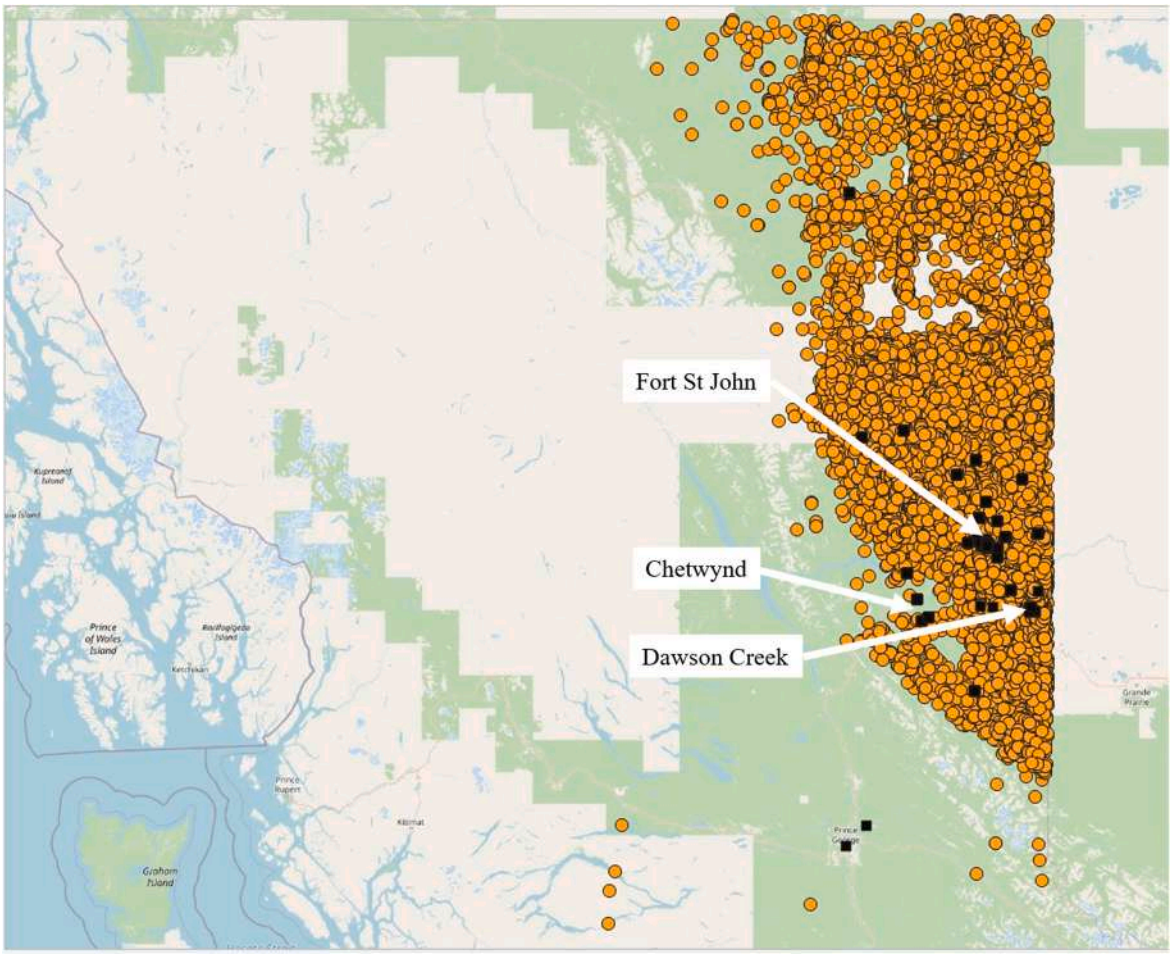

Fig. 1. Map of British Columbia with oil and gas wells (yellow circles) located in Northeastern British Columbia and postal code centroids (black squares) in 2016 (Open Data from BC Oil and Gas Commission). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Information regarding substance use was obtained from a self-reported prenatal registration questionnaire. This included questions about the use of the following substances at any time during the index pregnancy or before the mother knew they were pregnant: heroin/opioids, cocaine, methadone, solvents, cannabis, and other.

The following covariates were also obtained from the Perinatal Data Registry, and compiled by Northern Health: use of tobacco and exposure to second-hand smoke before and during the index pregnancy, mother's age at delivery, mother's residential six-digit postal code at delivery, prior adverse pregnancy outcomes (e.g., prior neonatal death, prior low birthweight baby, prior stillbirth), complications during the index pregnancy (e.g., hypertension, preeclampsia, gestational diabetes, bleeding before 20 weeks gestation), number of previous pregnancies, still birth, singleton, multiple birth count for the index pregnancy, infant's birth date, infant's biological sex assigned at birth, gestational age at delivery, Apgar scores (1, 5 and 10 min), birthweight and head circumference.

2.4. Statistical analyses

Distributions of covariates were calculated, and bivariate analyses were conducted stratifying covariate distributions by all IDW exposure metrics. Logistic regression models were constructed to investigate the association between well density/proximity quartiles within the four buffer zones and each of depression, anxiety, and substance use. Covariates potentially associated with exposure and health outcomes were selected *a priori* based on the current literature (Biaggi et al., 2016; Caron-Beaudoin et al., 2021a; Casey et al., 2019; Schetter et al., 2016). Final models were adjusted for maternal age at delivery (continuous), parity (primiparous, multiparous), and smoking (ever smokers (before or during the current pregnancy) or never-smokers). Census-level socioeconomic data are considered unreliable in regions with many Indigenous reserves/settlements since the reserves/settlements are not included in the Census. As such, the models did not include any socio-economic status (SES) variables, and instead used smoking and parity as proxies for SES (Frohlich et al., 2006; Glenn et al., 2017; Hajizadeh et al., 2020; Restrepo-Méndez et al., 2015). As a sensitivity analysis, we re-ran the models after excluding individuals with gestational diabetes, gestational hypertension, preterm births (defined as a live birth delivered before 37 completed weeks of gestation), stillbirths, and infants with congenital anomalies to account for potentially medically-complex pregnancies (N = 1794). Individuals with missing data for these conditions were also excluded (N = 127).

To account for potential spatial auto-correlation, we conducted a visual examination of the data using QGIS (Desktop 3.10.8) to see if the postal code centroids of pregnant individuals with and without mental health diagnosis would be evenly distributed in the region: we did not visually detect any obvious spatial patterns in the distribution of outcomes.

All analyses were conducted in R 3.6.1 (R Core Team, 2020).

3. Results

Of the 6278 individuals included in our analysis, those in the fourth quartile of the 10 km IDW were slightly older (28.5 years versus 27.6 years in the lowest quartile). Pregnant individuals were more likely to be multiparous in the highest and lowest quartiles of the 10 km IDW (60.1% in Q1 versus 54.8% in Q2, 53.7% in Q3, and 61.4% in Q4), and more likely to be smokers in the second quartile of the 10 km IDW (9.9% versus 8.6% in the lowest quartile) (Table 1). Pregnant individuals were also more likely to have gestational diabetes, gestational hypertension and preterm birth in the fourth quartile of the 10 km IDW. Conversely, depression, anxiety, and substance use during pregnancy were highest in the second and third quartiles of the 10 km IDW. There were 28,629 UNG wells in the region, and their distribution of well density/proximity within the 50, 10, 5, and 2.5 km buffers are presented in Table 2. All pregnant individuals lived in postal codes with at least one well within 50 km of the postal code centroid during her pregnancy, and only 27 pregnant individuals lived in postal codes with zero wells within the 5 km of the centroid during their pregnancy.

In adjusted models, there were greater odds of depression among pregnant individuals in the 10 km IDW second and third quartiles compared to the first quartile (Q2: aOR 1.30, 95% CI 1.03–1.64; Q3: aOR 1.35, 95% CI 1.07–1.70). Although not significant, the associations between the second and third quartile of the 5 km IDW scores and depression were also stronger than the fourth quartile compared to the first, similar to the trend of associations using the 10 km buffer (Q2: aOR 1.21, 95% CI 0.96–1.53; Q3: aOR 1.24, 95% CI 0.98–1.57). No

Table 1
Study population characteristics by 10 km IDW quartiles.

	Total	Q1	Q2	Q3	Q4
N	6278	1611	1536	1565	1566
Maternal age (mean (SD))	27.9 (5.3)	27.6 (5.6)	27.7 (5.1)	27.6 (5.2)	28.5 (5.4)
Multiparous (%)	3610 (57.5)	968 (60.1)	841 (54.8)	840 (53.7)	961 (61.4)
Male fetus (%)	3223 (51.3)	844 (52.4)	795 (51.8)	799 (51.1)	785 (50.1)
Smoking status (%)					
Non-smoker	4823 (76.8)	1239 (76.9)	1127 (73.4)	1178 (75.3)	1279 (81.7)
Former smoker	892 (14.2)	234 (14.5)	257 (16.7)	250 (16.0)	151 (9.6)
Current smoker	563 (9.0)	138 (8.6)	152 (9.9)	137 (8.8)	136 (8.7)
Gestational diabetes (%)	194 (3.1)	52 (3.2)	39 (2.5)	50 (3.2)	53 (3.4)
Gestational hypertension (%)	225 (3.6)	61 (3.8)	44 (2.9)	52 (3.3)	68 (4.3)
Preterm birth (%)a	399 (7.7)	103 (6.4)	88 (5.7)	94 (6.0)	114 (7.3)
Stillbirths (%)	45 (0.7)	11 (0.7)	13 (0.8)	10 (0.6)	11 (0.7)
Congenital anomalies (%)	17 (0.3)	5 (0.3)	5 (0.3)	2 (0.1)	5 (0.3)
Depression during pregnancy (%)	656 (10.4)	146 (9.1)	181 (11.8)	187 (11.9)	142 (9.1)
Anxiety during pregnancy (%)	505 (8.0)	116 (7.2)	136 (8.9)	132 (8.4)	121 (7.7)
Substance use during pregnancy (%)	388 (6.2)	96 (6.0)	110 (7.2)	106 (6.8)	76 (4.9)

a Missing N = 1090 (17.4%).

Table 2
Inverse distance weighting (IDW) median by quartile.

Characteristic	Median (5th, 95th percentile)
IDW Well density/proximity – 50 km buffer	
Q1	100.1 (9.6, 107.6)
Q2	113.3 (108.9, 117.6)
Q3	122.9 (118.4, 128.3)
Q4	135.0 (129.6, 143.6)
IDW Well density/proximity – 10 km buffer	
Q1	20.5 (1.1, 31.8)
Q2	39.2 (34.1, 41.1)
Q3	42.1 (41.1, 44.3)
Q4	49.6 (45.1, 56.8)
IDW Well density/proximity – 5 km buffer	
Q1	8.2 (0.3, 8.5)
Q2	11.8 (10.9, 12.4)
Q3	13.1 (12.6, 14.3)
Q4	16.0 (14.6, 29.6)
IDW Well density/proximity – 2.5 km buffer	
Q1	1.7 (0.6, 1.9)
Q2	2.1 (2.0, 2.6)
Q3	3.8 (2.7, 4.7)
Q4	6.7 (5.6, 16.7)

Table 3
Associations between well density/proximity metrics and depression, anxiety, and substance use during pregnancy among women with singleton pregnancies in the Fort St John hospital, BC, Canada, from December 30, 2006 to December 29, 2016 in unadjusted and adjusted models.

	Depression		Anxiety		Substance Use	
	OR (95% CI)	aOR (95% CI)	OR (95% CI)	aOR (95% CI)	OR (95% CI)	aOR (95% CI)
10 km IDW						
Q1	1.00	1.00	1.00	1.00	1.00	1.00
Q2	1.34 (1.07–1.69)*	1.30 (1.03–1.64)*	1.25 (0.97–1.62)	1.20 (0.92–1.56)	1.22 (0.92–1.62)	1.15 (0.86–1.53)
Q3	1.36 (1.08–1.71)*	1.35 (1.07–1.70)*	1.19 (0.92–1.54)	1.16 (0.89–1.51)	1.15 (0.86–1.53)	1.12 (0.83–1.50)
Q4	1.00 (0.79–1.28)	1.02 (0.80–1.31)	1.08 (0.83–1.41)	1.10 (0.85–1.44)	0.80 (0.59–1.10)	0.89 (0.64–1.21)
5 km IDW						
Q1	1.00	1.00	1.00	1.00	1.00	1.00
Q2	1.23 (0.98–1.55)	1.21 (0.96–1.53)	1.10 (0.85–1.43)	1.07 (0.82–1.39)	1.10 (0.83–1.46)	1.08 (0.81–1.45)
Q3	1.24 (0.99–1.57)	1.24 (0.98–1.57)	1.10 (0.85–1.44)	1.09 (0.84–1.43)	0.91 (0.67–1.22)	0.93 (0.69–1.26)
Q4	1.04 (0.82–1.32)	1.03 (0.81–1.31)	1.08 (0.83–1.40)	1.07 (0.83–1.40)	1.02 (0.76–1.36)	1.04 (0.78–1.41)
2.5 km IDW						
Q1	1.00	1.00	1.00	1.00	1.00	1.00
Q2	1.04 (0.83–1.30)	1.08 (0.86–1.35)	0.94 (0.73–1.22)	0.98 (0.76–1.27)	0.79 (0.59–1.06)	0.85 (0.63–1.14)
Q3	0.83 (0.66–1.04)	0.86 (0.68–1.09)	0.97 (0.75–1.26)	0.97 (0.75–1.26)	0.74 (0.56–1.00)*	0.81 (0.60–1.08)
Q4	0.87 (0.70–1.09)	0.88 (0.70–1.11)	0.98 (0.76–1.26)	1.00 (0.78–1.29)	0.86 (0.65–1.14)	0.89 (0.67–1.18)

N = 6278.
* p-value < 0.05.
OR: dds ratio; aOR: Adjusted odds ratio. Models adjusted for maternal age, smoking status, and parity.

association was observed between the 2.5 km IDW scores and depression (Table 3, Supplementary Fig. S1). No meaningful differences were observed between unadjusted and adjusted models.

There were no associations observed between well density/proximity using the 10, 5, or 2.5 km buffers and anxiety and substance use. There were no major differences between the adjusted and unadjusted associations.

Associations between the 50 km IDW and outcomes differed from the results using smaller buffers. In adjusted models, the associations between the 50 km IDW were strongest in the fourth quartile compared to the first for depression (aOR 2.71, 95% CI 2.13–3.49), anxiety (aOR 3.02, 95% CI 2.31–3.99), and substance use (aOR 2.20, 95% CI 1.62–3.02) (Supplementary Table 4 and Fig. S1).

After excluding individuals with potentially medically-complex pregnancies, the associations between the fourth quartiles of the 10 and 5 km IDW metrics and anxiety became stronger compared to the main models (aOR 1.37, 95% CI 0.97–1.93; aOR 1.36, 95% CI 0.97–1.90) (Supplementary Table 5).

4. Discussion

This exploratory analysis of a retrospective cohort study examined the association between UNG well density/proximity metrics and prevalence of mental illness prior to or during pregnancy, and substance use during pregnancy. The study region is densely populated with UNG wells, as such, the associations reflected mental illness risk in areas with higher density/proximity to wells compared to lower density/proximity to wells rather than a true "unexposed" control group. Despite this, we detected a positive association between UNG well density/proximity within a 10 and 5 km radius and prevalence of depression among pregnant individuals who gave birth at the Fort St John hospital between 2006 and 2016, but these associations did not follow a monotonic dose-response relationship as no association were observed in the highest quartile of exposure. There were no associations observed with anxiety and substance use. The results were stronger after excluding participants with medically-complex pregnancies. In contrast, no associations were observed between UNG well density/proximity and mental health when buffer zones were restricted to a smaller radius of 2.5 km. Our results suggest that mental illness may be a potential adverse outcome associated with UNG operations surrounding local communities, but more studies are required.

