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Acute cardiovascular responses of wildland firefighters to working at prescribed burn

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ABSTRACT

Wildland firefighters at prescribed burns are exposed to elevated levels of wildland fire smoke (WFS) while performing physically demanding tasks. WFS exposure has been linked to increases in hospital and emergency admissions for cardiovascular disorders in the general population. However, knowledge about the cardiovascular effect of occupational WFS exposure among wildland firefighters is limited. To provide a better understanding of the effect of this exposure scenario on acute hemodynamic responses, resting systolic/diastolic blood pressure (SBP/DBP) and heart rate (HR) of wildland firefighters were measured before (pre-shift), after (post-shift), and the morning (next morning) immediately following prescribed burn shifts (burn days) and regular work shifts (non-burn days). A total of 38 firefighters (34 males and 4 females) participated in this study and resting BP and HR were recorded on 9 burn days and 7 non-burn days. On burn days, HR significantly increased from pre-to post-shift (13.25 bpm, 95% CI: 7.47 to 19.02 bpm) while SBP significantly decreased in the morning following the prescribed burns compared to pre-shift (−6.25 mmHg, 95% CI: −12.30 to −0.20 mmHg). However, this was due to the decrease of SBP in the firefighters who were hypertensive (−8.46 mmHg, 95% CI: −16.08 to −0.84 mmHg). Significant cross-shift reductions (post-shift/next morning vs. pre-shift) were observed in SBP on burn days compared to non-burn days (−7.01 mmHg, 95% CI: −10.94 to −3.09 mmHg and −8.64 mmHg, 95% CI: −13.81 to −3.47 mmHg, respectively). A significant reduction on burn days was also observed from pre-shift to the following morning for HR compared to non-burn days (−7.28 bpm, 95% CI: −13.50 to −1.06 bpm) while HR significantly increased in pre-to post-shift on burn days compared to non-burn days (10.61 bpm, 95% CI: 5.05 to 16.17 bpm). The decreased BP observed in wildland firefighters might be due to a high level of carbon monoxide exposure and exercise-induced hypotension. The increase in HR immediately after prescribed burns might be attributable to WFS exposure and physical exertion in prescribed burn shifts. The results suggest that wildland firefighting exposure might cause a distinct hemodynamic response, including SBP reduction and HR increment, especially for those who have pre-existing hypertension.

1. Introduction

Millions of acres of forest lands are burned by tens of thousands of wildfires each year (NIFC, 2020), and release an enormous amount of wildland fire smoke (WFS) into the ambient air. In the United States, WFS has increasingly become an important source of air pollutants, especially particulate matter with aerodynamic diameter ≤ 2.5 μm ($PM_{2.5}$) and carbon monoxide (CO). The United State Environmental Protection Agency reported that the contributions of these two pollutants by wildfire events to the total national emissions have increased from ~16 to ~30% over the last decade (USEPA, 2019). Therefore, exposure to air pollutants from WFS emissions can cause substantial impact to public and occupational health.

Wildland firefighters, who are primarily responsible for wildfire suppression and prescribed burning, are more directly and frequently exposed to WFS compared to the general population. WFS contains numerous particulate and gaseous phase pollutants with the potential to cause health effects among exposed individuals (Adetona et al., 2016; Naeher et al., 2007). Positive associations between wildfire events and hospital or emergency room admissions for cardiorespiratory outcomes have been reported in previous population-based studies (Arbex et al., 2010; Johnston et al., 2007; Rappold et al., 2011). Acute respiratory

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symptoms and decline in lung function have also been observed in wildland firefighters following WFS exposures (Adetona et al., 2011b; Gaughan et al., 2008; Swiston et al., 2008; Wu et al., 2019). However, knowledge about potential cardiovascular responses as a result of WFS exposure in this occupational group is rather limited (Navarro et al., 2019). Positive associations between wildland firefighting career length and self-reported hypertension and heart arrhythmia were observed in a recent cross-sectional survey (Semmens et al., 2016).

Moreover, exposure to ambient air pollutants has been linked to increased risks of many cardiovascular diseases, and elevation in blood pressure (BP) is among the physiological responses that are triggered by the exposure and that have been proposed as underlying mechanisms (Paolo et al., 2016; Rajagopalan et al., 2018). Smoke emission from burning solid fuels (e.g., wood, charcoal, and crop residues) is reported to be associated with increased BP (Baumgartner et al., 2011, 2018; Norris et al., 2016; Quinn et al., 2017; Young et al., 2019). In stove intervention studies, research participants had a lower BP level after receiving a clean or improved cookstove intervention to reduce smoke exposure from cooking with biomass fuels (Alexander et al., 2015; Clark et al., 2013; Quinn et al., 2017). The acute effect of cookstove smoke exposure on BP was also demonstrated in a controlled human exposure experiment (Fedak et al., 2019), though other studies did not find similar effects (Hunter et al., 2014; Unosson et al., 2013).

In addition to BP elevation, increased resting heart rate (HR) has also been suggested as an indication of cardiovascular strain in response to exposure to air pollutants (Brook et al., 2014; Morishita et al., 2015). Significant increase in HR among people who were exposed to woodsmoke compared to those who inhaled filtered air was observed in a controlled human exposure study (Unosson et al., 2013). In a firefighting simulation study, the HR of firefighters increased significantly after exposure to artificial smoke while performing firefighting activities in a smoke-diving room (Hemmatjo et al., 2018). A causal association between cigarette smoking and higher resting HR has also been reported in a meta-analysis study (Linneberg et al., 2015).

Results of the abovementioned studies, along with the toxicological properties of woodsmoke (Naeher et al., 2007), raises concerns about the impact of WFS exposure on the cardiovascular health among wildland firefighters (Adetona et al., 2016; Navarro et al., 2019). During wildland fire events, their personal exposure concentrations of $PM_{2.5}$ in WFS is often at least an order of magnitude higher than the 24-hr US National Ambient Air Quality Standard (NAAQS) (35 μg/m^3) (Adetona et al., 2011a; Reinhardt and Ottmar, 2004). As wildland firefighters work prolonged shifts while performing physically demanding tasks and without appropriate respiratory protection, their cardiovascular health might be impaired by such exposures. Between 2007 and 2016, heart attack was the leading cause of on-the-job deaths among wildland firefighters in the United States accounting for 24% of the total number (NWCG, 2017). A recent risk assessment also concluded that wildland firefighters have an increased risk of cardiovascular mortality due to WFS exposure (Navarro et al., 2019).

Given the elevated levels of WFS exposure experienced by wildland firefighters and the scientific evidence suggesting its adverse cardiovascular effects, we hypothesize that WFS exposure induces an acute subclinical change in hemodynamic function among the firefighters. In this study, BP and HR of wildland firefighters were measured before, after, and the morning immediately following prescribed burn shifts and regular work shifts. Acute cardiovascular responses were then assessed by studying changes of the hemodynamic parameters across the prescribed burn shifts compared to corresponding changes across regular work shifts.

2. Material and methods

2.1. Study location and wildland firefighters

In this study, a total of 38 wildland firefighters were recruited from the United States Department of Agriculture–Wayne National Forest (WNF) and Ohio Department of Natural Resources–Division of Forestry (ODNR-DF). The demographic information about the wildland firefighters, unadjusted hemodynamic parameter values, and the characteristics of the prescribed burn shifts are provided in Table 1. Each firefighter was briefed on the purpose, design, and procedure of the study. All firefighters were given adequate information to allow them to make a voluntary decision about participation in the study. Informed consent was then obtained from each of them.