4.1. UNG and mental health

One other study examined the impacts of UNG operations on maternal mental illness. A study in Pennsylvania, U.S. examined the association between UNG and maternal outcomes, including the mediating effect of maternal depression and anxiety on the relationship between UNG and birth outcomes in 7715 mothers (Casey et al., 2019). Maternal residential exposure to UNG was estimated using an inverse distance-squared method that incorporated information such as well location, depth, dates of development, and volume of gas produced. There were no set buffers for this study's exposure metric, and exposed mothers lived a median distance of 11.2 km from the nearest well and unexposed mothers in the 1st-3rd quartiles lived a median of 24 km from the nearest wells. This study reported an additional 4.3 cases of antenatal anxiety or depression per 100 pregnant individuals among those in the fourth quartile of UNG operations versus pregnant individuals in the 1st-3rd quartiles. A major strength of this study is that the maternal address as used to estimate exposure, whereas we relied on postal code centroids.

Although we observed stronger associations with increasing quartiles of exposure using the 50 km exposure metric, the 10 km IDW exposure metric did not yield monotonic associations with mental illness, and no strong associations were observed using 5 and 2.5 km IDW. Interestingly, another study conducted by our research team also observed associations with the 10 km IDW second and third quartile scores and adverse birth outcomes compared to the first, but not in association with the fourth quartile (Caron-Beaudoin et al., 2021a). We included all wells present (active, dormant and orphaned) at any time during pregnancy; however, we were unable to consider the different production phases of the wells even though the emission of contaminants may vary greatly by phase (Walker Whitworth et al., 2018). Future studies should consider incorporating details on the phase of wells, and directly measuring contaminant concentrations to improve exposure assessment. Indeed, Hill et al. (2022) recently assessed the infant health risks associated with drinking water contamination in the context of UNG: they found that drilling near an infant's public water was associated with poorer birth outcomes (e.g., low birth weight, preterm birth) and more UNG contaminants in public drinking water.

4.2. Exposure to UNG chemicals and potential effects on mental health

UNG operations are a source of air and water pollution, emitting

volatile organic compounds, particulate matter, polycyclic aromatic hydrocarbons, diesel exhaust, methane, and chemical additives to fracking fluid, among other contaminants throughout the preproduction, production, and inactive phases (Bamber et al., 2019; Gonzalez et al., 2020; Martin et al., 2021; Wollin et al., 2020). UNG operations were also more strongly associated with birth outcomes in rural versus urban settings, likely due to an increased reliance on wells for drinking water that may be contaminated by UNG (Tran et al., 2021). A study in 29 pregnant individuals from Northeastern British Columbia reported elevated concentrations of a benzene metabolite in urine (*trans, trans*-muconic acid) (Caron-Beaudoin et al., 2018) and trace metals such as barium, aluminum, strontium, and manganese in urine and hair samples, particularly among Indigenous women, compared to women in the general Canadian population (Caron-Beaudoin et al., 2019); however, the exposure sources are unknown. Although the results were mixed, two reviews found some evidence of an increased risk of depression with increasing levels of ambient particulate matter and nitrogen oxides, potentially via inflammatory processes and oxidative stress (Fan et al., 2020; Thomson, 2019). More specifically, investigators of a study among 509 women in Mexico observed an 83% increased risk of postpartum depression associated with a 5-$\mu g/m^3$ increase in average fine particulate matter ($PM_{2.5}$) exposure during pregnancy, and a 158% increased risk after restriction to late-onset postpartum depression (Niedzwiecki et al., 2020). Our study was unable to examine the impact of living near UNG operations on postpartum mental illness, but the results of the study in Mexico provide insight on mental health surrounding pregnancy.

Worries surrounding toxicants and carcinogens from UNG-related air pollution and water contamination can lead to an increase in stress and anxiety in nearby communities (Hirsch et al., 2018; Soyer et al., 2020). Psychosocial stressors can lead to acute and/or chronic stress, and eventually lead to allostatic overload (Stewart, 2006). Pregnancy is in itself a stressful event with fluctuating hormonal and immunological states (Valsamakis et al., 2019). As such, pregnant individuals are at increased risk of developing mental illnesses under conditions of excess psychological stress (Biaggi et al., 2016; Hobel et al., 2008).

4.3. Psychosocial impacts of UNG at the community level

We observed stronger association between well density/proximity and mental health using larger radii over smaller ones. First, our sample size is relatively small compared to other epidemiological studies of UNG and health outcomes, which can decrease precision in our effect estimates. This is particularly relevant in the smaller radii, where a larger number of pregnant individuals have null IDW metrics. Second, this could indicate that the associations observed are driven by the societal changes resulting from *numerous* UNG wells in an area and the overall impact of UNG activity and development in the widely dispersed region of Northeastern British Columbia, rather than release of toxicants. UNG operations are usually centered in rural areas, and many surrounding communities may express heightened psychosocial stress during UNG development and operation due to the increase in noise, vibrations, light pollution, traffic, crime, and stressed community infrastructures from the rapid industrial development (Adgate et al., 2014; Boslett et al., 2021; James and Smith, 2017; Malin, 2020; Richburg and Slagley, 2019; Soyer et al., 2020; Willow et al., 2014). Studies in the US identified feelings of reduced life satisfaction (Maguire and Winters, 2016), loss of community cohesion (Lai et al., 2017), an increase in perceived adverse health effects (Evensen and Stedman, 2017) and feelings of disempowerment (McDermott-Levy and Garcia, 2016). Moreover, these rural communities are more likely to be Indigenous communities or agricultural towns who have greater dependency on the land, and are more vulnerable to damages resulting from UNG (O'Brien and Hipel, 2016; Sangaramoorthy et al., 2016). In fact, several studies have pointed to the environmental injustices surrounding UNG development and operations whereby UNG sites are more likely to be situated near communities with predominantly racialized individuals or living in poverty, and more likely to conduct flaring in such communities (Clough, 2018; Fry et al., 2015; Johnston et al., 2016, 2020; Ogneva-Himmelberger and Huang, 2015). The increase in these psychosocial stressors that accompany UNG operations coupled with the psychosocial stressors specific to the environmental injustices could explain the increased prevalence of maternal depression we observed, particularly given the greater opposition to UNG among women versus men (Boudet et al., 2014; Mayer, 2016).

The non-monotonic associations between well density/proximity using the 10 and 5 km radii and mental health were surprising findings. While the lack of an association in the largest quartiles could be a chance finding, it may also point to other factors at play. Risk perception in UNG settings is complex and residents nearby operations may experience simultaneous feelings of concern as well as optimism regarding economic benefits (McElroy et al., 2020). Communities that are more accepting of such activities may experience lower levels of stress associated with UNG activities, and in turn, may experience less mental illnesses (Saberi et al., 2014). Mayer et al. (2021) asked residents in three Colorado communities how the intensity of local unconventional natural gas operations and trust in energy regulators influences their self-rated health. They found that while living in a community that hosts UNG operations was associated with lower self-rated health, trust in energy regulators seemed to improve self-rated health. However, we were unable to control for risk perception as well as socioeconomic status in our analyses, which may have both contributed to our non-monotonic findings, as well as the lack of an association using smaller radii.

4.4. Study limitations

We used postal code centroids for each woman's residence because we did not have information on their residential addresses, and this may have resulted in exposure misclassification. However, most postal codes were small in area, and in two earlier studies of 114 pregnant individuals in Northeastern British Columbia, IDW scores (10, 5 and 2.5 km buffers) calculated using residential addresses and postal code centroids were significantly and highly correlated (Spearman r = 0.83–0.89). We only included the wells located in British Columbia to calculate the IDW scores in this study. The neighboring province of Alberta has around 180,000 unconventional natural gas wells using hydraulic fracturing. For larger radii, especially the 50 km, this may have led to an underestimation of UNG activity exposure among participants living close to the Alberta border. For example, Dawson Creek is located at approximately 15 km from Alberta. The study did not differentiate between active, dormant, and orphaned wells due to a lack of precision regarding the well process phases, and our comparison group was still exposed to UNG wells which may have underestimated the effect estimates. Additionally, we were also unable to account for other characteristics, including socioeconomic status and ethnicity which may be important confounders and/or modifiers. Canadian census data exclude Indigenous reserves and settlements, and no reliable data exists for Northeastern British Columbia that include Indigenous communities. Our analysis included multiple tests, so chance findings are possible.

Finally, while the mental illness data obtained from the British Columbia Perinatal Data Registry represent diagnoses by a healthcare provider, this data source did not allow us to determine when a mother was first diagnosed (e.g. prior to or during her index pregnancy). Thus, we were unable to characterize associations between UNG and incidence of mental illness; rather, the outcomes in our study represent prevalent outcomes and thus, we are unable to make inferences about the impact of UNG on the development of mental illness. Although this exploratory study is the first of its kind in Canada, the limitations in both our exposure and outcome classification prevent us from disentangling the complex interplay between UNG and maternal mental health and substance use.

5. Conclusion

Among pregnant individuals who gave birth at the Fort St John hospital from 2006 to 2016, we observed some positive associations between UNG well density/proximity within 10 and 5 km and having a prevalent diagnosis of depression. However, no associations were observed with well density/proximity using the 2.5 km radius. Few studies have explored this association, and our study, although exploratory, adds to the growing evidence surrounding the adverse effects of living in proximity to UNG operations. Future research should explore the effects by different UNG well phases and include biological markers to assess exposure in regions where natural gas is extracted unconventionally by hydraulic fracturing.

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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.2022.113962.

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Temporal trend and cross-sectional characterization of urinary concentrations of glyphosate in Japanese children from 2006 to 2015

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ABSTRACT

Background: Over the past two decades, domestic shipments of glyphosate (Gly), in the form of an ionic salt, have been increasing steadily in Japan. This increase has raising concerns about the effects of chemical exposure on children. The International Agency for Research on Cancer classified Gly as a "probably carcinogenic to humans (Group 2A)" in 2015. The purpose of the current study was to analyze Gly in urine samples of Japanese children to determine temporal changes, seasonal changes, and gender differences.

Method: First-morning urine samples were obtained from 50 Japanese children (4–6-year-old) in October of 2006, 2011, and 2015 (total = 150) to investigate the temporal trends in urinary Gly concentrations. Additionally, first-morning urine samples were collected from 3-year-old children in August–September of 2012 (summer; n = 42) and in February of 2013 (winter; n = 42) to investigate the seasonal and gender differences, and the correlations between urinary Gly concentrations and insecticide exposure biomarkers. Urine samples were analyzed to measure for Gly using a liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Results: Detectable Gly concentrations were found in 41% of the 234 children. The 75th percentile and maximum concentrations of urinary Gly were 0.20 and 1.33 μg/L, respectively. The urinary Gly concentration in 2015 was significantly higher than in 2006, suggesting that the Gly exposure levels have been increasing. No seasonal or gender-specific differences in urinary Gly concentrations were observed, and no correlation with insecticide exposure biomarkers was found.

Conclusion: This study revealed that Gly exposure trends show an increase between 2006 and 2015, and that season and gender were not the exposure-determining factors. Overall, urinary concentrations of Gly were comparable with studies from other countries.

1. Introduction

Glyphosate [*N*-(phosphonomethyl) glycine] (Gly) was initially registered as a herbicide in the USA and Japan in 1974 and 1980, respectively (EPA, 2017; Ota, 2013). Various formulations of Gly including an acid, monoammonium salt, diammonium salt, isopropylamine salt, potassium salt, sodium salt, and trimethylsulfonium or trimesium salt are currently on the market. To optimize the Gly-based pesticides, the ionic salt compound is often modified to improve the stability, solubility, and the ease of use of these products. In Japan, over the last two decades, particularly since 1995, domestic shipments of Gly have progressively increased, from 1304 tons to 5156 tons (NIES, 2021). This is owing to the broad spectrum of its applications in agriculture, forestry orchards, plantation crops, amenities, home gardening, parks, and road tracks for controlling annual and perennial grasses. In 2014,

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Abbreviations

Gly	glyphosate
AMPA	aminomethylphosphonic acid
EFSA	European Food Safety Authority
HBM	human biomonitoring
LOQ	limit of quantification
LOD	limit of detection
OP	organophosphorus
PYR	pyrethroid
NEO	neonicotinoid
DEP	diethylphosphate
DETP	diethylthiophosphate
DMP	dimethylphosphate
DMTP	dimethylthiophosphate
tCDCA	1*R-trans*-chrysanthemum dicarboxylic acid
DCCA	*cis/trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid
3-PBA	3-phenoxybenzoic acid
SPE	solid-phase extraction
QC	quality control
ADI	acceptable daily intake

Gly sales to the agricultural sector in Europe and the USA were estimated at 48,549 tons and 113,356 tons, representing 7% and 15% of the global Gly sales to the agricultural sector (746,580 tons), respectively (Antier et al., 2020; Benbrook, 2016). The global consumption of Gly is expected to increase further, possibly up to 1 million tons per year by 2023 (Mertens et al., 2018).

The global use of Gly has resulted in Gly contamination in environmental media (land, water, and air) and its entry into the food chain from multiple sources. Gly was detected in 3.1% of 5329 samples of fruits, vegetables, and cereals tested in Europe in 2015 (European Food Safety Authority, 2017). There is limited data available on Gly residues in food and dietary intake in Japan. The degradation of Gly to aminomethylphosphonic acid (AMPA) is primarily a microbe-mediated process (Gimsing et al., 2004; Sprankle et al., 1975), with an estimated environmental half-life of 7–60 days (Giesy et al., 2000). Gly exposure may be caused by the ingestion of contaminated food or water, or by the usage of Gly during agricultural activities. A recent study of Gly in food commodities in Switzerland indicated that cereals and pulses (e.g., beans, lentils, and chickpeas) were the major sources of exposure for consumers (Zoller et al., 2018). Laboratory animals absorb 20%–30% of the dose of Gly administered orally (EFSA, 2015; Food Safety Commission of Japan, 2016). EFSA (2014) found that unmetabolized Gly is primarily excreted after its oral administration to rats, with low levels of AMPA detected. Recently, three studies demonstrated that approximately 1% of the Gly dose is excreted through urine, with a human biological elimination half-life of 5.5–10h (Connolly et al., 2019; Faniband et al., 2021; Zoller et al., 2020).

A controversy exists over whether Gly could have harmful effects on humans. Irrespective of whether the cause is direct or indirect, some scientific studies have reported genotoxicity, reproductive toxicity, and carcinogenicity (Ingaramo et al., 2020; Kier and Kirkland, 2013; Zanardi et al., 2020). On the contrary, the largest cohort study (Agricultural Health Study survey) indicated no apparent association between Gly and any solid tumors or lymphoid malignancies overall (Andreotti et al., 2018). The International Agency for Research on Cancer classified Gly as a "probably carcinogenic to humans (Group 2A)" in 2015, owing to sufficient evidence provided through animal studies in conjunction with "limited evidence" in human cohort studies (IARC, 2015). The European Food Safety Authority (EFSA) concluded that Gly is unlikely to pose a carcinogenic hazard to humans (EFSA, 2015). The U.S. Environmental Protection Agency (EPA) released the interim decision that there are no risks of concern to human health when Gly is used in accordance with its current label (EPA, 2020).

Recent reports have focused on the distinctive vulnerability of children to chemical exposures in their environment (EPA, 2002; Landrigan et al., 2004), and their exposure to pesticides has also attracted particular attention worldwide. Given the ubiquity of Gly present in the environment, and its increasing inclusion in experimental and epidemiological studies, the possible effects of Gly on developmental health is of particular concern.

Human biomonitoring (HBM) measures chemical levels via all exposure routes (dermal, ingestion, and inhalation) in daily life. HBM is an important tool to support environment and health policy-making, because it can provide useful quantitative information regarding the actual exposure of a population (exposure assessment) to environmental pollutants including Gly (WHO, 2015; Connolly et al., 2020). Some Gly HBM data from Germany (Conrad et al., 2017), Denmark (Knudsen et al., 2017), Sri Lanka (Jayasumana et al., 2015), the USA (Parvez et al., 2018), and Ireland (Connolly et al., 2017) have also been reported, and Connolly et al. (2020) reviewed Gly exposure assessment studies employing the HBM method. These results suggest that Gly exposure of people including vulnerable groups, such as children and pregnant women, occurs in occupational and environmental settings. More recently, Lemke et al. (2021) performed an HBM study of Gly with the highest number of subjects (n = 2144) to date. In 52% of the samples the urinary Gly concentrations were above the limit of quantification (LOQ). However, a limited number of HBM studies have been conducted for Gly; particularly, there are limited, reports of HBM data available in Japan, except for our previous report (Nomura et al., 2020).

The objective of the present study was to assess time-trend (2006–2015), seasonal changes, and gender-specific differences in Gly exposure levels using urinary Gly as an exposure biomarker in Japanese children exposed to environments unrelated to agricultural activity. The potential for exposures to multiple pesticides including organophosphorus (OP) and neonicotinoid (NEO) insecticides has been suggested in our previous report (Osaka et al., 2016). Therefore, the correlation between the urinary concentrations of Gly and insecticide exposure biomarkers, including OP, pyrethroid (PYR), and NEO insecticides, was also evaluated to assess the co-exposure of Gly and other pesticides. Moreover, the daily Gly intake was preliminarily calculated from the urinary concentrations of Gly for toxicological assessments.

Table 1
Demographic data of subjects in this study.

Groups	Group A			Group B	
Subgroups	2006	2011	2015	summer	winter
Sample collection, (month/year)	10/2006	10/2011	10/2015	8–9/2012	2/2013
Number of subjects (male, female)	50 (26, 24)	50 (24, 26)	50 (29, 21)	42 (21, 21)	42 (21, 21)
Years of age (mean ± SD)	4.7 ± 0.8	4.7 ± 0.8	4.8 ± 0.8	3[a]	3[a]
Height (cm, mean ± SD)	103.9 ± 7.5	102.6 ± 5.8	104.9 ± 6.3	–	–
Weight (kg, mean ± SD)	16.6 ± 2.3	15.9 ± 2.3	16.8 ± 2.4	–	–
Creatinine concentration (g/L, mean ± SD)	0.83 ± 0.42	0.92 ± 0.39	0.91 ± 0.37	1.10 ± 0.62	0.74 ± 0.36

SD, standard deviation.
[a] Urine samples of Group B were collected from 3-year-old subjects who participated in a municipal health checkup program. Since the date of birth information was unknown, age is reported as 3-year-old.

2. Methods

2.1. Study subjects and design

Table 1 shows the basic characteristics of the study subjects. Our study subjects were 4–6-year-old children from kindergarten (hereafter referred to as Group A) and 3-year-old children who attended a municipal health check program (hereafter referred to as Group B) in the suburban areas of Aichi, a central region of Japan, respectively (Fig. 1).

Group A was composed of 150 children (79 male and 71 female), whose samples were used to investigate the temporal trends and gender-specific differences in urinary Gly concentrations. From Group A, a total of 150 first-morning voids were collected. Wada et al. (2011) initially collected urine samples for a cross-sectional study at a kindergarten in 2006. The urine sample collection was conducted again in 2011 and 2015 from children studying in the same kindergarten. From the collected urine samples, 50 (26 male and 24 female) from 2006, 50 (24 male and 26 female) from 2011, to 50 (29 male and 21 female) from 2015 were randomly selected for the present study.

Group B was composed of 84 (42 male and 42 female) 3-year-old children who participated in a municipal health checkup program in August–September 2012 (summer; 21 male and 21 female) and February 2013 (winter; 21 male and 21 female). The participants were different children; the corresponding data related to this have been reported in a previous study (Osaka et al., 2016). From Group B, a total of 84 first-morning voids were collected. These samples were used to study the seasonal and gender-specific differences in urinary Gly concentrations and the correlations between urinary Gly concentrations and insecticide exposure biomarkers in urine.

The Ethics Committee of the Nagoya University Graduate School of Medicine approved the study protocol (2011-0026).

2.2. Determination of urinary Gly concentrations

All urine samples were stored in CryoTubes (polypropylene material) at −80 °C immediately after collection, but two freeze–thaw cycles were added before the Gly analysis. It has been confirmed that in urine Gly is stable through several freeze-thaw cycles (Nomura et al., 2020). The urinary Gly concentrations in each sample were measured using the protocol proposed by Nomura et al. (2020). First, based on the urinary concentration of creatinine, the urine samples were diluted with purified water to normalize the urine concentration using an OT-2 pipetting robot (Opentrons, NY, USA). After transferring 1 mL of the diluted urine samples to a 96-well plate, 10 μL of Internal Standard solution (0.1 mg/L Gly-$^{13}C_2$,^{15}N) was added and mixed. The urine samples were purified by solid-phase extraction (SPE) using the SCX cartridge and the NH_2 cartridge with the Extrahera™ automation system (Biotage, Uppsala, Sweden). The eluate from the SPE column was dried, and the residues were dissolved in 100 μL of a solution of methanol and H_2O (5:95, v/v) with 0.1% formic acid and 0.1% deactivator additive (5 μmol/L of medronic acid) (Agilent Technologies, CA, USA). The supernatant of the dissolution solution was measured using LC-MS/MS. The LC-MS/MS system was composed of an Agilent 1200 infinity LC coupled with an Agilent 6430 Triple Quadrupole LC/MS System (Agilent Technologies). The urinary Gly concentrations (μg/L) were calculated by multiplying the creatinine-adjusted value (μg/g creatinine) by creatinine concentration (g/L), based on urine standardization before Gly measurement (Nomura et al., 2020).

To avoid analytical bias, all the samples were analyzed in a randomized order. Quality control (QC) urine sample were prepared from five healthy volunteers (ranging from 20 to 24 years old) who had neither received medication not had been occupationally exposed to Gly beforehand. The urine samples were mixed and diluted to a creatinine concentration of 0.05 g/L, and then a Gly standard solution was added to prepare two final concentrations of 0.0185 μg/L (0.37 μg/g creatinine) and 0.065 μg/L (1.3 μg/g creatinine). The prepared QC samples and distilled water (blank check) were analyzed every eight samples.

The limit of detection (LOD) and LOQ obtained from pooled urine samples, having a creatinine concentration of 0.05 g/L, were 0.01 μg/L (0.17 μg/g creatinine) and 0.03 μg/L (0.51 μg/g creatinine), respectively. The LOD and LOQ were defined as the peaks with signal-to-noise ratio of 3 and 10, respectively. The between-run precision for Gly in the QC samples was evaluated as the percentage of relative standard deviation (%RSD), and it was found to be 8% in all cases. In addition, to investigate the degradability of Gly due to sample storage, we measured four samples in which urinary Gly was detected in 2019. We found the same quantification values in all samples; therefore, Gly in urine was stable at −80 °C for at least 1.5 years (data not shown).

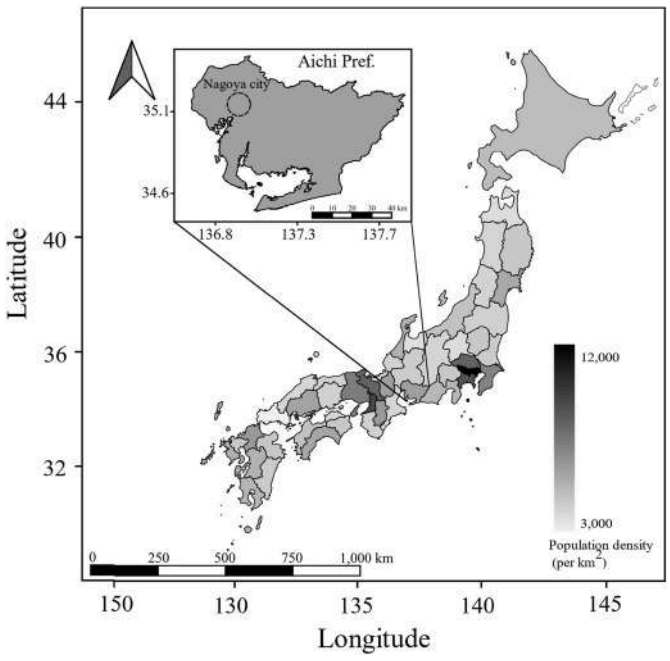

Fig. 1. Location for sample collection in this study. The dotted circle represents the prefectural capital of Aichi.

2.3. Determination of exposure markers of the other pesticides

In Group B, urinary exposure biomarkers for OP, PYR, and NEO were also assessed. The exposure biomarkers of the insecticides were as follows: diethylphosphate (DEP), diethylthiophosphate (DETP), dimethylphosphate (DMP), and dimethylthiophosphate (DMTP) for OP exposure; 1*R*-*trans*-chrysanthemum dicarboxylic acid (tCDCA), *cis*/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA), and 3-phenoxybenzoic acid (3-PBA) for PYR exposure, and the sum of the molar concentrations of seven neonicotinoids (acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam; ΣNEO) for NEO exposure. The LOD and LOQ were 0.07 μg/L and 0.3 μg/L for DEP, 0.05 μg/L and 0.2 μg/L for DETP, 0.15 μg/L and 0.5 μg/L for DMP, 0.05 μg/L and 0.2 μg/L for DMTP, 0.02 μg/L and 0.07 μg/L for all PYR exposure biomarkers, 0.03 μg/L and 0.10 μg/L for acetamiprid, 1.07 μg/L and 3.54 μg/L for clothianidin, 0.32 μg/L and 1.06 μg/L for dinotefuran, 0.31 μg/L and 1.03 μg/L for imidacloprid, 0.13 μg/L and 0.43 μg/L for nitenpyram, 0.32 μg/L and 1.06 μg/L for thiacloprid, and 0.22 μg/L and 0.73 μg/L for thiamethoxam, respectively. In a previous study, we reported insecticide exposure biomarkers in Group B (Osaka et al., 2016).

2.4. Measurement of urinary creatinine concentrations

Urinary creatinine concentrations were measured using the method

proposed by the Japan Society of Clinical Chemistry (2012). First, the urine sample was diluted 100-fold with water, and a creatinine standard solution was prepared using distilled water at concentrations of 0, 2, 4, 6, and 8 mg/dL. The prepared solution was then analyzed using a high-performance liquid chromatography instrument equipped with a UV detector. The analyses were performed using an Agilent HPLC 1100 series (Agilent Technologies, CA, USA). The within-series and between-day precisions were 0.24% and 1.27%, respectively.

2.5. Data analysis

All statistical analyses were conducted using the JMP Pro Statistical Software, Version 15, and two-sided p values < 0.05 were considered statistically significant. Nonparametric continuous variables were compared using the Kruskal–Wallis test for a three-group comparison, followed by Dunn's test for post-hoc multiple comparisons. In addition, the Jonckheere–Terpstra trend test was conducted to evaluate the tendencies of urinary Gly concentration from 2006 to 2015. Finally, the correlations between urinary Gly concentrations and urinary exposure biomarker concentrations of OP, PYR, and NEO insecticides in Group B was evaluated using Spearman's rank correlation coefficient. Undetectable urinary concentrations of Gly (values below LOD) were substituted by LOD/$\sqrt{2}$ (Hornung and Reed, 1990).

For toxicological assessment, the daily intake of Gly (*DI*) was calculated according to the following formula for Group A.

$$DI(\mu g / kg\ bw / day) = (UGC \cdot CE)/(UER \cdot bw)$$

UGC is the urinary Gly concentration (μg/g creatinine), *CE* is the gender- and body height-specific daily creatinine excretion, as reported by Remer et al. (2002), *UER* is the urinary excretion rate, extrapolated from previous reports as 1% (Faniband et al., 2021; Zoller et al., 2020), and *bw* is the body weight of the participant. Unfortunately, the *DI* was not calculated for Group B, owing to the unavailability of weight and height data of the children.

3. Results

The volume-based and creatinine-adjusted concentrations of urinary Gly in Group A are summarized in Table 2. The detection frequency of urinary Gly has increased every year since 2006 (18%), 2011 (30%), and 2015 (50%). The Gly concentration and creatinine-adjusted Gly concentration in 2011 and 2015 were significantly higher than those in 2006 ($p < 0.001$, post-hoc Dunn's test). In addition, a tendency test (Jonckheere–Terpstra test) showed an increasing trend in urinary Gly concentrations between 2006 and 2015. The same result was obtained even with the use of LOQ-based data set that was substituted for a LOQ/$\sqrt{2}$ in the <LOQ samples (Table 1S). Fig. 2 illustrates the box-plots of urinary Gly concentrations in 2006, 2011, and 2015, which are superimposed on a line graph with the shipment weights of total Gly (sum of various salt formulations) over the past two decades in Japan. The graph revealed that the increase in urinary Gly concentration was correlated with the increase in domestic shipments. Based on the increasing trend of domestic shipments, the urinary concentration of Gly is expected to increase in the future.

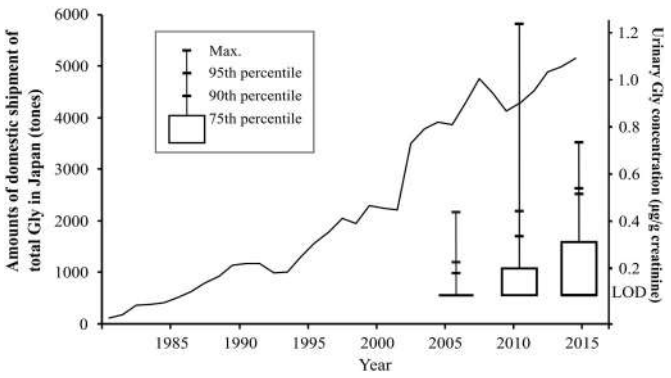

Fig. 2. Amount of domestic shipment of total glyphosate (Gly) in Japan (line graph) and urinary Gly concentration (box-plot) in Group A (2006, 2011, and 2015). The total Gly shipment was calculated as the sum of the weights of various Gly salt formulations.

In Group B, the difference in urinary Gly concentration was examined by season (summer and winter) and gender (Table 3). The detection frequencies in the four subgroups were ≥55%. The detection frequency in the summer group, was slightly higher than that in the winter group. Although there was some variation in the measured values for each selected percentile, there was no statistical difference in urinary Gly concentrations by gender. There was no difference in urinary Gly concentrations by gender. Gender-specific differences were also not found in Group A (data not shown).

In Group B, the urinary concentrations of Gly were analyzed in relation to those of the other pesticide exposure biomarkers. The correlations between Gly concentrations and eight insecticide exposure biomarkers, including DEP, DETP, DMP, DMTP, tCDCA, DCCA, 3-PBA, and ΣNEO, were examined. None of the pesticide exposure biomarkers correlated significantly with the urinary Gly concentrations.