A baseline questionnaire was administered to each firefighter at the beginning of participation to obtain information about demography (e.g., age, sex, height, weight), firefighting career (e.g., career length, numbers of past prescribed burns and wildfires), relevant health history (e.g., pre-existing and family history of respiratory, cardiovascular, and metabolic diseases), etc. Two questionnaires were also provided to the firefighters immediately after (work task questionnaire) and the morning immediately following the work shift (morning-after questionnaire) to obtain information about factors (e.g., smoking, second-hand smoke exposure, medication, and wood burning for residential heating) that could confound the association between WFS exposures and cardiovascular effects. No firefighter reported using any blood pressure medication, while two firefighters reported being smokers. This study was reviewed and approved by The Ohio State University Institutional Review Board (2017H0075).

2.2. BP and HR measurements and WFS exposure assessment

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured before (pre-shift, approximately at 8–10am), at least 20 min after (post-shift, roughly at 4–6pm), and the morning (next morning between 8 and 10am and about 16 h after the post-shift measurements) immediately following prescribed burn shifts (burn days) and regular work shifts (non-burn days). Resting BP and HR were measured simultaneously using the Welch Allyn Spot Vital Signs monitor (Welch Allyn, Skaneateles Falls, NY) either in the forest area or at the office. Before the measurements, firefighters were allowed to rest for at least 5 min and sit in a chair with their backs supported. Their legs were uncrossed, and their feet were held flat on the floor. The measurement was taken in accordance with the American Heart Association (AHA) guideline, and cuff size was determined based on AHA recommendation following the "small adult" (22–26 cm), "adult" (27–34 cm), "large adult" (35–44 cm)

Table 1
General characteristics of wildland firefighters, unadjusted hemodynamic parameters, and prescribed burn shifts.

Characteristics	Mean ± SD or[b]	
Wildland firefighters (N = 38)		
Age (yr)	35.63 ± 9.31	
Gender	Male: 34, Female: 4	
BMI (kg/m^2)	27.91 ± 5.04	
Career length (yr)	8.94 ± 7.88	
Current Smoker	Yes: 2, No: 36	
Hemodynamic parameters	*Burn days*	*Nonburn days*
SBP (mmHg)	133.56 ± 17.42	125.22 ± 12.60
DBP (mmHg)	80.82 ± 9.87	78.71 ± 7.89
HR (bpm)	73.87 ± 14.57	71.39 ± 13.35
Pollutant concentrations on burn days[a]		
$PM_{2.5}$ (mg/m^3)	1.43 ± 0.13	
CO (ppm)	7.02 ± 0.69	
BC (μg/m^3)	58.79 ± 5.46	
Prescribed burns[a]		
Work shift duration (hr)	5.09 ± 1.57	
Size of burned area (acre)	308.88 ± 193.13	
Averages of weather parameters at burns[a,b]		
Ambient temperature (°F)	84.17 ± 6.81	
Relative humidity (%)	26.16 ± 6.45	

[a] These measures were only recorded on burn days.
[b] These were measured by the real-time sensors on the aerosol monitors.

and "adult thigh" (45–52 cm) categories of the arm circumference (Pickering et al., 2005).

Wildland firefighters who participated in this study provided at least one measurement on burn or non-burn days. Some firefighters were represented on up to 6 and 4 burn and non-burn days, respectively. The number of firefighters who had at least one set of completed measurement is 26 on burn days (55 person-days) and 11 on non-burn days (11 person-days). Ten firefighters (31 person-days) had at least one complete set of samples on both burn and non-burn days. No significant differences were observed in age, BMI, and career length between firefighters who had at least one complete set and those who did not. In addition, the results of statistical models using data with and without firefighters with incomplete BP and HR measurement are similar.

Personal exposure to $PM_{2.5}$ during prescribed burns was measured in the breathing zone of the wildland firefighters using the lightweight MicroPEM aerosol sensor (RTI International, Research Triangle Park, NC). PAC7000 single gas detector (Draeger, PA) was also carried by the firefighters in the breathing zone to measure time-integrated CO concentrations in WFS emissions. Both monitors were calibrated in accordance with the manufacturer's instructions before and after each prescribed burn day. The concentration of black carbon (BC) in WFS particulates was determined using a SootScan™ Model OT21 Optical Transmissometer (Magee Scientific, Berkeley, CA). The concentrations of air pollutants in WFS are presented in Table 1.

2.3. Statistics

BP and HR at the time of measurement (pre-shift, post-shift, next morning) and according to the types of workday (burn days or non-burn days) were determined using linear mixed effect model (LMM) while controlling for firefighting career length, smoking status, and body mass index (BMI). These covariates could influence the hemodynamic responses and were associated with BP and HR among the study participants. To account for within-subject correlation in the repeated measurements collected per wildland firefighter, participant ID and the date of measurement were included as random effect variables in the model. The normality of the data was verified using the goodness of fit test (Shapiro-Wilk and Kolmogorov-Smirnov tests) and the appropriateness of the LMM was confirmed from the residual plot.

Cross-shift changes (post-shift or next morning vs. pre-shift) in BP and HR were assessed using LMM while controlling for previously mentioned covariates. Adjusted *p*-values based on the Tukey's HSD multiple comparison tests were used to determine if the cross-shift changes were significant. Confidence intervals were also constructed using Tukey's HSD tests. The differences in the cross-shift changes in BP and HR between burn and non-burn days were tested using the same model.

According to the BP thresholds defined by AHA, firefighters were classified into the normotensive/elevated BP (SBP $<$ 130 mmHg and DBP 80 $<$ mmHg; N = 16 [N = 7 normotensive; N = 9 elevated BP]) or the hypertension (SBP $\geq$ 130 mmHg or DBP 80 $\geq$ mmHg; N = 22) group. The classification of the firefighter participants into BP categories is based on the averages of multiple pre-shift measurements as was done in prior studies (Burkard et al., 2018; O'Neal et al., 2015; Sharman et al., 2015). An alternative classification is based on the consistency of pre-shift measurements with placement of the firefighters in the category within which greater than 50% of their multiple pre-shift measurements fell. Results of analyses using the alternative criterion are similar to those that are from models using the classification based on average BP measurements with 92% of the firefighter participants remaining in the same categories. The cross-shift changes in both groups are analyzed using the LMMs previously described. All the analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC) and a *p*-value less than 0.05 was considered significant.

3. Results

BP and HR according to the measurement time and adjusted for career length, smoking status, and BMI are presented in Table 2. Cross-shift changes in BP and HR of wildland firefighters working on burn and non-burn days are shown in Fig. 1. Overall, SBP significantly decreased in the morning following the prescribed burn shift compared to pre-shift while HR significantly increased from pre-shift to post-shift on burn days. No significant cross-shift changes of BP or HR were observed on non-burn days. For normotensive and elevated BP firefighters, HR significantly increased from pre-to post-shift without any cross-shift changes in BP on prescribed burn days. Contrarily, SBP significantly decreased from pre-shift to the next morning in the hypertensive group while HR significantly increased from pre-to post-shift on burn days.

The differences in cross-shift changes in BP and HR between burn and non-burn days are presented in Fig. 2. With all participants included, SBP from pre-shift to either post-shift or the next morning was significantly reduced on burn days compared to non-burn days. A similar trend was observed for the change in HR from pre-shift to the next morning. However, pre-to post-shift change in HR was significantly higher on burn days than on non-burn days. When the wildland firefighters are categorized, a significant increase of HR from pre-to post-shift but no other difference in cross-shift changes was observed on burn days compared to non-burn days in normotensive and elevated BP firefighters. Among the hypertensive firefighters, SBP significantly declined across the work shift (pre-to post-shift or the next morning), and HR increased from pre-to post-shift but declined from pre-shift to next morning on burn days relative to non-burn days.