Among the latest urine samples (collected in 2015 in Group A), the maximum daily intake of Gly was 1.2 μg/kg bw/day. This was less than 0.12% of the acceptable daily intake (ADI) of 1 mg/kg bw/day, as determined by the Food Safety Commission of Japan (2016).

4. Discussion

This study investigated time trends, seasonal changes, and sex differences in Gly exposure, and correlation between exposure of Japanese children to Gly and other pesticides, employing the HBM method. Our

Table 2
Detection frequency, and percentiles of urinary Gly concentrations in Group A (n = 150).

Sampling year	>LOD (%)	Selected percentile				Max.	*p*-value for difference[a]	*p*-value for difference vs. 2006[b]	*p*-value for trend[c]
		50th	75th	90th	95th				
Gly concentrations (μg/L)									
2006	18	<LOD	<LOD	0.16	0.25	0.68			
2011	30	<LOD	0.15	0.33	0.40	1.33	**<0.001**	**<0.001**	**<0.001**
2015	50	<LOD	0.24	0.37	0.50	0.61		**<0.001**	
Creatinine-corrected Gly concentrations (μg/g creatinine)									
2006	18	<LOD	<LOD	0.19	0.23	0.44			
2011	30	<LOD	0.20	0.34	0.44	1.26	**<0.001**	**<0.001**	**<0.001**
2015	50	<LOD	0.31	0.52	0.57	0.74		**<0.001**	

LOD, limit of detection.
[a] Kruskal–Wallis test.
[b] When a significant difference was determined using a Kruskal–Wallis test, a post-hoc Dunn's test was performed.
[c] Jonckheere–Terpstra test for trends, statistically significant correlation coefficients ($p < 0.05$) are highlighted in bold.

Table 3
Seasonal and gender differences of urinary Gly concentrations in Group B (n = 84).

Subgroups	>LOD (%)	Selected percentile				Max.	*p*-value for difference[a]
		50th	75th	90th	95th		
Gly concentrations (μg/L)							
Season							
Summer (n = 42)	60	0.17	0.35	0.54	0.65	1.20	0.159
Winter (n = 42)	55	0.07	0.20	0.38	0.43	0.55	
Gender							
Male (n = 42)	57	0.15	0.32	0.54	0.65	1.20	0.635
Female (n = 42)	57	0.10	0.28	0.36	0.50	0.54	
Creatinine-corrected urinary Gly concentrations (μg/g creatinine)							
Season							
Summer (n = 42)	60	0.22	0.30	0.36	0.41	0.95	0.621
Winter (n = 42)	55	0.19	0.26	0.42	0.46	2.20	
Gender							
Male (n = 42)	57	0.21	0.29	0.37	0.42	1.01	0.845
Female (n = 42)	57	0.20	0.28	0.38	0.46	2.20	

LOD, limit of detection.
[a] Mann–Whitney *U* test.

study yielded three significant findings. First, urinary Gly concentration have increased significantly between 2006 and 2015 in Japanese children. Second, no gender- or season-specific differences were observed in urinary Gly concentrations. Third, an association between Gly concentration and the insecticide exposure biomarkers in urine samples was not found in children.

The increasing Gly concentrations in children are potentially due to the growing use of Gly in Japan (Fig. 2). However, since imported products, such as grains, also use Gly in many other countries, exposure to Gly via imported products should also be considered. Conrad et al. (2017) reported a temporal trend (2001–2015) of urinary Gly concentrations in German young adults (20–28-year-old), suggesting that the Gly exposure levels increased until 2013, and declined gradually thereafter. The study findings suggested that the possible reduction in exposure since 2013 might be owing to changes in the application of Gly in agriculture: Austria, for example, banned the pre-harvest use of Gly in 2013. Thus, HBM studies can also reflect the effects of policy changes by governments. Hence, emphasizing the need for continuous efforts for HBM studies to understand Gly exposure are needed in Japan.

Seasonal variations can occur as Gly is commonly used by farmers around their fields, gardens, and households for weed control, from spring to summer. Carles et al. (2019) reported the seasonal fluctuations in Gly concentrations in surface water in France. The concentration of Gly in winter season was lower than those in other seasons. Therefore, the level of exposure to Gly was expected to be higher in summer than in winter. However, in the present study, no changes were observed in the urinary Gly concentrations. This result is supported by findings from a previous report (Stajnko et al., 2020). Moreover, that our result might have been due to the fact that summer and winter surveys did not include the same children, which might have biased the concentrations because of the different life style and dietary habits of the participating children.

In addition to the seasonal variation, gender differences were also examined, and we found that there were no differences in urinary Gly concentrations based on gender. Gender-specific differences in urinary Gly concentrations in children have been examined in some previous studies. Stajnko et al. (2020) showed that Gly exposure among children did not differ by gender, whereas among adolescents, Gly exposure tended to be higher in boys. The authors concluded that one of the reasons why the gender-specific differences were observed in adolescents but not in children could be due to the greater developmental and hormonal activities in adolescents.

Studies have indicated a possibility for adverse health effects from exposures to multiple pesticides. Osaka et al. (2016) reported a positive association between the excretion levels of urinary NEOs and OP metabolites but not between those of NEOs and PYR metabolites.In this study, on the contrary, the urinary Gly concentrations did not correlate with any of the exposure biomarkers for the three major insecticide lines, indicating that the characteristics of chronic exposure level and/or the route of exposure in children to Gly may be different from those of insecticides. Moreover, the timing of exposure and the difference in the elimination half-life of Gly may have contributed to this result.

For comparison, the Gly HBM data from other studies focusing on children's (under 16 years old) exposure are summarized in Table 4, including the data from the present study. Japanese children Gly concentrations were approximately the same or lower than in children from other countries. Hoppe (2013) set a tentative reference value of 0.8 μg/L for urinary Gly concentration. In the present study, three urine samples showed values exceeding the tentative reference values (1.3% of all urine samples in Groups A and B).

Urinary Gly concentration measurement enables the estimation of the systemic intake of Gly. The German Federal Institute for Risk Assessment had reported that a test person with the highest Gly level of roughly 4 ng/mL in urine had ingested less than 1% of the EFSA ADI (% ADI) of 0.5 mg/kg of bodyweight (BfR, 2016). Lemke et al. (2021) also evaluated the %ADI for children and found it to be 3.1%. The ADI value of 1 mg/kg bw/day was determined by the Food Safety Commission of Japan (2016). Based on our measurement results in 2015 in Group A, the maximum daily intake of Gly in more recent subjects was 0.12% of the ADI. Moreover, the median daily intake of Gly in Group A in 2015 was 0.3 μg/kg bw/day, which was only 0.03% of the ADI. The Gly *UER* is an important factor in the *DI* arithmetic formula. Previous studies have estimated the Gly *UER* at 20%–30% (EFSA, 2015; European Commission, 2002), whereas recent studies suggest that the Gly *UER* may be as low as 1% (Faniband, 2020; Zoller et al., 2020). Although these *UER* were obtained from subjects who were non-Japanese, and the *UER* remains under discussion, a *UER* of 1% was tentatively adopted in this study. Further investigation is needed to elucidate the Gly *UER* in Japanese adults and children.

There are several limitations to this study related to Gly concentration measurement and urine samples. First, certain aspects of the urinary Gly concentrations, such as storage degradation and absorption to storage tubes, remain unclear. Although we have confirmed that urinary Gly is stable for at least 1 year at −80 °C (data not shown), the 15-year stability of urinary Gly has not yet been clarified. The adsorption of Gly on the storage tube was minimized by using a polypropylene material. Second, the spot urine sample was not the ideal sample to reflect the chronic exposure status of Gly. Future HBM studies of Gly should be conducted on 24-h urine samples. Third, we were unable to measure the urinary concentrations of the Gly metabolite, AMPA. Some previous studies have shown that AMPA presents a similar toxicological profile as Gly (EFSA 2015; Li et al., 2013; Mañas et al., 2009). Fourth, the detection frequency of urinary Gly in this study was less than 50% in Group A, and thus a more sensitive analytical technique might provide more insight into the time-trend of the human exposure. Finally, questionnaire data that could potentially identify factors that contribute to urinary Gly concentrations were not collected.

In conclusion, this study revealed that Gly exposure tended to increase between 2006 and 2015, and that season and gender were not exposure-determining factors. Moreover, the concentrations of urinary Gly were similar to or lower than those in other countries. These results can be considered when designing future epidemiological studies to clarify the risk of Gly exposure.

Table 4
Comparison of glyphosate (Gly) concentrations in the present study and those from other similar studies focusing on children's exposures.

Study	Country	Sampling period	Age	*n*	DF (%)	GM (μg/L)	90th (μg/L)	95th (μg/L)
Present study	Japan	2006–2015	3–6	234	41	–	0.36	0.51
Curwin et al. (2007)	USA	2001	<16	51	88	2.5–2.7		
Trasande et al. (2020)	USA	2013–2017	0–8	108	11	0.28[b]		
Lemke et al. (2021)	Germany	2015–2017	3–5	358	51[a]	0.11	0.42	0.64
Sierra-Diaz et al. (2019)	Mexico[c]	2016	5–15	192	73[a]	0.36[b]		
	Mexico[d]	2016	5–13	89	100[a]	0.61[b]		
Stajnko et al. (2020)	Slovenia	2018	7–15	246	27[a]	–	–	0.19

DF, detection frequency above limit of detection; GM, geometric mean.
[a] Detection frequency above limit of quantification.
[b] Arithmetic mean.
[c] Mexico Agua Caliente.
[d] Mexico Ahuacapan.

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Declaration of competing interest

The authors declare 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.2022.113963.

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Wastewater-based epidemiology for early warning of SARS-COV-2 circulation: A pilot study conducted in Sicily, Italy

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ABSTRACT

There is increasing evidence of the use of wastewater-based epidemiology to integrate conventional monitoring assessing disease symptoms and signs of viruses in a specific territory. We present the results of SARS-CoV-2 environmental surveillance activity in wastewater samples collected between September 2020 and July 2021 in 9 wastewater treatment plants (WTPs) located in central and western Sicily, serving over 570,000 residents. The presence of SARS-CoV-2, determined in 206 wastewater samples using RT-qPCR assays, was correlated with the notified and geo-referenced cases on the areas served by the WTPs in the same study period. Overall, 51% of wastewater samples were positive. Samples were correlated with 33,807 SARS-CoV-2 cases, reported in 4 epidemic waves, with a cumulative prevalence of 5.9% among Sicilian residents. The results suggest that the daily prevalence of SARS-CoV-2 active cases was statistically significant and higher in areas with SARS-CoV-2 positive wastewater samples. According to these findings, the proposed method achieves a good sensitivity profile (78.3%) in areas with moderate or high viral circulation (≥133 cases/100,000 residents) and may represent a useful tool in the management of epidemics based on an environmental approach, although it is necessary to improve the accuracy of the process.

1. Introduction

The coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) brought an unprecedented impact worldwide. According to the World Health Organization data, as of January 2022, about 336 million cumulative cases have been reported worldwide, including more than 5.5 million deaths (World Health Organization dashboard at,). In Italy, the pandemic has led to the implementation of extraordinary public health measures ranging from the closure of schools and services/activities to local and national lockdown (Ministero della Salute - Istituto Superiore di Sanità, 2020). Because of these special measures, the COVID-19 outbreak has disrupted old habits, routines, and lifestyles, affecting human relationships and the productivity of the entire country (Cerami et al., 2020). In the pandemic context, real-time analyses of epidemiological data played an eminent role to address prompt interventions (Nahla Khamis, 2020) whereas the

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SARS-CoV-2 circulation needed continuous and rapid microbiological detection to track and isolate cases, also including testing individuals who do not turn to the health system (eg. paucisymptomatic or asymptomatic). According to some authors, the frequency and infectivity of asymptomatically infected persons could be the main reasons why COVID-19 has become a pandemic (Nikolai et al., 2020). The integration between epidemiological/microbiological and environmental surveillance systems may help to create an ''early warning'' strategy able to anticipate the viral spread in these territories and to support decision-making processes based on the risk analysis defined at the regulatory level. Surveillance on wastewater, namely wastewater-based epidemiology (WBE), is based on the principle that pathogens could be excreted by infected subjects through faeces and body fluids, for long or short periods, reaching the purification plants through the sewer system (Hamouda et al., 2021). Therefore, raw sewage entering the wastewater treatment plants (WTPs) constitute an important observation point on the circulation of pathogenic agents in the population, through the analysis of aggregate wastewater samples obtained from entire urban centres and/or differently aggregated urban areas through appropriate sampling strategies (Murakami et al., 2020). Even though SARS-CoV-2 is a respiratory pathogen, the persistence and replication of the virus in the gastrointestinal tract and shedding through faeces has been well documented (Jones et al., 2020; Guo et al., 2021; Mohan et al., 2021; Parasa et al., 2020; Wang et al., 2020a, 2020b). Some people who are infected have higher levels of virus particles in their faeces than others (about 50%). These levels are not determined by whether a person has many or few symptoms, or entirely asymptomatic (National Institute for Public Health and the Environment NIPHE – Ministry of Health, Welfare and Sport, 2021). Many factors can impact the shedding rate of viruses in the faeces, including viremia, the duration, severity and the stage of the disease, or age (Chen and Li, 2020). Moreover, SARS-CoV-2 seems to persist longer in the stool than in the respiratory tract (nearly 22 days) (Zhang et al., 2021). During the current pandemic, traces of SARS-CoV-2 genomes have been identified in wastewater in many areas of the world (Bonanno Ferraro et al., 2021), including Europe (Castiglioni et al., 2021; Hillary et al., 2021; La Rosa et al., 2020; Medema et al., 2020; Randazzo et al., 2020; Westhaus et al., 2021), USA (Gonzalez et al., 2020; Sherchan et al., 2020; Peccia et al., 2020; Wu F. et al., 2020), Australia (Ahmed et al., 2020), China (Mao et al., 2020), Japan (Haramoto et al., 2020; Hata et al., 2021), United Arab Emirates (Albastaki et al., 2021; Hasan et al., 2021) and Africa (Johnson et al., 2021; Jmii et al., 2021). In Italy, a retrospective WBE study coordinated by the Italian National Health Institute (NHI) showed that SARS-CoV-2 was already circulating in Northern Italy at the end of 2019, before the country's first confirmed cases in mid-February (La Rosa et al., 2021a). These bodies of evidence highlight the importance of sewage water as a sentinel tool to monitor the presence of epidemic viruses circulating in the general population and to identify outbreaks even before cases are reported to the healthcare system. To this end, in July 2020 the NHI launched a nationwide wastewater pilot monitoring program to investigate the spread of SARS-CoV-2 during the summer in tourist locations and then in autumn and winter seasons. Our study was conducted in the framework of the national surveillance program and reports the results of SARS-CoV-2 surveillance in wastewater samples from 9 conventional treatment plants in place in 8 Sicilian cities, during four different waves characterized by the different spread of the virus. Moreover, the relationship between the presence of the pandemic virus in wastewater and the trend of cases of infection in the population of Western Sicily was investigated to evaluate the application of this method as a possible early warning of SARS-CoV-2 circulation in the general population.

2. Materials and methods

2.1. Study design and sample collection

This observational study was carried out in Sicily (Italy), the largest island in the Mediterranean Sea accounting for about 5 million inhabitants. Nine wastewater treatment plants (WTPs) located in 8 cities and four different provinces of central and western Sicily were selected, serving a total of 574,107 inhabitants (ranging from 7062 to 241,206; 11.2% of total residents of the island). Wastewater samples (N = 206) were collected every 15 days for approximately 12 months (between July 21, 2020, and August 16, 2021). The position and the characteristics of the WTPs are shown in Fig. 1. For each WTP, 1 L of a 24-h composite sample of raw sewage was collected by an automatic sampling device. Collected samples were transferred on ice to the laboratory, stored at +4 °C, and analyzed for the detection of SARS-CoV-2 RNA within 12 h from sampling. In four cases, in which it was not possible to carry out the transport quickly, samples were immediately stored at −20 °C and transported to the laboratory still frozen within 15 days from the date of collection.

2.2. Laboratory methods

2.2.1. Virus concentration

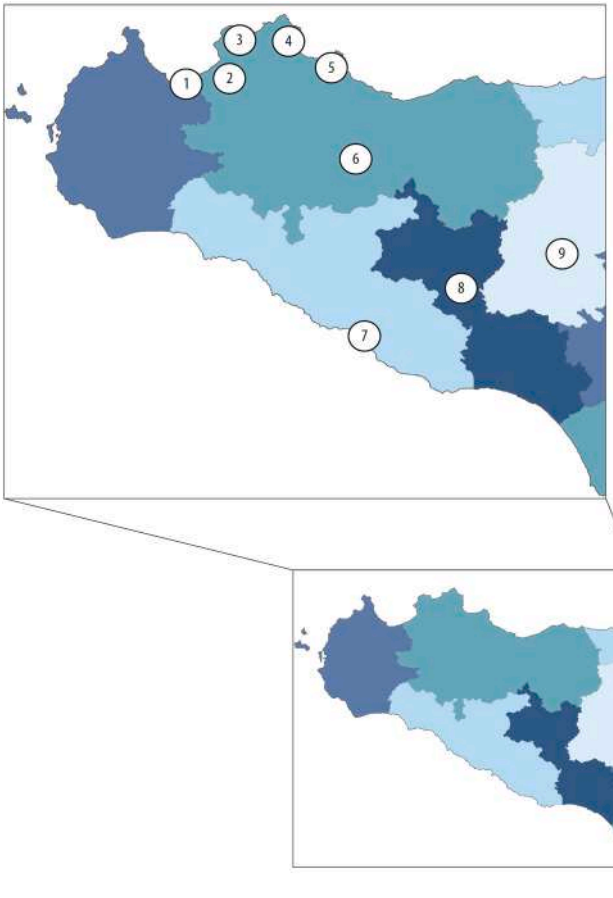
Before the concentration, 10 μL of a standard murine norovirus (MNV-1) suspension (supplied by NHI) was added to each sample (250 mL) as extraction control to evaluate the efficiency of the whole process. Sample concentration took place using a two-phase (PEG-dextran method) separation as detailed in the 2003 WHO Guidelines for Environmental Surveillance of Poliovirus protocol (World Health Organization, 2003a) with modifications to adapt the protocol to enveloped viruses (La Rosa et al., 2020).

2.2.2. RNA extraction

Viral RNA extraction was carried out using a semi-automated extraction method with buffer lysis and magnetic silica. In detail, the lysis phase was performed using 10 mL of lysis buffer (NucliSENS, bioMerieux, Marcy l'Etoile, France) with 5 mL of a concentrated sample, after an incubation of 20 min at room temperature, 100 μL of magnetic silica beads (bioMerieux, Marcy l'Etoile, France) were added and, after further incubation of 10 min, an automated procedure was performed by nucleic acid purification system (Auto-Pure96, All Sheng Instruments, Zhejiang, China). Before molecular tests, the extracted nucleic acids were purified from potential PCR inhibitors using the OneStep PCR Inhibitor Removal Kit (Zymo Research, CA, USA).

2.2.3. RT-qPCR

All RT-qPCR assays for SARS-CoV-2 were performed in the same laboratory with a QuantStudio 7K Flex Real-Time PCR System (Thermo Scientific). The reaction mixture (15 μL) consisted of 3.9 μL of Master Mix (QuantiNova Pathogen kit - Qiagen, CA, USA), 0.45 μL of 30 μM each primer, 0.3 μL of 10 μM of probe, water to the final volume of 10 μL and 5 μL of RNA template. The oligonucleotide sequences of primers and probe and thermal RT-qPCR conditions were described in La Rosa et al. (2020). All reactions were performed in quadruplicate. Molecular biology water served as non-template control. Verification of PCR inhibition was performed as a quality parameter of the determinations. To verify the inhibition, the PCR Ct (cycle threshold) value obtained from the sample added with 1 μL of a Ct 18 clinical positive control RNA was compared with the PCR Ct value of a water for molecular biology sample added with 1 μL of the same RNA according to the following formula: $\Delta Ct = Ct$ (sample + control RNA) − Ct (water + control RNA). The sample was considered acceptable if ΔCt was ≤ 2. The limit of detection (LoD) of the assay targeting ORF1ab gene was also evaluated. To assess the concentration/extraction efficiency of the method, prior to concentration, 100 μL of a process control virus solution (Murine Norovirus



Wastewater treatments plant	Inflow (m^3/die)	Served population (N)
1) Balestrate	2,592	7,062
2) Partinico	6,363	30,794
3) Carini	9,768	52,511
4) Palermo WTP1	18,907	78,401
5) Palermo WTP2	95,931	267,713
6) Lercara Friddi	1,687	7,607
7) Agrigento	7,000	22,762
8) Caltanissetta	12,500	80,846
9) Enna	5,2222	26,411
Total		574,107

Fig. 1. Map of Sicily showing the locations of the wastewater treatment plants.

at 2.0×10^3 genomic copies/μL) were added to 250 mL of each wastewater sample. The samples were then concentrated and extracted. For the PCR assays, serial ten-fold dilutions of a stock solution of the process control virus were used to produce standard curves. The Ct values of the reaction of samples spiked with process control virus were compared to the Ct value of the reaction containing the undiluted process control virus and the concentration/extraction efficiency (%) was calculated according to the following formula $= 10^{(\Delta Ct/m)}$ x F x 100 (ΔCt = Ct sample – Ct undiluted solution of control process virus; m = slope of standard curve of control process virus; F = fraction of the initial sample processed). The sample was considered acceptable if the concentration/extraction efficiency was ≥1%, as suggested by the protocol of NHI.

2.2.4. Clinical data sources

Sicilian SARS-CoV-2 cases were recorded according to the protocol shared by the integrated national surveillance system established by NHI (Istituto Superiore di Sanità, 2020). SARS-CoV-2 patients were considered eligible if they met the following inclusion criteria: being a resident of Sicily (or temporarily domiciled in Sicily) and having a laboratory-confirmed SARS-CoV-2 positive result using a reverse transcriptase real-time polymerase chain reaction (Real-Time RT-PCR) of nasal, pharyngeal, or nasopharyngeal swabs between January 1st, 2020, and August 31st, 2021. For each case, the following data were extracted: demographics (birth date, sex, place of residence); collection date of the first SARS-CoV-2 PCR positive test, collection date of the first SARS-CoV-2 PCR negative test (or end of quarantine), and clinical status. Subjects included in the analyses were anonymized and geocoded by an automated information algorithm performed in accordance with the privacy law. Thus, cases were attributed to the Sicilian areas served by the 9 wastewater treatment facilities. The number of resident populations for each area was calculated by considering the census sections included in that area as reported by the last available census report carried out by the Italian National Institute of Statistics (ISTAT) in 2011. Each SARS-CoV-2 case was considered as an active case during the window period between the collection date of the first SARS-CoV-2 PCR positive test and the collection date of the first SARS-CoV-2 PCR negative test (or end of quarantine). Otherwise, a SARS-CoV-2 case was considered as a new SARS-CoV-2 case only on the day when they got the first SARS-CoV-2 PCR positivity.

2.3. Statistical analyses

Categorical variables were summarized as percentages, whereas quantitative variables were presented as median (and interquartile range). For each area, the following occurrence measures per day of the study period were calculated:

- prevalence of active SARS-CoV-2 cases: number of active SARS-CoV-2 cases/residents*100,000
- incidence of new SARS-CoV-2 cases: number of new SARS-CoV-2 cases/residents*100,000.

Active SARS-CoV-2 incidence rates were compared by Poisson regression analysis. A logistic regression model was calculated to evaluate the association between the active SARS-CoV-2 incidence rates and the probability of positive PCR results of wastewater samples. The logistic regression model results were used to calculate the fitted predicted values for positive and negative PCR results of wastewater samples. A receiver operating characteristic (ROC) curve was used to assess the

active SARS-CoV-2 incidence rates for each PCR sample and to identify an optimal cut-off value that could predict the active SARS-CoV-2 incidence rate (ROCR Package and Optimal Cutpoints Package). Youden's index has been used for calculating the best cut-off value in the ROC curve. All the analyses were performed with the R software package and a p-value < 0.05 was considered statistically significant.

3. Results

The LoD was determined by spiking wastewater extracts with solutions of concentrations of approx. 1,000, 100, 50, 20, 10, 2 and 1.0 g.c./μL. Ten replicates of each dilution were tested. The LoD was determined as the lowest concentration to which all ten replicates were positive. The assay had a LoD of 2 g.c./μL. The recovery rate of SARS-CoV-2 from sewages may have varied according to the physico-chemical properties of wastewater samples (Mean = 11.31 ± 14.79%; Range = 1.0–61.0%; CI 95% = 3.58)". Overall, 51% (n = 105/206) of wastewater samples were positive to SARS-CoV-2 (range 23.1%–70% in the 9 different facilities). For each WTP, SARS-CoV-2 was detected as follows: 23.1% (n = 6/26) in WTP1, 58.3% (n = 14/24) in WTP2, 53.6% (n = 15/28) in WTP3, 72.7% (n = 16/22) in WTP4, 57.1% (n = 16/28) in WTP5, (n = 10/22) 45.4% in WTP6, 42.8% (n = 9/21) in WTP7, 70.0% (n = 14/20) in WTP8, and 25.0% (n = 5/20) in WTP9. SARS-CoV-2 cumulative prevalence was 5.9% considering the whole Sicilian area studied (p = NS). Prevalence of SARS-CoV-2 active cases was statistically significant higher in areas with SARS-CoV-2 positive wastewater samples than in area with negative SARS-CoV-2 wastewater samples (273.8 cases/100,000 residents vs. 46.9 cases/100,000 residents; $p < 0.001$) (Table 1). Differences were also observed either considering only symptomatic patients (87.4/100,000 vs. 18.9/100,000; $p < 0.001$) or asymptomatic active cases (170.9/100,000 vs. 25.5/100,000; $p < 0.001$).

Fig. 2 depicts the SARS-CoV-2 incidence rate (primary y-axis) and the relative frequency of PCR positive samples on all 9 WTPs (secondary y-axis) with respect to the observation time (x-axis). Overall, the cumulative SARS-CoV-2 epidemic curve observed in all WTPs overlapped the prevalence of SARS-CoV-2 positive wastewater samples. A ROC curve was applied to identify the level of SARS-CoV-2 cases/100,000 inhabitants able to predict the wastewater sample results (Fig. 3A and B). The analysis showed a best cut-off value of 133 active cases/100,000 residents (sensitivity = 80.3%, specificity = 76.5%, accuracy = 78.3%) that should be considered equal to 9.5 new cases/day/100,000 inhabitants. Finally, a logistic regression analysis was implemented to evaluate the association between wastewater positivity and SARS-CoV-2 cases. The probability of wastewater positivity was found to increase by about 0.86% per active case/100,000 inhabitants ($p < 0.001$). The fitted probability of positive (and negative) wastewater samples according to SARS-CoV-2 prevalence was reported in Fig. 4. In depth, the probability of a positive sample is quite low (<24.6%) when SARS-CoV-2 active cases were below 50 active cases/100,000, whereas it was relatively high (>91.9%) when SARS-CoV-2 active cases were above 400 active cases/100,000.

Table 1
Variables involved in determining PCR positivity in samples collected from wastewater treatment plants.

Variable	PCR positive wastewater samples	PCR negative wastewater samples	*p*-value
- SARS-CoV-2 active cases [median (IQR) *100,000]	273.8 (118.6–408.2)	46.9 (14.0–125.7)	<0.001
- SARS-CoV-2 active cases (only symptomatic patients) [median (IQR) *100,000]	87.4 (53.2–128.9)	18.9 (3.8–64.9)	<0.001
- SARS-CoV-2 asymptomatic active cases [median (IQR) *100,000]	170.9 (61.3–273.4)	25.5 (4.4–66.0)	<0.001

4. Discussion

To date, four epidemic waves of SARS-CoV-2 were recorded in Sicily, the first of which showed very low intensity, probably underestimated due to a low number of diagnostic tests performed, while the others presented at a significantly higher intensity. Although the research of SARS-CoV-2 in wastewater was performed occasionally during the first wave, it has been evaluated systematically since the beginning of the second wave of the pandemic in Sicily which occurred in July 2020. The aim of searching SARS-CoV-2 in WTPs of cities with a variable number of inhabitants (varying between approximately 6200 and 670,000) of central and western Sicily is to evaluate whether WBE could represent a good proxy of the early spread of the virus. The study findings have shown that SARS-CoV-2 was detected in all monitored sites, both in small and large treatment plants. Furthermore, the probability of detecting the viruses in wastewater samples changed in relation to the number of SARS-CoV-2 cases detected in the population. The analysis allowed estimation with 78.3% accuracy in the presence of more than 133 active cases/100,000 inhabitants (equal to 9.5 new cases per day per 100,000) when a positive wastewater sample was detected. According to this data, a proportional increase in the frequency of sampling could be needed when the expected SARS-CoV-2 active cases could be very low. The sensitivity reported in our research may appear to be lower than that reported in other studies (De Giglio et al., 2021; Hewitt et al., 2021). However, several factors may have contributed to these discrepancies, including the recovery rate of method, the variation in the number of asymptomatic and pre-symptomatic individuals, the COVID-19 testing rates, the incidence rates observed during the study period, the WTPs or environmental characteristics as plant daily capacity, and the rainfall events. Nevertheless, despite the global clinical surveillance for COVID-19 has been established, remain several cases of asymptomatic individuals, and those with very mild symptoms, that would not be identified, and therefore, together with contacts not traced, a large part of the real transmission could be potentially missed (Angulo et al., 2021; Havers et al., 2020; Larsen et al., 2020; Wu S.L. et al., 2020). The wastewater surveillance of SARS-CoV-2 has proven to be a powerful tool to evaluate disease incidence at the community level, but it still needs to be integrated into other public health initiatives (e.g., campaign-based and randomized testing of individuals such as the presence of pathogen or antibodies, clinical case reporting, and mobile-based contact-tracking and self-reporting systems) (Larsen et al., 2020). This represents a significant challenge considering the poor integration of the environmental and clinical science communities (Boulos and Geraghty, 2020).