4. Discussion

In contrast to a relatively large number of studies investigating the effects of WFS exposure on acute pulmonary and respiratory responses in wildland firefighters, there is very limited information about the acute cardiovascular effects of such exposure in this population. As wildland firefighters are more directly exposed to WFS across extended periods during wildfire suppression and prescribed burn activities, they might have a distinct cardiovascular response to WFS exposure compared with the general population. Therefore, we initiated this pilot study to investigate acute hemodynamic responses to WFS exposure by measuring changes in BP and HR among the firefighters before and following work at prescribed burns.

The decreases in BP that were observed in this study (~1–2 mmHg immediately after and ~3–6 mmHg the morning following prescribed burn shifts) are similar to those reported in previous controlled human exposure studies. Small decreases in both SBP and DBP (~1–2 mmHg) were observed between 10 and 40 min after 1-h woodsmoke exposure (~1 mg/m^3 PM_1) with moderate exercise among 16 healthy firefighters; their BP further decreased non-significantly (~5 mmHg) at 24 h after the exposure (Hunter et al., 2014). In another controlled exposure study, SBP was ~2–5 mmHg lower at 10–60 min after 3-h woodsmoke exposure (mean PM_1 of 314 μg/m^3) with intermittent exercise and DBP was consistently ~1–3 mmHg lower until 50 min after the exposure was terminated compared to the pre-exposure measurements (Unosson et al., 2013). In a recent study, SBP was 0.2–2.3 mmHg lower when measured 30 min after a 2-h exposure to woodsmoke ($PM_{2.5}$ from 10 to 500 μg/m^3) but subsequently was 2–3 mmHg higher 24 h following the exposure (Fedak et al., 2019).

The decreased BP observed in wildland firefighters might partially be attributable to the time of measurement. In previous studies of controlled exposure to diesel exhaust particulates or concentrated ambient particulates, both systolic and diastolic pressures increased consistently during a 2-h exposure period but decreased immediately after the cessation of exposure (Byrd et al., 2016; Cosselman et al., 2012). BP measurement was performed at ~20 min and ~16 h after completion of prescribed burns due to logistical considerations for

Table 2
Blood pressure and heart rate (estimate with 95% confidence interval) of wildland firefighters (N; N: # of unique firefighters; person-days) by the time of measurement (pre-shift, post-shift, or next morning), types of workdays (burn or non-burn days), and blood pressure categories (normal/elevated or hypertensive).

Overall	N; N (# of unique participants; # of person-days)	SBP (mmHg)	DBP (mmHg)	HR (bpm)
		Est. (95% CI)	Est. (95% CI)	Est. (95% CI)
Burn days				
Pre-shift	33; 71	133.47 (130.13–136.81)	80.67 (78.59–82.76)	73.27 (69.86–79.68)
Post-shift	33; 71	131.69 (128.35–135.03)	78.05 (75.96–80.14)	86.52 (83.10–89.93)
Next morning	27; 55	127.22 (123.44–131.01)	77.09 (74.73–79.46)	73.13 (69.26–76.99)
Non-burn days				
Pre-shift	24; 41	124.93 (121.94–127.93)	78.63 (76.08–81.19)	71.48 (67.64–75.31)
Post-shift	22; 33	129.45 (126.11–132.79)	74.53 (71.68–77.38)	74.41 (70.13–78.68)
Next morning	14; 14	128.50 (123.36–133.63)	77.04 (72.67–81.41)	78.36 (71.80–84.93)
Normal/Elevated	N; N (# of unique participants; # of person-days)	SBP (mmHg) Est. (95% CI)	DBP (mmHg) Est. (95% CI)	HR (bpm) Est. (95% CI)
Burn days				
Pre-shift	12; 29	120.12 (116.99–123.26)	74.85 (72.38–77.31)	69.34 (65.13–73.55)
Post-shift	12; 29	120.67 (117.54–123.81)	72.81 (70.35–75.27)	85.03 (77.82–86.24)
Next morning	10; 25	117.24 (113.85–120.62)	73.24 (70.58–75.90)	69.65 (65.11–74.20)
Non-burn days				
Pre-shift	12; 20	115.83 (111.75–119.91)	73.61 (70.37–76.85)	69.03 (64.60–73.46)
Post-shift	11; 15	119.74 (115.17–124.32)	69.08 (64.45–72.72)	69.08 (64.11–74.05)
Next morning	6; 6	118.59 (111.15–126.03)	72.41 (66.50–78.32)	76.02 (67.95–84.10)
Hypertensive	N; N (# of unique participants; # of person-days)	SBP (mmHg) Est. (95% CI)	DBP (mmHg) Est. (95% CI)	HR (bpm) Est. (95% CI)
Burn days				
Pre-shift	21; 42	143.45 (139.35–147.55)	85.10 (82.18–88.02)	76.40 (72.19–80.62)
Post-shift	21; 42	139.98 (135.88–144.08)	82.05 (79.13–84.97)	90.05 (85.84–94.27)
Next morning	17; 30	134.99 (130.16–139.83)	79.86 (76.42–83.30)	75.34 (70.37–80.31)
Non-burn days				
Pre-shift	12; 21	133.60 (129.58–137.63)	83.46 (79.57–87.36)	74.00 (68.42–79.57)
Post-shift	11; 18	138.55 (134.10–143.01)	79.48 (75.17–83.79)	78.50 (72.32–84.68)
Next morning	8; 8	135.99 (129.38–142.60)	80.76 (74.36–87.15)	81.56 (72.41–90.72)

*The three measures (DBP, SBP, and HR) were collected in each measurement instance.

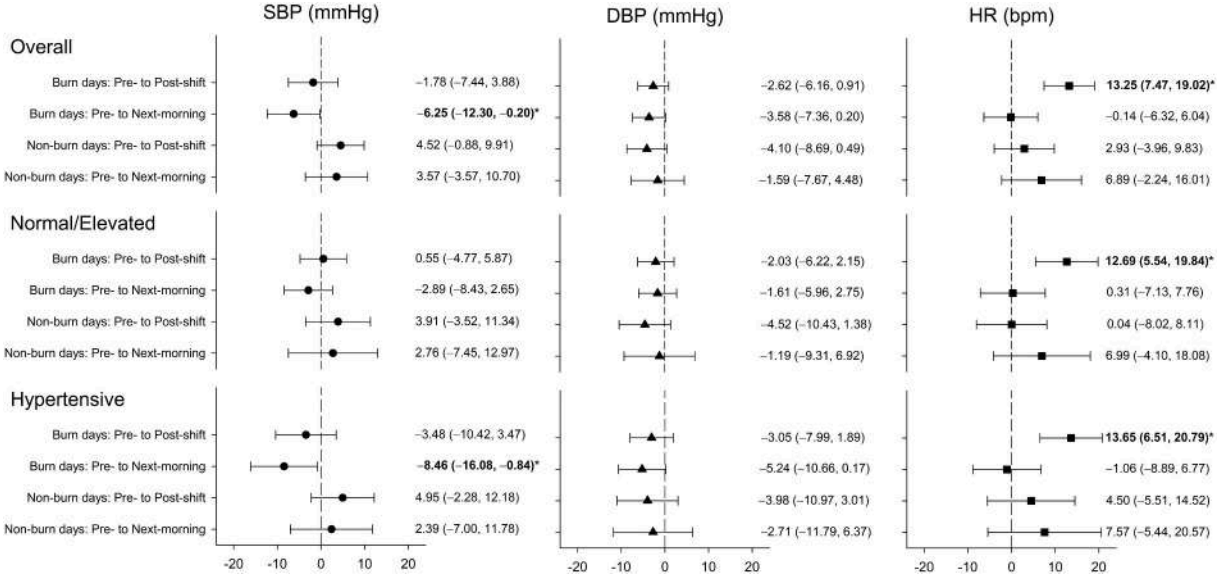

Fig. 1. Cross-shift changes (from pre-shift to post-shift or next morning) of blood pressure and heart rate on burn and non-burn days.

research personnel and less interruption to the work and routine of the firefighters. Therefore, the timing of increased BP increases due to wildland firefighting, if any, might be missed because of the choice of measurement time-points.