WBE was theorized in 2001 (Daughton and Ternes, 2001), then implemented to trace illicit drug and pharmaceutical consumption in communities (Lopardo et al., 2018; Maida et al., 2017; Nikolaos et al., 2017; van Nuijs et al., 2011; Zuccato et al., 2005) and human pathogens during the global polio eradication program (Hovi et al., 2012; Ndiaye et al., 2014; Roberts, 2013). Today, we know that human pathogens in wastewater can represent a good proxy of the concentrations excreted by the population afferent to the treatment plant if they persist long enough (2–4 days) to be detected (Brouwer et al., 2018; Carducci et al., 2020; Kitajima et al., 2020). Therefore, monitoring temporal changes in viral concentrations and diversity in community wastewater samples can be used not only to determine the true extent of the infection in the population but also the emergence of new viral strains and the early detection of new viral outbreaks (Ahmed et al., 2020; Daughton, 2012; Johnson et al., 2021; Hart and Halden, 2020; La Rosa et al., 2021b; Monteiro et al., 2022). While retrospective studies have already demonstrated the feasibility as an alert system, WBE for real-time early warning cannot be realized without frequent sampling, rapid sample delivery, analytical turnaround, and reporting. For this reason, based on

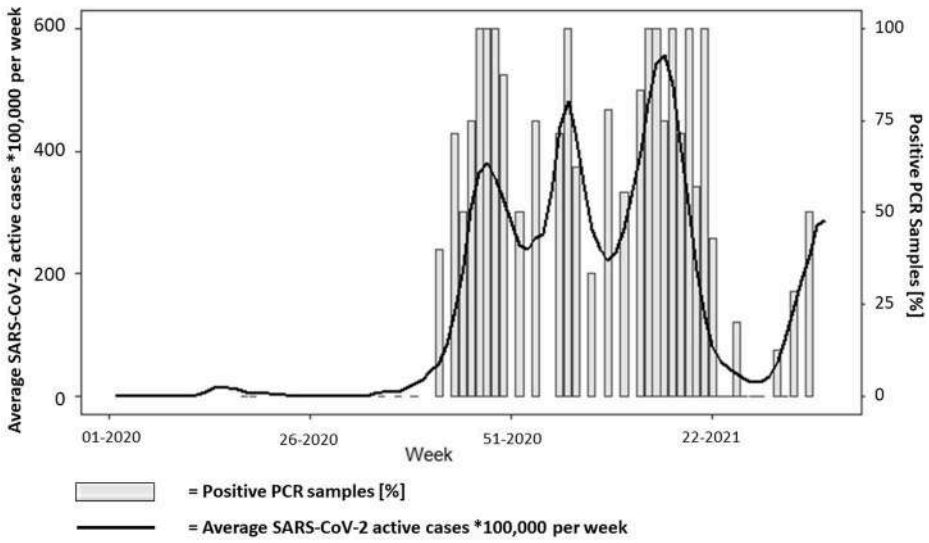


Fig. 2. PCR results and average active cases observed on a weekly basis in the areas connected with the WTPs.

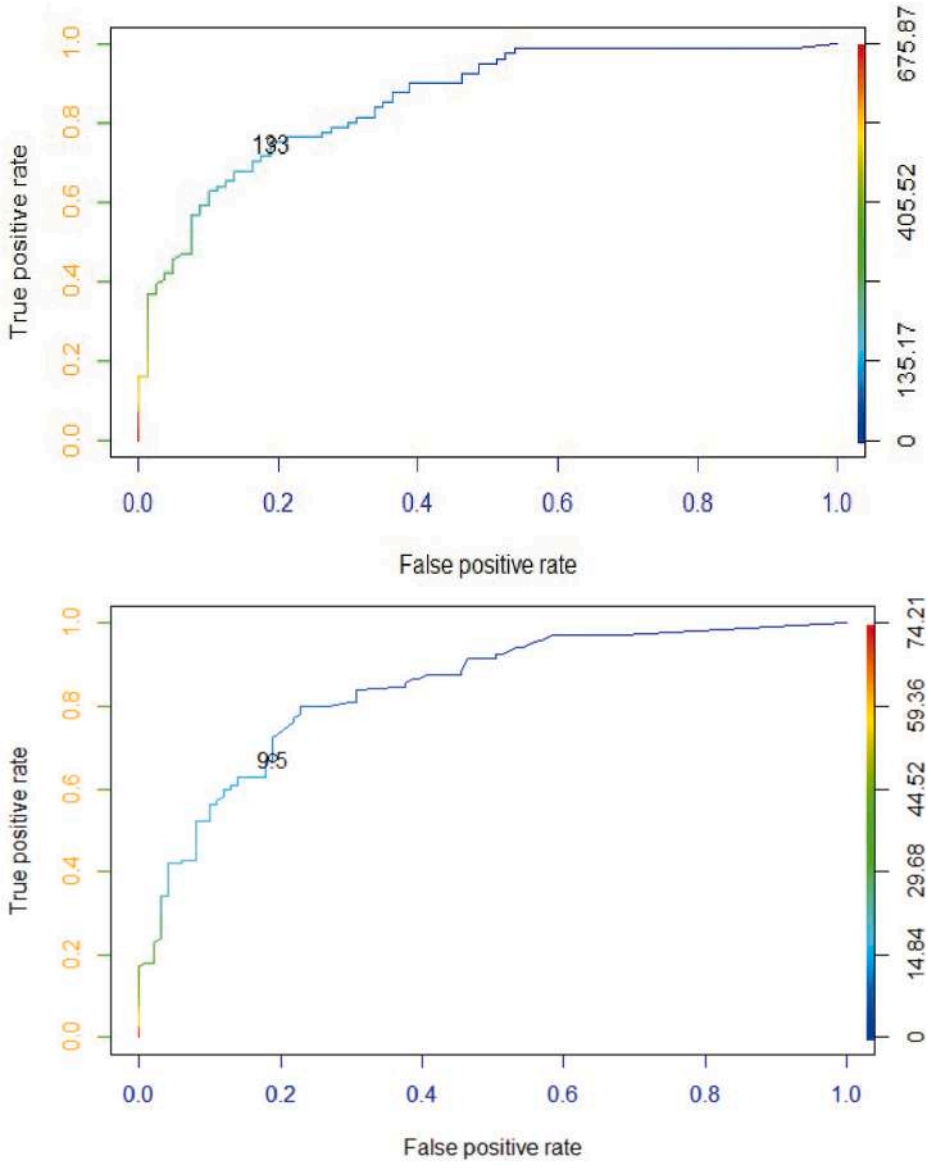


Fig. 3. ROC curve to predict the number of SARS-CoV-2 active cases/100,000 inhabitants (A) and SARS-CoV-2 new cases/100,000 inhabitants (B).A: SARS-CoV-2 active cases/100,000 inhabitants: best cut-off point 133/100,000 residents; sensitivity = 80.3% (69.2%–88.2%); specificity = 76.5% (65.8%–84.7%); AUC = 78.3% (70.9%–84.2%).B: SARS-CoV-2 new cases/100,000 inhabitants: best cut-off point 9.5/100,000 residents; sensitivity = 78.5% (69.3%–85.6%); specificity = 78.8% (69.2%–86.1%); AUC = 78.6% (72.3%–83.9%).

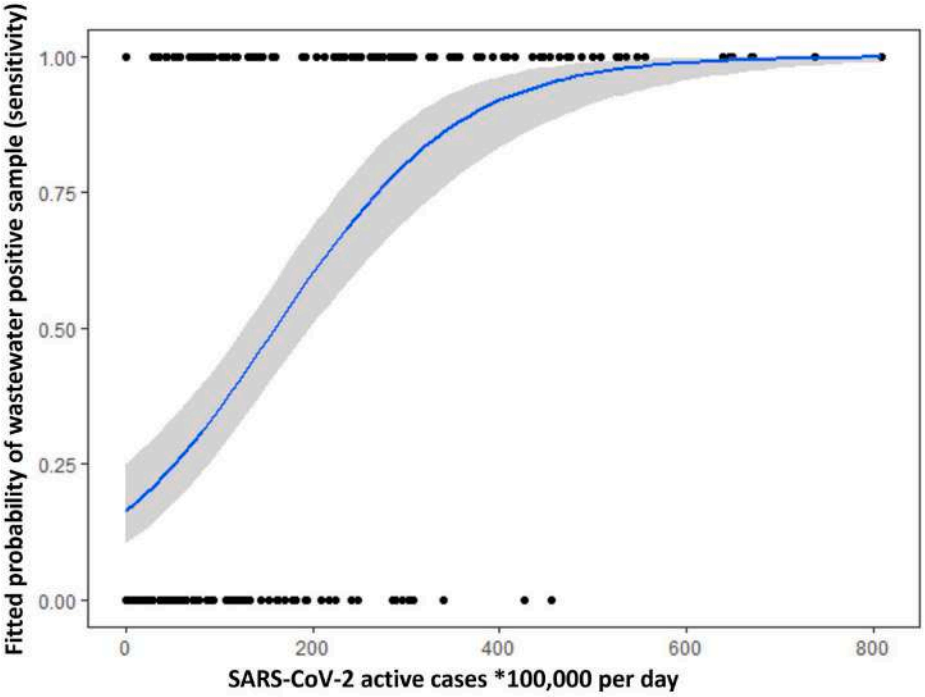


Fig. 4. Predicted probability of positive wastewater according to the active SARS-CoV-2 cases.

the EU Commission recommendation n. 2021/472 (European Commission, 2021), the EU Member States are strongly encouraged to implement as soon as possible, and no later than October 1st, 2021, a national wastewater surveillance system targeted at data collection of SARS-CoV-2 and its variants with the minimum sampling frequency on two samples per week for large cities with over 150,000 inhabitants. In Italy, cities with more than 50,000 inhabitants will also be monitored due to the low presence of cities with more than 150,000 inhabitants by a collection of one sample per week. Nevertheless, the findings presented in this study have some limitations to be further investigated to clarify their potential impact on sensitivity of the method starting from its recovery rate that seems lower than other described. As discussed by McMahan et al. (2021), the following factors are currently being studied in the new wastewater-based surveillance approach: the quantification of viral RNA to better evaluate the presence of cases in the population; the impact of physical-chemical characteristics of wastewater on viral recovery; the sewage flow rate; the presence of COVID hospitals; the size of the population residing in the area served by WTP; differences between urban and suburban centres. A further limitation could be given to the accuracy in the geo-localization due to errors in addresses input or in the exclusion of tourists who were temporary present in Sicily at the time of SARS-CoV-2 positivity, and to the unavailability of information on the location address. The effectiveness of the WBE early-warning approach, as proved by the outcome of this research, is prompting the implementation of the Italian nationwide wastewater monitoring system to monitor for the SARS-CoV-2, by effectively integrating the conventional clinical surveillance.

5. Conclusions

The detection of SARS-CoV-2 RNA in wastewater may provide a good picture of the trend of infections in a community, providing valuable real-time data on the viral spread within a spatial unit, hence, putatively requiring fewer resources than individual diagnostic testing.

To our knowledge, this is one of the first studies aimed to correlate the number of geo-referenced COVID-19 cases, within the area served by a wastewater treatment plant, with SARS-CoV-2 detection in wastewaters adducted to that plant to estimate the specificity of the method applied. Although it is necessary to improve both the sensitivity and the accuracy of the environmental analysis, according to our findings, the current method already seems to achieve a good sensitivity profile in areas with moderate or high viral circulation and may represent a useful tool for integration in the management of epidemics. The integration between clinical and environmental surveillance systems may constitute an appropriate decision-support tool to put in place public health intervention to prevent the spreading of the epidemic virus in the general population and guide public health decisions.

CRediT authorship contribution statement

Carmelo Massimo Maida: Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Microbiological characterization. **Emanuele Amodio:** Formal analysis, Writing – review & editing. **Walter Mazzucco:** Supervision, Writing – original draft, Writing – review & editing. **Giuseppina La Rosa:** Conceptualization, Writing – review & editing. **Luca Lucentini:** Conceptualization, Writing – review & editing. **Elisabetta Suffredini:** Conceptualization, Writing – review & editing. **Mario Palermo:** Writing – review & editing. **Gina Andolina:** Microbiological characterization. **Francesca Rita Iaia:** Microbiological characterization. **Fabrizio Merlo:** Samples collection, Writing – review & editing. **Massimo Giuseppe Chiarelli:** Samples collection, Writing – review & editing. **Angelo Siragusa:** Samples collection, Writing – review & editing. **Francesco Vitale:** Supervision, Writing – review & editing. **Fabio Tramuto:** Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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

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What is required to combine human biomonitoring and health surveys?

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ABSTRACT

Obtaining holistic information about health and health determinants at the population level should also include data on environmental risk factors of health. So far, only a few countries have combined, at the national level, health and human biomonitoring (HBM) surveys to collect extensive information on health, lifestyles, biological health determinants and environmental exposures. This paper will provide guidelines on how to combine health and HBM surveys and what is the added value of doing so. Health and HBM surveys utilize similar infrastructure and data collection methods including questionnaires, collection and analysis of biological samples, and objective health measurements. There are many overlapping or comparable steps in these two survey types. At the European level, detailed protocols for conducting a health examination survey or HBM study exists separately but there is no protocol for a combined survey available by now. Our recommendations for combined health and HBM surveys focus on a cross-sectional survey on general population aged 6–79 years. To avoid unnecessary participant burden, for the selection of included measurements basic principle would be to ensure that results of the measurements have a public health relevance and clear interpretation. Combining health and HBM surveys into one survey would produce an extensive database for research to support policy decisions in many fields such as public health and chemical regulations. Combined surveys are cost-effective as only one infrastructure is needed to collect information and recruit participants.

1. Introduction

Epidemiological studies are a well-established scientific tool to obtain information about health and health determinants of a population and population sub-groups. Randomized control trials (RCTs), cohort and case-control studies, and systematic reviews, are the most appropriate study designs for aetiological research in clinical medicine, public health including environmental health, and health policy. However, other study designs can be also considered. Cross-sectional studies provide a snapshot of the situation of the target population at a given time. Although, they do not allow causal inference to be drawn between environmental exposure and health effects, they can provide indications of possible associations (Levin, 2006). Population-based surveys are a type of observational studies that provide descriptive information about the population (Kumar Yadav et al., 2019) and can be used for evidence-informed decision making.

Cross-sectional studies can be transferred to cohort or longitudinal studies, if study participants are followed-up either through linkage to administrative registers such as hospitalizations and mortality or repeated survey measurements or questionnaires are conducted. This

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would allow for more in dept analysis of associations between observed risk factors and lifestyles and their health effects. For causal inference, the gold standard has been RCTs. From observational studies such as cross-sectional surveys with follow-up and longitudinal studies, a quasi-experimental research design based on observational data can be set up to draw causal inference (Nichols, 2007; Shadish et al., 2002). The choice between different study designs depends on feasibility aspects related to time, and technical and financial resources which are context specific.

Dahlgren and Whitehead (2007) have outlined a holistic perspective for the social determinants of health. These determinants include individual level lifestyle factors, social and community networks, and socio-economic, cultural, and environmental conditions (e.g., traffic related air pollution, occupational or residential chemical exposures, living near an industrially contaminated site). Within this perspective, health surveys are often used to collect information about individual level lifestyle factors and socio-economic status as well as about health status and biological risk factors such as obesity, hypertension, and diabetes. However, due to the focus on health-related aspects, health surveys might not automatically be feasible to reveal all the diverse and complex details of human exposure. In connection with the increased environmental awareness over the past decade, in parallel to existing health surveys, human biomonitoring (HBM) studies have been established as an important tool for investigating human exposure to chemicals and for quantifying body burden (internal dose). Compared to health surveys, HBM studies are specifically designed to investigate all relevant aspects of human exposure and hence more specific data on exposure relevant aspects are recorded to uncover exposure routes and adverse outcome pathways (Human biomonitoring: facts and figures, 2015; Sexton et al., 2004).

For the collection of information, questionnaires are commonly used in survey research. At first inspection, these questionnaires may differ between health surveys and HBM studies. Health surveys, often also called health interview surveys (HIS), use questionnaires to collect information on lifestyles including smoking habits and diet, diagnosed diseases, and use of medications and health care services. At the European Union (EU) level, the European Health Interview Survey (EHIS) is mandatory health interview survey for the EU Member States defining data collection periods, sample size and questionnaire items (European Commission, 2018). More extensive health surveys called health examination surveys (HES), also include health measurements such as anthropometric measurements (e.g. heigh and weight) and blood pressure, and collection of biological samples to obtain more objective information about health and its determinants (European Health Examination Survey, 2021).