Additionally, it is possible that the dilatory and BP-lowering effects of other exposures at the fireline counteract the expected BP-raising effect of WFS-associated PM exposure (Cunha et al., 2020; Giorgini et al., 2015; Halliwill, 2001; Halonen et al., 2011; Wu et al., 2015). For example, post-exercise hypotension often occurs after dynamic exercise/training and the magnitude of BP reduction is associated with exercise intensity, exertion duration, and hypertension (Cornelissen and Smart, 2013; Eicher et al., 2010; Gomes Anunciação and Doederlein Polito, 2011; Halliwill, 2001; Rezk et al., 2006).

Wildland firefighters usually undertake rigorous fire tasks during prescribed burns. On a typical prescribed burn day, they are engaged in multiple physically demanding activities including burn preparation,

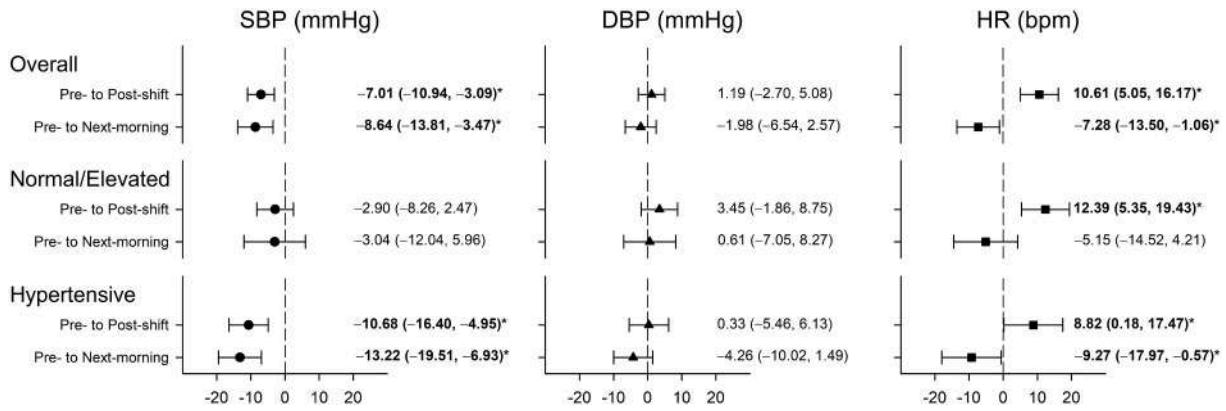

Fig. 2. Comparison of cross-shift changes (from pre-shift to post-shift or next morning) in blood pressure and heart rate between burn and non-burn days.

lighting of fires, and the maintenance of fires within predetermined burn areas. Apart from hand tools, each firefighter also carries a backpack that weighs ~45-pound for most of the work shift (5.09 ± 1.57 h and 308.88 ± 193.13 acres of burned area, Table 1). On the contrary, wildland firefighters in this study usually worked in the office (e.g., attended meetings or annual fire refresher) during a regular 8-h non-burn day work shift.

As previous studies demonstrate about the association between BP reduction level and hypertension (Cornelissen and Smart, 2013; Halliwill, 2001), we hypothesize that the observed hypotension effect in this study was particularly apparent because of the predominant number of pre-hypertensive and hypertensive firefighters (58%) among the participants. This inference is supported by the result of cross-shift changes according to the different BP categories, showing that a significant decrease in BP was observed in hypertensive but not normotensive or elevated BP firefighters (Figs. 1 and 2).

Moreover, ambient temperature is inversely associated with BP (Giorgini et al., 2015; Halonen et al., 2011; Wu et al., 2015), and exercising in hot environments, such as would be experienced by wildland firefighters at prescribed burns (84.17 ± 6.81 °F in this study, Table 1), can exaggerate the acute hypotensive effect of exercise (Cunha et al., 2020; Halliwill, 2001). In a previous study investigating post-exercise hypotension among 7 men with elevated blood pressure (127.4/83.7 mmHg), SBP measured following two bouts of cycling exercise in hot environment (35 °C) was consistently lower (−0.3 to −4.7 mmHg) compared to measurements following the same exercise regime in normal environment (21 °C) across a 21-hr recovery period (Cunha et al., 2020). The authors of this study suggested that the post-exercise hypotension could be further aggravated by vasodilatory effect in response to heat load after the exercise (Cunha et al., 2020).

It should be noted that the observed acute lowering of BP may be harmful. Hypotension and BP below a nadir in treated hypertensives, especially with the presence of coronary artery disease, are associated with myocardial ischemic events (Divisón-Garrote et al., 2016; Divisón-Garrote et al., 2020; Messerli et al., 2006; Messerli and Panjrath, 2009; Owens and O'Brien, 1999). Furthermore, previous systemic reviews conclude that decrease in SBP during exercise stress testing is associated with increased risks of multiple cardiovascular events (Barlow et al., 2014; Schultz et al., 2017). Incidentally, myocardial infarction is the leading cause of non-accident on-the-job fatality among wildland firefighters, and exposure to PM, such as is contained in WFS, is associated with subclinical coronary artery disease (Butler et al., 2017; Jilani et al., 2020; NWCG, 2017).

Unlike $PM_{2.5}$ and BC that are commonly linked to BP increase, CO exposure seems to have a relaxation effect on blood vessels (Penney and Howley, 1991; Rezk-Hanna et al., 2019; Stec et al., 2008). Following exposure, CO interacts with soluble guanyl cyclase (sGC) and subsequently results in cyclic guanosine 3′-5′ monophosphate (cGMP) increase, leading to a vasodilatory effect (Stec et al., 2008). In previous controlled exposure studies, non-significant decreases in BP were observed following exposure to woodsmoke which contained 16 ppm of CO for 1 h or 25 ppm of CO for 3 h (Hunter et al., 2014; Unosson et al., 2013). BP was similarly reduced following 45-min in-vehicle exposures to CO (30.2–72.4 ppm) compared to when there was no CO exposure (0 ppm) (Lee et al., 2017). Personal CO exposure concentration across prescribed burns was 7.02 ± 0.69 ppm in this study (Table 1). However, actual exposure (inhaled amount) may be higher in the firefighters than for participants in the previous studies due to the increased breathing rates of firefighters while working at wildland fires that may rise up to 5–8 times above the resting rate (Navarro et al., 2019). While lower SBP was primarily observed among the hypertensive firefighters up to ~16 h following exposure to WFS, we were unable to determine from prior studies whether people with elevated BP or hypertension are more sensitive to this effect, and whether the vasodilatory and BP-lowering effects of CO exposure last beyond the first few hours of exposure.

Contrary to the BP results, HR significantly increased by ~13 bpm in post-shift and subsequently returned to pre-shift levels in the mornings following prescribed burn days (Fig. 1). The pre-to post-shift change in HR on burn days was ~10 bpm higher compared to the changes on non-burn days (Fig. 2). Similar observations were also made in prior studies of controlled exposure to woodsmoke. Resting HR increased ~1–2 bpm within 30 min after a 1-h woodsmoke exposure (~1 mg/m^3 PM_1) with intermittent exercise (Hunter et al., 2014). A significant increase in HR from ~2 to 6 bpm at 1-h post-exposure following 3-h exposure to woodsmoke (~300 μg/m^3 PM_1) that did not occur following exposure to filtered air was observed in another study (Unosson et al., 2013).

Interestingly, the magnitude of the increase in HR observed in this study was much higher than what was observed in the other studies that are referenced. This could partly be due to the relatively prolonged exposure of the wildland firefighters to elevated level of WFS exposure at prescribed burn shifts ($PM_{2.5}$: 1.43 ± 0.13 mg/m^3 and BC: 58.79 ± 5.46 μg/m^3, Table 1). Detail of exposure assessment for wildland firefighters in this study has been reported elsewhere (Wu et al., 2021). Furthermore, the average duration of prescribed burn shifts was about 2–5 times longer than the duration in the controlled exposure studies (Hunter et al., 2014; Unosson et al., 2013).

Nonetheless, it should be noted that wildland firefighters were usually involved in more strenuous activities compared to the moderate exercise (on a bicycle ergometer with rest at 15-min intervals) performed in the controlled exposure studies (Hunter et al., 2014; Unosson et al., 2013). A recent firefighting simulation study also showed that structural firefighters' HR significantly increased from ~70 to ~160 bpm at the end of firefighting operation in a smoke-diving room (Hemmatjo et al., 2018). Since exercise intensity is also associated with HR increase (Eicher et al., 2010; Rezk et al., 2006), the elevated HR observed in wildland firefighters in this study might in part be due to

their physical exertion during prescribed burn shifts.

No association between air pollutants ($PM_{2.5}$, CO, and BC) in WFS emissions and cross-shift changes in BP or HR was observed in this study (data not shown). Nevertheless, a significant exposure-response was often observed in prior studies investigating the effect of biomass smoke exposure in an indoor environment (Baumgartner et al., 2011, 2018; Norris et al., 2016; Quinn et al., 2017; Young et al., 2019). The discrepancy between our results and those of others might be because the exposure-response associations in our study is obscured by a divergence between the external concentration and the dose of WFS exposure.

Several limitations should be noted when the result of this study is interpretated. First, there was no direct measurement of physical activity level. However, it was observed that firefighters were involved in more arduous tasks during prescribed burns whereas they typically worked in the office (e.g., attended meetings or annual firefighting refresher trainings) on a non-burn day. Secondly, schedule and preparation for a prescribed burn activity is often uncertain due to the dependence of its conduct on suitable weather conditions. Consequently, wildland firefighters were selected into this study by convenience sampling method rather than through randomization process.

In addition, the measurements in the morning following the work shifts were more difficult to obtain from the firefighters. Sixteen and 28 next morning measurements were missed on burn and non-burn days, respectively. To address concerns due to incomplete data, LMMs including only complete sets of measurements on burn and non-burn days were also used to evaluate acute cardiovascular responses. Therefore, any person-day with missing measurement points (before, after, and/or the next morning) were excluded in this alternate set of analyses. The results of analysis are similar to the findings presented in the table and figures (data not shown).

5. Conclusions

Reductions in SBP and increases in HR were observed in wildland firefighters immediately after and the morning following work at prescribed burns, presumably due to multiple fireline-related factors (e.g., WFS exposure, arduous tasks, and hot environment). Wildland firefighters might have a distinct cardiovascular response, especially in those who are hypertensive, following WFS exposure during prescribed burn shifts.

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Declaration of competing interest

The authors of this paper declare no conflict of interest.

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Alterations to the urinary metabolome following semi-controlled short exposures to ultrafine particles at a major airport

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ABSTRACT

Background: Inflammation, oxidative stress and reduced cardiopulmonary function following exposure to ultrafine particles (UFP) from airports has been reported but the biological pathways underlying these toxicological endpoints remain to be explored. Urinary metabolomics offers a robust method by which changes in cellular pathway activity can be characterised following environmental exposures.
Objective: We assessed the impact of short-term exposures to UFP from different sources at a major airport on the human urinary metabolome.
Methods: 21 healthy, non-smoking volunteers (aged 19–27 years) were repeatedly (2–5 visits) exposed for 5h to ambient air at Amsterdam Airport Schiphol, while performing intermittent, moderate exercise. Pre- to-post exposure changes in urinary metabolite concentrations were assessed via ^{1}H NMR spectroscopy and related to total and source-specific particle number concentrations (PNC) using linear mixed effects models.
Results: Total PNC at the exposure site was on average, 53,500 particles/cm^{3} (range 10,500–173,200) and associated with significant reductions in urinary taurine (−0.262 AU, 95% CI: −0.507 to −0.020) and dimethylamine concentrations (−0.021 AU, 95% CI: −0.040 to −0.067). Aviation UFP exposure accounted for these changes, with the reductions in taurine and dimethylamine associating with UFP produced during both aircraft landing and take-off. Significant reductions in pyroglutamate concentration were also associated with aviation UFP specifically, (−0.005 AU, 95% CI: −0.010 – <0.000) again, with contributions from both landing and take-off UFP exposure. While non-aviation UFPs induced small changes to the urinary metabolome, their effects did not significantly impact the overall response to airport UFP exposure.
Discussion: Following short-term exposures at a major airport, aviation-related UFP caused the greatest changes to the urinary metabolome. These were consistent with a heightened antioxidant response and altered nitric oxide synthesis. Although some of these responses could be adaptive, they appeared after short-term exposures in healthy adults. Further study is required to determine whether long-term exposures induce injurious effects.

1. Introduction

Global air transport has grown strongly over the past decades (Lee

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et al., 2009). In 2019, scheduled passenger numbers reached more than 4.5 billion and 61.3 million tonnes of cargo were transported by plane

secondary organs within hours of pulmonary exposure (Kreyling et al., 2002; Oberdörster et al., 2004; Brown et al., 2002).

Abbreviations

^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
ADMA	Asymmetric dimethylarginine
AU	Arbitrary unit
BC	Black carbon
CI	Confidence interval
CO	Carbon monoxide
CPC	Condensation particle counter
DDAH	Dimethylarginine dimethylaminohydrolase
DEP	Diesel exhaust particle
DMNA	Dimethylnitrosamine
FeNO	Fractional exhaled nitric oxide
FEV_1	Forced expiratory volume
FVC	Forced vital capacity
HOCl	Hypochlorous acid
HSQC	Heteronuclear single quantum coherence
LAX	Los angeles international airport
NO	Nitric oxide
NOS	Nitric oxide synthases
NO_x	Nitrogen oxides
Nrf2	Nuclear factor erythroid 2-related factor 2
PEF	Peak expiratory flow
PM	Particulate matter
PM2.5	PM2.5 fine particles (0.1–2.5 μm)
PM10	PM10 course particles 2.5–10 μm
PMF	Positive matrix factorization
PNC	Particle number concentrations
PPM	Parts per million
PURGE	Pre-saturation utilising relaxation gradients and echoes
REFs	Road traffic particulates
ROS	Reactive oxygen species
SEM	The standard error of the mean
SMPS	Scanning mobility particle sizer
SO_x	Sulphur oxides
STOCSY	Statistical total correlation spectroscopy
TBEs	Tertiary-butyl ethers
TMAO	Trimethylamine-N-Oxide
TOCSY	Total correlation spectroscopy
TSP	Trimethylsilylpropanoic acid
UFP	Ultrafine particles
VOCs	Volatile organic compounds

(International Air Transpo, 2020). Encouraged by our developing understanding of road emissions toxicity, concern has developed over the impacts that aviation and other airport emissions could have on human health. In addition to manoeuvring aircraft, auxiliary power units, ground service equipment and ground access vehicles are strong sources of nitrogen oxides (NO_x), carbon monoxide (CO), volatile organic compounds (VOCs), sulphur oxides (SO_x) and particulate matter (PM) at airports (Yang et al., 2018; Winther et al., 2015; Pirhadi et al., 2020; Simonetti et al., 2015).

Much of airport-originating PM falls within the ultrafine size range (PM $<$ 0.1 μm) (Pirhadi et al., 2020) and is dominated by particles $<$20 nm in diameter based on the particle number concentrations (PNC) counts. This fraction is apportioned to primarily aircraft emissions (Pirhadi et al., 2020; Keuken et al., 2015). Concentrations of aviation-related PNCs have been detected at significantly elevated levels as far as 18 km downwind of airports (Keuken et al., 2015; Hudda et al., 2014, 2018; Rivas et al., 2020) affecting both total indoor and outdoor PNCs (Hudda et al., 2014, 2018). Resultantly the number of individuals exposed to aircraft -related emissions far exceeds airport personnel and passengers, extending to residents and workers in the urbanisations that neighbour airports.