HBM studies, on the other side, use questionnaires to obtain more detailed information about exposure-related behaviours such as living conditions and area, food consumption, occupational exposure, and lifestyles. In addition, HBM studies collect biological samples (e.g. whole blood, plasma and urine) for the analysis of health and/or environment-related chemicals and/or their metabolites.

Health surveys and HBM studies both use a survey methodology including questionnaires and collection of biological samples on general population or population sub-groups and the required infrastructure to operate are similar. Since both study types are also investigating the determinants of health, one would expect that combining health surveys and HBM studies would have added value for health and exposure monitoring, research, and policymaking.

Until now, only few countries such as Germany (Kolossa-Gehring et al., 2007), France (Balicco et al., 2017), Israel (Berman et al., 2017), the USA (Centers for Disease Control and Prevention (CDC), 2021), and Canada (St-Amand et al., 2014) have successfully combined their national health survey and HBM study. All three European examples (Germany, France and Israel) have been focused on the general population, but age groups covered by them have varied as well as included health measurements and analysed environmental biomarkers. This makes the comparison of the results between these studies possible for only a limited number of common outcomes and age groups (Tolonen et al., 2018). In many European countries, only a small-scale, research driven studies combining health measurements and HBM analysis have been conducted or samples collected in health surveys have been used to investigate selected environmental biomarkers (Tolonen et al., 2021). Comparability of these small-scale studies is difficult due to differences in used study protocols. Especially when biobanked samples from health surveys are used for analysis of environmental biomarkers, we may be lacking relevant supporting information on exposures, living and working conditions, and lifestyles.

The most common reasons why in Europe health surveys and HBM studies are rarely combined were investigated within the European Human Biomonitoring Initiative (HBM4EU) (HBM4EU, 2021; Ganzleben et al., 2017). It turned out that in addition to the lack of or difficulty to secure funding, there is a lack of knowledge and capacities for sample handling and analysis, difficulties in managing large and diverse data from combined studies which possibly include several study visits, and a lack of flexibility between health and HBM part of the combined survey (Tolonen et al., 2018).

To tackle the reported lack of knowledge on conducting a combined health and HBM survey, this paper aims to provide a general guideline on how to proceed. An overview of the different phases of the organization of a cross-sectional survey will be provided with a special focus on steps where needs and/or approaches between health and HBM surveys may differ. Until now, at the European level guidelines and recommendations for health and HBM surveys exist separately, and guidelines for combined studies are not available so far. For international comparison, protocols from the National Health and Nutrition Examination Survey (NHANES) from the USA (National Center for Health StaticsNHANES, 2019–2020) and Canadian Health Measures Survey (NHMS) (Statistics Canada, 2019, 2020), which have a well-established biomonitoring module included to the national health examination survey, are available. These may not be directly applicable for European situation due to cultural and societal differences.

2. Material and methods

This overview and guidelines were prepared in the framework of the HBM4EU. HBM4EU is a joint effort of 30 countries, the European Environment Agency, and the European Commission, co-funded under Horizon 2020. HBM4EU generates evidence of the actual exposure of citizens to chemicals and the possible health effects to support policy-making (HBM4EU, 2021; Ganzleben et al., 2017).

The guidelines for combining cross-sectional health surveys and HBM studies presented here are based on existing European level recommendations and standardized operating procedures (SOPs) for both health surveys and HBM studies.

Standardized procedures for health examination surveys with the main focus being on cardiovascular disease epidemiology, have been available at the international level since 1968 (Rose and Blackburn, 1968) and have been updated several times over the years (Luepker et al., 1066; Rose et al., 1982). The World Health Organization (WHO) has also set up the 'STEPwise Approach to non-communicable disease (NCD) Risk Factor Surveillance (STEPS)', which provides detailed guidelines for conducting a health survey (World Health Organization, 2021). For health surveys, we have used both EHIS methodological guidelines (European Health Interview Survey (EHIS wave 3), 2018) and European Health Examination Survey (EHES) guidelines (Tolonen, 2013, 2016). EHES guidelines are in line with WHO STEPS guidelines.

For HBM studies, we have used guidelines and SOPs prepared under the HBM4EU initiative (Fiddicke et al., 2021; Esteban Lopez et al., 2021; Vorkamp et al., 2021; Santonen et al., 2019). The HBM4EU online library (HBM4EU Online library. 2021) includes SOPs for study design, recruitment of participants, collection and handling of biological samples, and chemical analysis and quality assurance. Also, guidelines

developed in COPHES and DEMOCOPHES projects (Becker et al., 2014; Fiddicke et al., 2015; Schindler et al., 2014; Exley et al., 2015) have been reviewed for these recommendations.

The following recommendations and guidelines for combined health survey and HBM study are focused on a general population survey. With some modifications, these recommendations and guidelines can also be used for targeted studies, where the focus will be on a specific population group, chemical or health outcome. For targeted studies, the definition of target group, and included chemical analysis and health measurements needs to be adjusted based on the study aims.

3. Results

The survey process can be divided into different phases (Fig. 1): 1) Design; 2) Planning and preparation; 3) Pre-testing and piloting; 4) Final survey design, planning and preparation; 5) Fieldwork and data collection; and 6) Data file construction, analysis and reporting. Quality control measures go across all the phases. In surveys from the domains of health and HBM, later referred to as health module and HBM module, many of these phases are identical or similar and only in some phases, domain specific features need to be considered. These guidelines will focus only on the phases where requirements for health and HBM module may deviate and decisions between them need to be made.

Since both survey types follow a general epidemiological approach, for combined health and HBM survey, several general requirements are applicable. However, differences in needs between the two modules also exist. Therefore, during the design phase, and planning and preparation of the fieldwork, analysis of specific requirement of the different needs with respective decisions should be made. The key differences in existing standardized protocols and guidelines for health and HBM surveys, that require decisions when two modules are combined, are described below.

3.1. Scope, objectives, and design of the study

The first task in the elaboration of any survey protocol is to define its specific scope and objectives. Pertaining to a combined health and HBM survey to provide a coherent scope and clear objectives covering the needs of both modules, is very important for subsequent implementation steps.

For decision-making on the study design of a combined health and HBM survey, the following questions should be considered:

- Is there an existing health survey into which an HBM module can be included or vice versa? Or is there any other type of cross-sectional study such as a nutritional survey into which both HES and HBM can be added? Or are you planning a completely new combined health and HBM survey?

As part of the study design, it is required to decide if all the survey components should be conducted during one study visit or will participation in the survey require several visits. For example, health module components may be conducted at first and as an add-on to this, participants are asked to attend the second examination visit/home visit for the HBM module within the coming weeks.

3.2. Population sample selection and sample size

To combine two study types, target population and selected sample should serve the needs for both health and HBM modules. The main questions are: What is the target population? What is the required sample size (i.e. number of invited persons)? What sampling frame (i.e. a list of all individuals from the target population) should be used? How should the sample be drawn? What is the minimum required participation rate?

At the European level, recommendations for health examination surveys focus on the adult population. Children and adolescents are equally important but their inclusion in health surveys would require some additional considerations, e.g. legal and ethical issues, use of specific equipment for measurement of children and age group specific health measurements. Therefore, the EHES recommendations, are focused on adults only. From a health survey perspective, also the elderly without an upper age limit would be of interest to be able to capture the impact of functional limitations and morbidity for disease burden and health care needs of the population. In contrast, the inclusion of children and adolescents is of great interest for HBM research since many chemicals might interfere with developing organisms. For this reason, it is recommended to focus on age group 0–70 years, but if

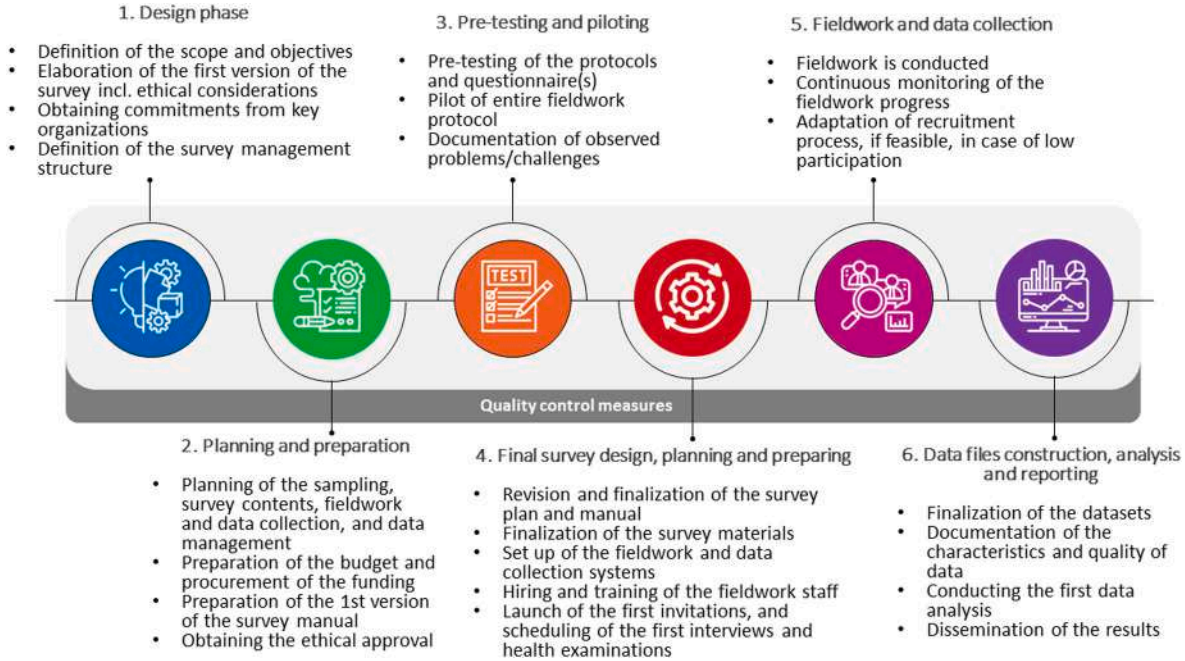

Fig. 1. Different phases of the survey process.

including small children is difficult, at least ages 6–79 years should be covered with a combined health and HBM survey.

All population groups, including institutionalized persons, are of interest and none of them should be excluded even though they would be difficult to recruit/examine. For more targeted studies of specific population sub-groups, people with specific profiles may need to be excluded.

National health surveys require a large sample size (number of persons invited). For example, based on a power calculation done within EHES, when the target group is the population aged 25–64 years and the interest is to estimate outcomes by 10-year age group, sex and educational level, the required sample size is 4000 persons, 500 persons per 10-year age group-sex domain. If results are to be presented also regionally, sample size needs to be increased to allow large enough sample for each region to ensure representativeness of the results. A survey statistician should always be consulted to ensure a large enough sample size based on power calculations and expected participation rate. (Tolonen, 2013). It may not always be feasible to conduct a HBM module on an entire sample of 4000 persons. However, since a smaller sample could be sufficient for HBM research questions, the HBM module could be conducted in a sub-sample. This sub-sample should include at least 500 randomly selected persons (expecting that at least 300 participate) in the age group(s) relevant for the substances of interest to ensure required representativeness. In this case, the sub-sample for the HBM module should be selected from the full sample of 4000 persons selected for the health module, not only among participants, to avoid double selection bias for the results.

The best available sampling frame, ideally a population register, should be used to ensure good coverage of the target population. In case a population register is not available or cannot be used for survey sampling, other available sampling frames which are frequently updated and include sufficient contact information for individuals can be used.

Two-stage sampling supports the logistics and fieldwork organization and ensures that both rural and urban areas will be coved with selected sample by large enough samples to allow inference by area.

Each study should aim for as high a participation rate as possible, ideally at least 60%, although participation rates this high are not common in Europe any longer (Tolonen, 2013; Mindell et al., 2015). For representativeness, it is better to target available resources to the recruitment activities rather than increasing sample size, as survey participation is often selective for socio-demographic factors as well as for health status and lifestyles (Karvanen et al., 2016; Christensen et al., 2015; Knapstad et al., 2016; Tolonen et al., 2010).

3.3. Survey content

Combined health and HBM surveys have three main data collection tools: questionnaire(s), collection and analysis of biological samples, and objective health measurements. Questionnaires and collection of biological samples are used for both health and HBM modules, while objective health measurements are more relevant for the health module.

3.3.1. Questionnaires

While questionnaire items in both health surveys and HBM studies overlap in many aspects, there are still certain differences, and some topics may be asked from a different perspective. HBM module requires specific questions related to the exposure to the substance(s) of interest as well as from occupational activities, household environment, and domestic exposures which are usually not included in health surveys. On the other hand, the health module usually requires more detailed information about health, health determinants including lifestyle and health behaviour information, and use of medication and access to health care services.

When preparing a questionnaire for the combined health and HBM survey, a balance between the needs of both domains should be found, keeping at least the key questions for both domains. The main questionnaire, which is provided for all participants, should include at least socio-demographic and socio-economic background questions on sex, age, marital status, labour status and occupation, household composition and income unless these can be obtained from the sampling frame or through record linkage to other data sources. From health status, at least general health, medical history and use of health care services should be asked. Lifestyle questions should cover smoking, alcohol consumption, physical activity, sedentary behaviours, and diet, and information on height and weight should be asked even though they may also be measured later during the health examination. Additional to this, more detailed questionnaires may be provided for example for a sub-sample to obtain better characterization of different sub-groups, to reduce participant burden, and to keep the main questionnaire within reasonable limits to promote a high participation rate.

For HBM studies, HBM4EU has prepared a set of standardized questionnaires (HBM4EU Online library. 2021) which are recommended to be used in future HBM studies conducted in Europe. For health surveys, a set of standardized questions is available through European Health Interview Survey (EHIS) regulations (European Commission, 2018) as well as through European Health Examination Survey (EHES) guidelines (Tolonen, 2016).

Both surveys can use a variety of questionnaire administration modes; self-reported either by paper-and-pen or online through a web questionnaire, and interviews conducted either face-to-face, by telephone or through a virtual format (video interview), or a mixture of these. The selection of used questionnaire administration mode(s) depends on national practices, setting of the fieldwork (availability of staff and premises), and by literacy level and coverage of internet access within the respective country.

3.3.2. Collection of biological samples and analysis of biomarkers

Biological samples are needed for both health and HBM modules. For health surveys, EHES recommends collecting at least blood samples to analyse lipids and glucose but also a collection of urine samples, especially 24h urine, is highly recommended to analyse sodium intake. For HBM studies, the required biological matrix depends on the compounds to be measured. The most frequently used sample types are either blood or blood compartments (serum, plasma) and/or urine.

For combined health and HBM survey, prioritization of biomarkers and/or compounds of interest needs to be done during the design phase of the survey since only a limited amount of blood can be drawn from an individual. For urine, the available quantity is not as critical. Some biomarkers and/or compounds can also be measured from other, non-invasive samples such as hair, saliva, nails, or breast milk or cord blood for women, and therefore, it should also be considered if reliable and validated methods exist to analyse some of the biomarkers and/or compounds of interest from other matrices than blood (Esteban and Castano, 2009).