Historically, studies of PM toxicity have focused primarily on the adverse impacts of exposure to PM10 and PM2.5 (Park et al., 2018; Gerlofs-Nijland et al., 2007, 2019; Kim et al., 2015; Khan and Strand, 2018). However, expansion of these studies to incorporate traffic-related UFPs has demonstrated the potential for UFPs to elicit greater toxicity (based on mass concentrations) and a higher likelihood to induce systemic effects compared with larger particles of the same composition. This is due to their small diameter, high surface area- to- mass ratio and high number concentration. These properties allow UFPs to adsorb greater quantities of redox-active metals and organic compounds and to deposit efficiently within the alveoli where they generate oxidative stress and inflammation, inhibit antimicrobial mechanisms and rapidly enter the surrounding tissue, avoiding clearance by airway macrophages (Kwon et al., 2020; Geiser et al., 2005; Lundborg et al., 2006; Li et al., 2003). Furthermore, small quantities of UFPs can cross the alveolar-capillary barrier, enter the bloodstream and translocate to

Consistent with these observations from UFPs emitted by road-traffic, aviation-related UFPs have been shown to induce both airway and systemic inflammation *in vivo*. In mice, particles collected from a commercial airport and non-commercial airfield caused dose-dependent infiltration of the airways by neutrophils, lymphocytes and eosinophils during the first 24h of exposure (Bendtsen et al., 2019). Heightened concentrations of pro-inflammatory mediator IL-6 were also observed in the blood of asthmatic adults following a 2-h walk in the high UFP-zone surrounding Los Angeles International Airport (LAX) (Habre et al., 2018). Supported by evidence that exposure to UFP from Amsterdam Airport Schiphol associates with mild reductions in lung and cardiac function (decreased forced vital capacity and prolonged QTc intervals) in healthy young individuals (Lammers et al., 2020). While studies of adverse health effects and UFP exposure in airport workers remain scarce and inconclusive (Merzenich et al., 2021), the adverse effects that we see in these interventional and *in vitro* studies are consistent with those induced by diesel exhaust particles (DEP) (Bendtsen et al., 2021). Together with evidence that ultrafine DEP and aviation UFP have similar physicochemical properties (Bendtsen et al., 2021), these findings confirm the credibility of concern regarding aviation-related UFP exposure and health.

Expanding upon the observations from Amsterdam Airport Schiphol, this study employed untargeted metabolomics to identify response biomarkers that inform identification of potential causal adverse outcome pathways. Metabolomics captures the profile of small molecules that exist within a sample, and has been used to identify changes in cellular activity following exposure to fuel exhausts produced by road vehicles and ships (Oeder et al., 2015; Walker et al., 2019; Surowiec et al., 2016; Brower et al., 2016). Being global in design, these analyses identified components of adverse responses that were not captured previously by hypothesis-driven studies, including fuel- specific differences (Selley et al., 2019). For humans, urine is an especially favourable sample for metabolomic study of environmental exposures. As the primary route of excretion for cellular waste, urine is rich in metabolites and inclusive of pathway dysfunction markers from across the body (Bouatra et al., 2013). As a non-invasive and easily accessible sample, it also lends itself well to studies of large cohorts or repeat sampling.

Employing metabolomic urinalysis, this study explored the hypothesis that UFP from different airport-related sources (aviation, ground service vehicles and feeder highways) induces distinct changes to the urinary metabolome. Aiming to identify mechanistically informative markers of cellular responses to airport UFP exposure, we analysed the metabolic content of urine produced before and after short-term exposures to ambient air at Amsterdam Airport Schiphol, Netherlands.

2. Materials and methods

2.1. Study design and population

The design of this prospective, interventional study is detailed in Fig. 1. Healthy adults (n = 21) underwent 5h exposures to ambient air within a mobile laboratory at Amsterdam Airport Schiphol (Amsterdam, the Netherlands). Two to five repeat visits were made per participant, leaving a minimum of two weeks between visits to enable ablation of biological responses to exposure. Participants were university students, living in Amsterdam, > 2 km away from Schiphol airport and not within 300m away of a highway or road that was trafficked by > 10,000 vehicles per day. Participants were aged between 20 and 23 years, were predominantly female (81%) and had BMIs within healthy range (22.6 kg/m^2 ± 2.4). All participants had normal cardiopulmonary function as determined by measurements of forced expiratory volume (FEV_1), forced vital capacity (FVC), peak expiratory flow (PEF), fractional exhaled nitric oxide (FeNO), blood pressure, heart rate and oxygen saturation (presented previously in Table 2 of Lammers et al. 2020). Each participant provided first morning urine samples the day of exposure and again the next morning, an average of 18h after the end of exposure (minimum 12h, maximum 27h). Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) profiles were acquired for each urine sample to characterise changes in the urinary metabolome that related to UFP exposure at the airport.

2.2. Exposure

Detailed methods for participant exposures are provided by Lammers et al. (2020). Briefly, individuals remained for 5h within a mobile laboratory (14 m^3), situated next to the airside of Amsterdam Airport Schiphol, ~300m away from two runways, ~500m from two highways, 10 km from the city and close to several large car parks. The laboratory was fitted with an airflow system to refresh the flow of ambient air in a uniform manner (~400m (Yang et al., 2018)/h) for the duration of the exposure. Extensive air quality measurements were produced from the flow to characterise exposures for each individual at each visit. This varied due to differences in meteorological conditions (especially wind direction) and in runway use. By considering forecasted weather, we were able to schedule visits to include variation in UFP levels and source contributions (e.g. aviation and road traffic) for each individual. During the exposures, participants cycled at low intensity for 20 min/h and rested for the remainder of the time. Prior to exposures, participants were instructed not to consume alcohol or caffeine (for 24 and 12h respectively) as well as tobacco and non-pharmaceutical drugs for the duration of the study. Food and drinks were provided on the day of exposure to minimise intake of nitrate-rich foods but individual intake was not standardised or limited.

2.3. Exposure characterisation

Individual exposures to UFP emissions from aviation emissions (total, take-off only and landing only), non-aircraft airport vehicles (such as passenger buses, fuel tankers, baggage trucks and local airport traffic) and non-airport (urban background and road) sources were calculated as a 5h average for each exposure date using a Positive Matrix Factorization (PMF) source apportionment model. Details of the model and instruments used to collect the input data are provided by Pirhadi et al. (2020) (Pirhadi et al., 2020) and Lammers et al. (2020) (Lammers et al., 2020). To summarise the sources of the UFP, air within the exposure chamber was sampled continuously and subjected to measurement for particle number concentrations (PNCs) and PM mass (gravimetrically), carbon monoxide (CO), black carbon (BC) and nitrogen oxides (NO_x). A water-based condensation particle counter (CPC) provided PNCs for total PM of ≤ ~2.5 μm in diameter and a scanning mobility particle sizer (SMPS) was fitted to measure PNCs for size

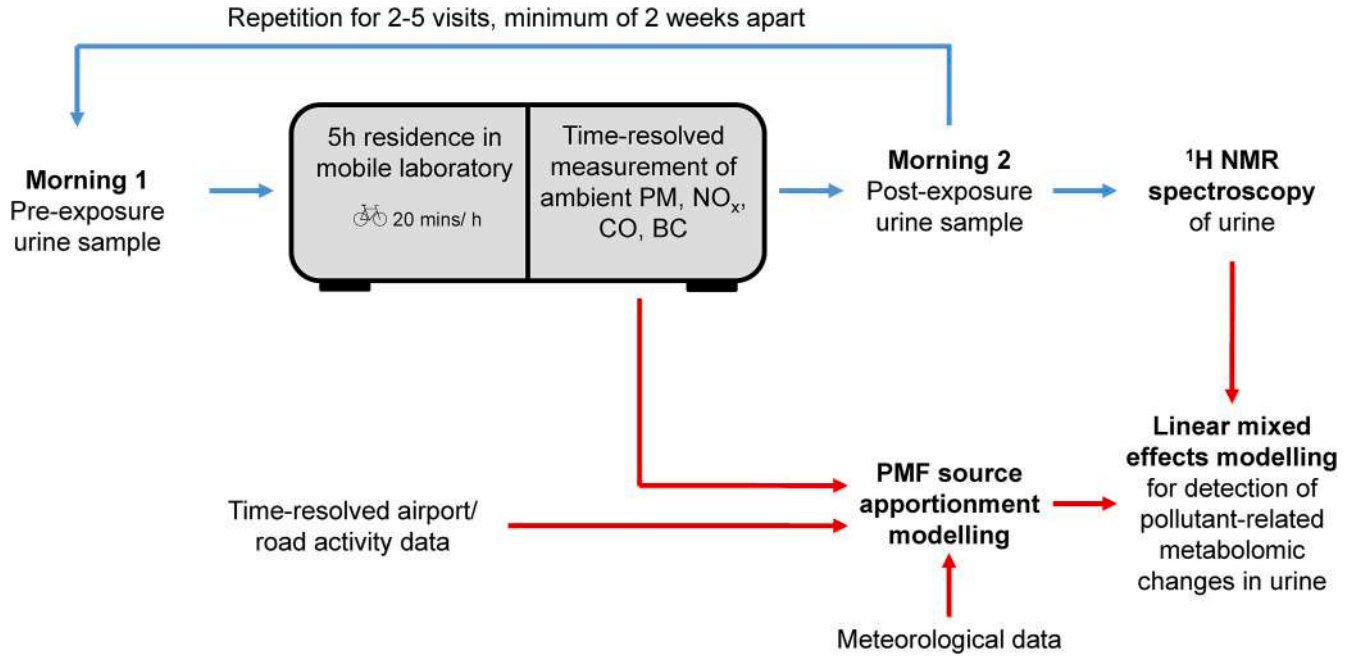

Fig. 1. Study design. On 2 – 5 occasions, participants provided urine samples prior to spending 5h in a mobile laboratory at Amsterdam Airport Schiphol. A second urine sample was given at the 24h time point and the metabolomic content of each sample was characterised via ^{1}H NMR. The output was combined with source apportioned pollutant measurements in a linear mixed effects model to identify changes in urinary metabolic content that relate to different air pollutant exposures at the airport. Exposure and experimental steps are denoted with blue arrows while data interrogation is represented by red arrows.

fractions between 6 and 225 nm in diameter. Particle masses were established using a tapered element oscillating microbalance while NO_x was measured with a chemiluminescence NO_x analyzer, CO with a gas filter correlation analyser and BC via optical absorption using an aethalometer. Meteorological conditions (temperature, wind speed and relative humidity) for the times of sampling were provided by the Royal Netherlands Meteorological Institute.

2.4. ^{1}H NMR spectral acquisition

Samples were prepared by combining 540 μl urine with 60 μl phosphate buffer containing 0.1M trimethylsilylpropanoic acid (TSP) in 5 mm NMR tubes. Spectra were acquired with a 600 MHz AV-NEO spectrometer equipped with a triple resonance cryoprobe with ^{1}H/^{13}C/^{15}N channels and a SampleJet for automation (all Bruker, UK). For each sample, a 1D ^{1}H spectrum was acquired using the presaturation utilising relaxation gradients and echoes (PURGE) pulse sequence, optimised to reduce the effects of non-ideal gradients (Le Guennec et al., 2017). 64 scans were used, with an acquisition time of 2.62 s, a spectral width of 20.8 parts per million (ppm), 4 dummy scans and a relaxation delay of 4 s. Additionally, a Total Correlation Spectroscopy (TOCSY) spectrum and a heteronuclear single quantum coherence (HSQC) spectrum were acquired on one of the samples for identification purposes. For TOCSY, the Bruker pulse sequence "dipsi2gpphzs" was used, slightly modified to include presaturation, which 16 scans, 512 t1 increments, a spectral width of 13.7 ppm in both dimensions and a relaxation delay of 2 s. The HSQC spectrum was acquired using the Bruker pulse sequence "hsqcetgpsisp2.2", with 32 scans, 512 t1 increments, a spectral width of 210 ppm in the 13C dimension and 20.8 ppm in the ^{1}H dimension and a relaxation time of 2 s.

2.5. ^{1}H NMR spectral processing and peak assignment

After acquisition, the 1D ^{1}H spectra were processed with an exponential window function of 0.3 Hz before Fourier Transform, then phasing, calibration of the ppm scale to the TSP peak (0 ppm) and baseline correction with a polynomial function of order 2. Processed spectra were imported into Chenomx Profiler (Chenomx Inc, Canada) for annotation using a peak fitting technique. Statistical total correlation spectroscopy (STOCSY) analyses were performed using Matlab (Version R2019b, Mathworks, USA) to assist this process by identifying peaks that belonged to common parent molecules (r values > 0.8). Annotations were confirmed for feature metabolites by comparing peak signals within the HSQC spectrum with reference values published in the Human Metabolome Database (Human Metabolome Database, 2021). Integrals were calculated for individual peaks using Matlab, employing code that determined the size of the signal based on the area under the curve between peak minima and maxima. Peak integrals were normalised to those of creatinine signals from the same spectrum to account for variations in urinary concentration. All Matlab codes were developed within the Section of Computational and Systems Medicine, Imperial College, London.

2.6. Statistical analysis

Linear mixed effects models were used to (A) detect confounding variables in the dataset and (B) identify changes in urinary metabolite content that are related to pollutant exposure. These were performed in R Studio (version 1.1.463, USA) using the 'lmer' function of Package 'lme4'. Throughout the analysis, relationships between metabolite signals and variables of interest (presented as regression coefficients) were considered statistically significant where the 95% confidence interval did not contain zero.

2.7. Confounding variables

To identify whether use of over-the counter pharmaceuticals (acetaminophen and ibuprofen, as detected within the spectra) induced changes to the urinary metabolome independent of pollutant exposure, pre-exposure data (pre) for each individual (i) and visit (j) was input into the following model:

$$Y_{i,j} = \beta_0 + Y_{i,j,pre} + \beta_1 E_j + U_{0i} + \varepsilon_i$$

With Yi,j referring to the relationships between non-target variable and metabolomic change across the study, Ej represents a vector of the potentially confounding variables and Yi,j,pre, the metabolite signals produced from the pre-exposure spectra of each participant at each of their visits. β refers to population-average fixed effects; specifically, the average metabolite signal where all other co-variates are zero (β0) and the average signal relative to a 5-95th percentile (5-95p) increase in the variable of interest. The U0i is a random intercept produced from each individual's deviation from the study population's average metabolite signal with ε1 as the accompanying error term.

2.8. Pollutant-related metabolomic changes

Alterations were made to the confounding variable identification model to focus on changes in metabolite concentration that were caused by pollutant exposure and to correct for confounding co-exposures.

$$Y_{i,j} = \beta_0 + Y_{i,j,pre\text{-}post} + \beta_1 E_j + \beta_2 V_{1,i,j} + \beta_3 V_{2,j} + \beta_4 V_{3,j} + U_{0i} + \varepsilon_i$$

Here, the model calculated the difference in metabolite signals for each individual at each visit ($Y_{i,j,pre\text{-}post}$) whilst adjusting for vectors of pharmaceutical signals produced from the ^{1}H NMR spectra ($V_{1,i,j}$), environmental conditions (room temperature and humidity) for different visits ($V_{2,j}$) and where appropriate, concentrations of secondary pollutants during different visits ($V_{3,j}$), β_2, β_3 and β_4 represent population- averages for these variables.