For some health biomarkers such as glucose or insulin, or lipoprotein factions, fasting blood samples are required. However, fasting can be problematic for some of the compounds measured in the HBM module, especially for short-term exposure of non-persistent chemicals for which the diet is the major exposure route (Fromme et al., 2007; Koch et al., 2013; Preau et al., 2010). One solution to overcome the impact of fasting is to collect fasting blood samples on a sub-sample. This may also help survey logistics since fasting samples are often collected in the morning after overnight (8–12 h) fasting. Limiting the collection of fasting samples to a sub-sample of those who come to the examination in the morning also allows the use of afternoon and evening times for appointments. If this type of arrangement is made, it should be ensured that those assigned for the fasting samples, and who therefore are assigned for morning appointments, are a random sample from the total sample and represent the target population.

For blood samples in combined health and HBM survey, serum, plasma, and whole blood should be collected if feasible. For plasma samples, both citrate and EDTA as anticoagulants are recommended for

many health-related biomarkers while sodium-heparin is recommended for some other biomarkers and e.g. for metals. At least cotinine, total and HDL cholesterol, and glucose and/or glycated haemoglobin (HbA_{1c}) should be analysed in the combined HBM and health survey additional to selected environmental substances.

Basic blood sample collection and handling procedure do not differ for samples used for health and HBM modules. It is recommended to collect as much blood (serum, plasma and/or whole blood) as possible to facilitate analysis of a wide range of biomarkers and/or compounds but also supporting storage of some aliquots for future use. When samples are stored for a long time, i.e. in a biobank, they should be stored at the lowest possible temperature to minimize any recrystallization that might negatively impact the sample integrity.

For urine, 24h pool would be preferred for the analysis of many biomarkers but often this is difficult to collect in a survey setting. Therefore, collection of the first-morning void, whenever logistically feasible, or at least a random spot urine is recommended as many of the biomarkers and environmental substances can also be measured from them. From urine samples, at least total urine volume (especially relevant for 24-h urine samples), and urinary creatinine and specific gravity, which is also used to normalize the concentrations of HBM parameters should be analysed (Lermen et al., 2019).

Pertaining to sample collection for HBM purposes, special attention must be paid to the sample collection materials, both for blood and urine samples, to avoid potential cross contamination. It is recommended to clean all sample collection and storage materials according to standardized procedures to minimize possible inorganic and/or organic contamination through the manufacturing process (Lermen et al., 2015). Also, the collection of field blank samples can be used to document the background concentrations (Centers for Disease Control and Prevention (CDC), 2018).

If feasible with available samples and other resources (e.g., financial resources), additional biomarkers of interest, which are relevant for both survey types, e.g., biomarkers of effect for different health outcomes, should be included. Biomarkers of effect could for example include reproductive hormones, thyroid hormones, liver enzymes, biomarker of renal function, and nutritional biomarkers such as vitamin D.

3.3.3. Health examinations

Health measurements are an integral part of health surveys but are also included in many of the HBM studies on a smaller scale. The number of health measurements depends on the extent of health information needed. For combined health and HBM survey, at least anthropometric measurements of height, weight and waist circumference, and blood pressure should be included. Other possible health measurements could, for example, be spirometry, body composition using a bioimpedance device, cognitive function tests, and physical activity/fitness tests.

All additional measurements which can be included in the combined survey will provide valuable information about the health and health determinants of the target group. The number and type of additional measurements should be considered carefully in each survey to avoid unnecessary burden for participants. For the selection of additional measurements, criteria presented in Table 1 could be used, all points applying.

Table 1
Criteria for the selection of health measurements for a survey.

Criteria
1. Measurement result has a public health relevance
2. Measurement has a clear interpretation of the results
3. There are international standards for the measurement protocol
4. Measurement is practical and easy to administer in the survey setting
5. Information cannot or is difficult to obtain from any other data source, i.e. survey is the primary source of information
6. Cost of the measurement is feasible within the survey budget
7. Measurement is ethically acceptable in the survey setting
8. Measurement is well accepted by the participants

3.4. Ethics and data protection

For both HBM studies and health surveys, approval from the ethics committee and in some countries, a separate approval from the data protection authorities is required. Even though ethics are the code of conduct, not legislation, there are several ethical documents which guide the preparation and organization of health surveys including the Declaration of Helsinki (World Medical Association, 2018), which is considered as the pillar for ethical standards in medical research. Also, the Belmont Report on "Ethical Principles and Guidelines for the Protection of Human Subjects of Research" (The National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research, 1979), the Recommendation of the Committee of ministers No. R(90) 3 concerning medical research on human beings (Council of Europe and Committee of Ministers, 1990), the Oviedo Convention on Human Rights and Biomedicine (Oviedo, 1997), the Council of Europe protocol to the convention on Human Rights and Biomedicine (Council of Europe, 2005), and the International Ethical Guidelines for Biomedical Research Involving Human Subjects by the Council of International Organizations of Medical Sciences and WHO (Council for International Organizations of Medical Sciences (CIOMS) and World Health Organization (WHO), 2016) are important documents to be considered when planning and implementing a survey.

Approval by the ethics committee must be obtained before any of the fieldwork can start. For the process of obtaining ethical approval, required documents may vary by country and/or ethics committee but in general, the following documents are required:

- detailed study protocol including a detailed description of recruitment protocol, eventual justification for including children and other vulnerable population groups as well as procedures to be used for the collection of biological samples and the objective health measurements,
- information material about the study to be provided for the invitees,
- informed consent form,
- questionnaire(s),
- respective data protection notification stating that data are stored in pseudonymised in which case the identification code is kept only by survey coordination, or in fully anonymised in which case the identification code is deleted, and
- eventual use of incentives.

From each survey participant, and in the case of children their parents/legal guardian, a written informed consent is needed before any biological samples can be collected or objective health measurements conducted (Tolonen, 2013; Lermen et al., 2015). Combined health and HBM surveys provide valuable information for later follow-up studies. Therefore, participants must be informed about eventual further handling of their data and samples together with information of the storage of data and samples in pseudonymised format. Procedures for approvals for future studies must be described.

The format of the informed consent may vary from traditional consent which is asked from the participants each time their data or stored biological samples are used for a new project, to a broad consent allowing future use by new investigators (secondary use) and/or exchange of data. A newer concept of dynamic consent fosters continuous contact with the study participants feeding back results and invitations to participate in new studies in collected data and/or samples (Teare et al., 2021).

From the legal side, several EU and national regulations/legislations need to be considered when preparing a survey protocol. These include the EU General Data Protection Regulation (Regulation (EU), 2016), national data protection regulation, national medical research

legislation, act of the rights of the patients, biobank act and archives act.

3.5. Training of the personnel

Training of the survey personnel both at the central office and those conducting the fieldwork is essential for all surveys. A training seminar or workshop at the beginning of the study is recommended for all staff members to make the procedures standardized across the teams but also for cross-country and over time comparability purposes. Entire survey personnel should be given training on the main components of the study process including.

- purpose and aims,
- legal and ethical aspects including privacy issues,
- design of the survey and survey organization,
- recruitment strategy,
- importance and methods of SOPs,
- hygiene and safety issues,
- quality assurance procedures,
- data management system, and
- publicity and communication strategy.

Additionally, fieldwork personnel should receive training in communication skills, interviewing techniques, motivating participants, and giving feedback. It is also important that they know when and how to consult survey physicians and supervisors and be aware of safety issues and protocols. Those conducting measurements and/or collecting biological samples and/or preparing/handling the samples must go through detailed training and certification. If questionnaires are administered through interviews, specific interviewer training should be organized to explain the purpose of each question and provide instructions on how the interviewer can probe the interviewee if needed, to prevent interviewer biases.

3.6. Requirements for fieldwork site

Basic requirements for the fieldwork sites are the same for health surveys and HBM studies, i.e., the fieldwork site should be organized so that it is easily accessible for participants also by public transportation. For persons with functional limitations, easy access by elevators/ramps is needed. Since some measurements have special requirements, these should be considered to ensure that measurements are not compromised due to the setting in which they are taken. Blood pressure measurement requires a quiet room with a comfortable temperature as any sudden loud sounds and too cold temperature may affect the blood pressure levels. Also, if blood pressure is measured using the auscultation method, all noise from the other rooms or corridor may disturb the measurement. For all anthropometric measurements, participants are asked to undress and therefore privacy is required. Body fluids like e.g., blood, plasma, serum, and urine are considered as potentially infectious materials and therefore are assigned biosafety level 2. The fieldwork sites must provide a corresponding biosafety level 2 containment in order to be able to process such samples (World Health Organization (WHO), 2004). In addition, special care must be put on the selected rooms to minimize any potential contamination. Also, for all measurements and discussions between survey personnel and survey participants, privacy and data protection need to be ensured.

3.7. Recruitment of participants

Successful recruitment of participants is of vital importance for any study. There are no one-size-fits-all solutions for a recruitment strategy. It must be adjusted based on the study design, target population, available funds, and previous experience. However, the same basic principles apply to both health surveys and HBM studies.

The recruitment strategy should be well planned, and sufficient resources should be allocated for the planning and recruitment process itself. The strategy should include plans on how to ensure the publicity of the study, what are the format and timing of contact attempts, what kind of supporting materials are needed during the recruitment, and by whom and when they are prepared. The use of incentives (financial or gifts) has been found to increase participation rates in many studies and could be considered if nationally allowed (Rao, 2020; Castiglioni et al., 2008). However, incentives may also introduce selection bias if some specific population groups, such as more deprived people, are more prone to participate due to offered incentives. However, this varies considerably between countries. In some countries, the use of incentives in population health surveys is not allowed by ethics committees/national legislation. Therefore, other formats of promotion should be considered. In this regard, personal study results can also be used as an incentive especially when there is a clear interpretation of the results such as BMI for obesity, blood pressure levels for elevated blood pressure, or in HBM module the interpretation of the analytical results in accordance with available health-based guidance values. Procedures for the feedback of study results to the study participants must be clarified in the protocol. This protocol should also include how to deal with cases on incidental findings, including who to involve in eventual medical follow-up.

It is important to raise people's awareness of the study through publicity. How this is done depends on study design and country. If the study is organized as a random sample of the population, announcements in media such as local newspapers and radio, as well as through social media are often good choices. Also providing posters about the study to public places such as health care centres, libraries, and community houses etc. could be used. Nowadays, the internet and social media are increasingly used for publicity and are valuable, low-cost tools for promotion. The survey should have its own website, and in some countries, communities/cities have their own Facebook/Twitter accounts which could be used to distribute information about the survey.

The first personal contact can be by mail, telephone or as a personal visit depending on the country. It is important that the invitee receives all relevant information to make an informed decision about participation. This usually means that an invitation letter together with an information leaflet is provided for the invitee. There must also be a possibility to ask for further information for example by phone. In some countries, the informed consent form is sent together with the information leaflet allowing invitees to read it at home before attending the survey visit. If a person cannot be reached by the first contact or he/she is reluctant to participate but does not refuse explicitly, re-contacts should be made. The number, format, and timing of the re-contact attempts depend on the survey and available resources. Sometimes also national regulations/legislation or ethics committees may limit the possible number and format of re-contacts.

3.8. Data access and reporting

As combined health and HBM surveys are often conducted in collaboration with several institutes, it will be important to agree on data ownership and use well in advance. Having a clear agreement of data ownership, possible embargo periods after data have been collected, when and how results can be published, and by whom and how data can be accessed for further research is important in order to avoid unnecessary delays in data use after data collection has ended.

4. Discussion

Non-communicable diseases (NCDs) such as cardiovascular disease and cancer cause a major health burden worldwide. In the EU, it has been estimated that about one third of the adult population aged 15 and over live with a NCD (OECD, 2016). Traditionally, NCD risk factors include metabolic syndrome including raised blood pressure,

overweight and obesity, high blood glucose level and high cholesterol levels together with lifestyle risk factors of smoking and heavy alcohol use, and socio-economic and socio-demographic position. These have been monitored through health examination surveys over the past decades. More recently, the scientific understanding of the health impact of environmental risk factors including air pollution, noise and chemicals has increased, and are today considered as plausible and most likely an important contributing factor to the observed increase in a range of NCDs (Pruss-Ustun et al., 2019; Chowdhury et al., 2018).

Obtaining holistic information about health and health determinants of the population should therefore also include data on environmental risk factors of health. In line with this, it is important to be able to monitor the evolution of exposure levels of the general population to support sound chemical regulation and health-related mitigation measures. HBM studies are seen as the "gold standard" for the measurement of environmental exposures of humans (Sexton et al., 2004).

Having extensive information on health, biological risk factors, lifestyles, socio-demographic characteristics, as well as environmental risk factors on the same individuals would generate a more complete database which can foster wide scientific research potentials. Moreover, when a wide range of biomarkers including biomarkers of effects are analysed in combined health and HBM surveys, the assessment of the association between exposure to chemicals and health effects could be more easily conducted. Through research on this extensive database, we could support policymaking in several fields such as public health including allocation of health care resources and setting up public health prevention measures, and chemical regulations. When some of the collected biological samples are stored in biobanks for future use with related informed consents allowing secondary use of these samples, samples could be used to answer many additional medical/clinical and health-related questions.

Examples from Germany (Kolossa-Gehring et al., 2007), France (Balicco et al., 2017), Israel (Berman et al., 2017), the USA (Centers for Disease Control and Prevention (CDC), 2021) and Canada (St-Amand et al., 2014) demonstrate that it is possible to combine health surveys and HBM studies at the national level. Introducing the HBM module as part of general health surveys has been discussed and considered in many European countries (Tolonen et al., 2018, 2021). In health surveys, biological samples are already commonly collected which would, in theory, make it easier to extend them to integrate the HBM module. In Europe, there are also a lot of health surveys (national, regional or targeted/disease specific) which have collected and stored biological samples for future use. Often, in these surveys, ethical approval already allows the measurement of environmental biomarkers/combounds from collected samples (Tolonen et al., 2018).

Even though combining health and HBM modules into one survey requires a lot of work and prioritization of identified needs for both domains, there are several identified advantages in this (Tolonen et al., 2018; Paalanen et al., 2019). The combined survey will provide a wide range of detailed data on several domains as well as the possibility to collect a larger sample. When the HBM module is added to the existing health survey, already existing logistical infrastructure can be used for recruitment of participants, collection of information through questionnaires and health measurements, and collection and handling of biological samples. The combined survey also benefits from joint public health relations for promotion of the survey during the fieldwork. All these contribute to the reduced cost of the surveys.

To ensure that this potential for a growing number of combined health and HBM surveys in Europe is best used for multinational research projects and monitoring purposes, it is essential to support standardization of used survey methods. These guidelines would be the first step towards standardized procedures to ensure comparability of surveys between different countries. The guidelines presented here are prepared in focus for national surveys but can well be implemented in regional or targeted surveys as well with small modifications for the target population, sample size, and possibly for included measurements as well.

5. Conclusions

Due to the similar logistic infrastructure required for both health surveys and HBM studies, combining them is feasible and cost-effective. The combination will provide a wider information base for more holistic analysis of human health and its determinants, including the effects of environmental substances.

Declaration of competing interest

The authors declare no conflict of interest.

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Abbreviations

BMI – Body mass index
EHES – European Health Examination Survey
EHIS – European Health Interview Survey
EU – European Union
HbA_{1c} – Glycated haemoglobin
HBM – Human biomonitoring
HBM4EU – European Human Biomonitoring Initiative
HDL – High density lipoprotein
HES – Health Examination Survey
HIS – Health Interview Survey
NCD – Non-communicable disease
RCT – Randomized Control Trial
SOP – Standardized Operating Procedure
STEPs – STEPwise Approach to NCD Risk Factor Surveillance
WHO – World Health Organization

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