Pearson's correlation analyses were performed using GraphPad Prism 8 (GraphPad, California, USA) to explore the strength of relationships between metabolites that associated with exposure (referred to as feature metabolites). Correlations were considered 'moderate' or 'strong' where the Pearson's r value was $\geq$0.60 and 0.80, respectively (Akoglu, 2018), and the p value was $\leq$0.05.

3. Results

3.1. Individual particle exposures were predominantly contributed to by aviation emissions

In total, samples from 21 of the exposed participants were included for metabolomic profiling. Spectra from 1 participant were withdrawn from the analysis following peak annotation due to the presence of ethanol peaks in the urine. The remaining samples represented participation on 32 exposure days with each individual undergoing 2–5 exposures during the period between May and October 2018. Only 2 participants undertook two exposures (finishing the study early for personal reasons), with 13 participants undertaking four exposures and 6 participants undertaking five exposures due to extremely low exposures occurring on their first visit.

As documented by Lammers et al. and Pirhadi et al., 5 h averages of total PNC ranged between 10,500 and 173,200/cm^3 at the exposure site (Lammers et al., 2020), with aviation activity contributing most to PNC exposure for the majority of the study period and individuals (Fig. 2A–B) (Pirhadi et al., 2020). Pearson's correlation analysis found no significant relationship between PNC from total aviation, airport traffic and non-airport traffic sources but as expected, total aviation PNC correlated strongly and positively with total PNC measurements, take – off PNC and landing PNC (r = 0.97, 0.97 and 0.89 respectively) (Table S1).

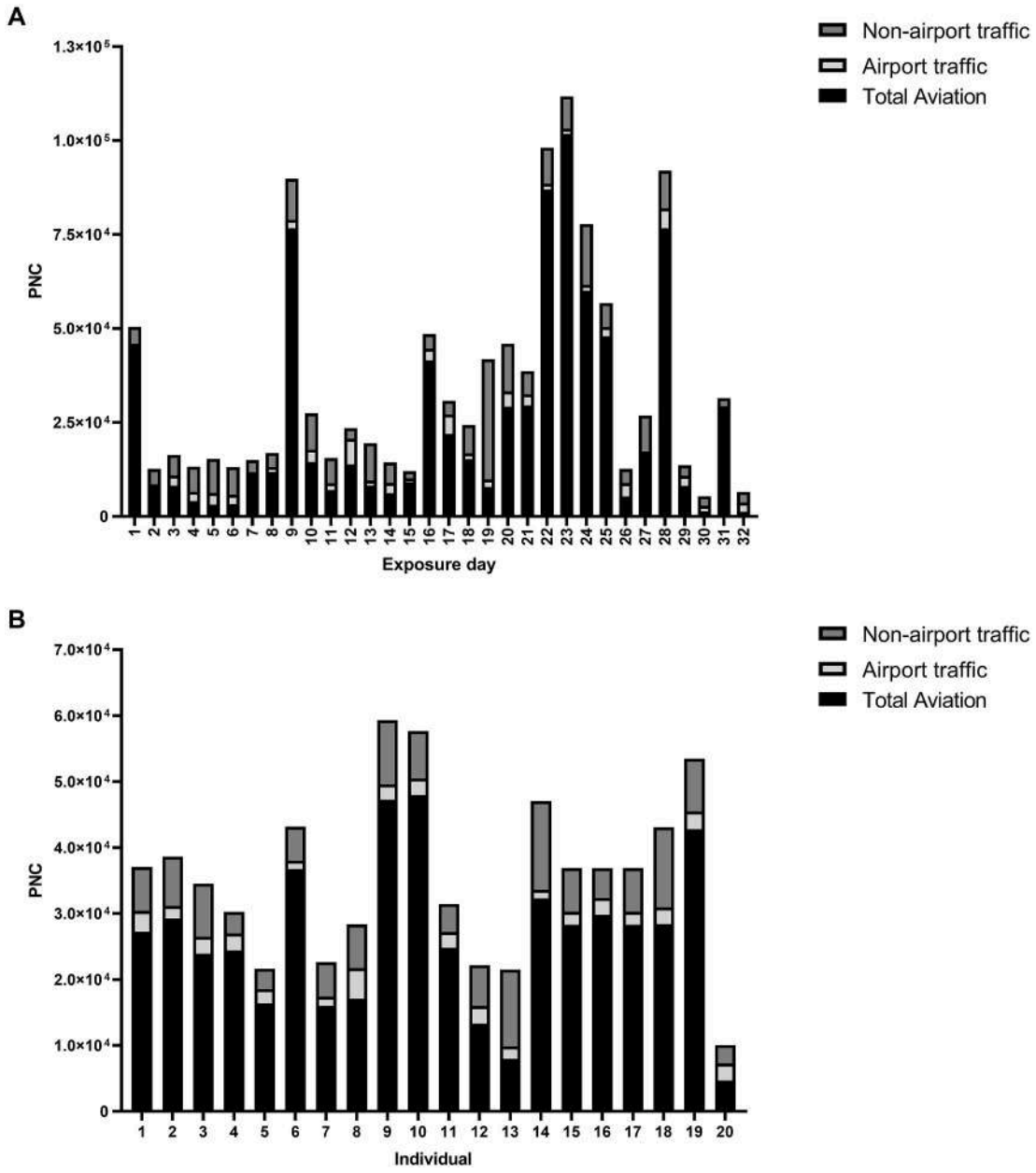

Fig. 2. PNC for total aviation, airport traffic and non-airport traffic sources during exposures. Source apportioned 5h mean values are provided for each exposure event (A) as determined by PMF modelling (Pirhadi et al., 2020) and for each participant (presented as the mean of these values across their 2 – 5 exposures) (B).

Moderately strong positive correlations also existed between take-off and landing PNCs (r = 0.76) (Table S1).

3.2. ^{1}H NMR spectra revealed substantial use of analgesics within the study population

Peaks from 68 distinct, assignable metabolites were detected within the ^{1}H NMR spectra alongside a further 54 peaks that could not be assigned using Chenomx profiling, HMBD searches or literature searches (Table S2). Of the assignable metabolites, 9 were produced during metabolism of commonly used, over-the-counter analgesics (ibuprofen and acetaminophen). As use of these analgesics were not exclusion criteria for the study, incidence of use by the study population was assessed using the visibility of the ibuprofen/ibuprofen-glucuronide peak at 0.74 ppm and the acetaminophen- glucuronide peaks at 5.10 ppm as markers of recent ibuprofen and acetaminophen consumption (Fig. 3). These peaks were selected because they did not exhibit overlap from other metabolites in the spectra. Visible ibuprofen/ibuprofen-glucuronide peaks, were present in 47 and 50% of pre- and post-exposure spectra (respectively), while visible acetaminophen glucuronide peaks were detected in 20 and 13% of pre- and post-exposure spectra. The presence of both analgesic peaks was detected in 12 and 5% of pre- and post-exposure spectra while only 32 and 41% of pre and post-exposure peaks contained no visible peaks for either metabolite.

Although pharmaceutical use is commonly observed in metabolomic analyses, the impact that therapeutic acetaminophen or ibuprofen use has on the endogenous urinary metabolome in humans has not been published. For the current study, linear modelling of the pre-exposure spectra demonstrated that urinary concentrations of trimethylamine-N-Oxide (TMAO), 3-aminoisobutyrate and glutamine were significantly elevated in association with acetaminophen use, and that citrate,

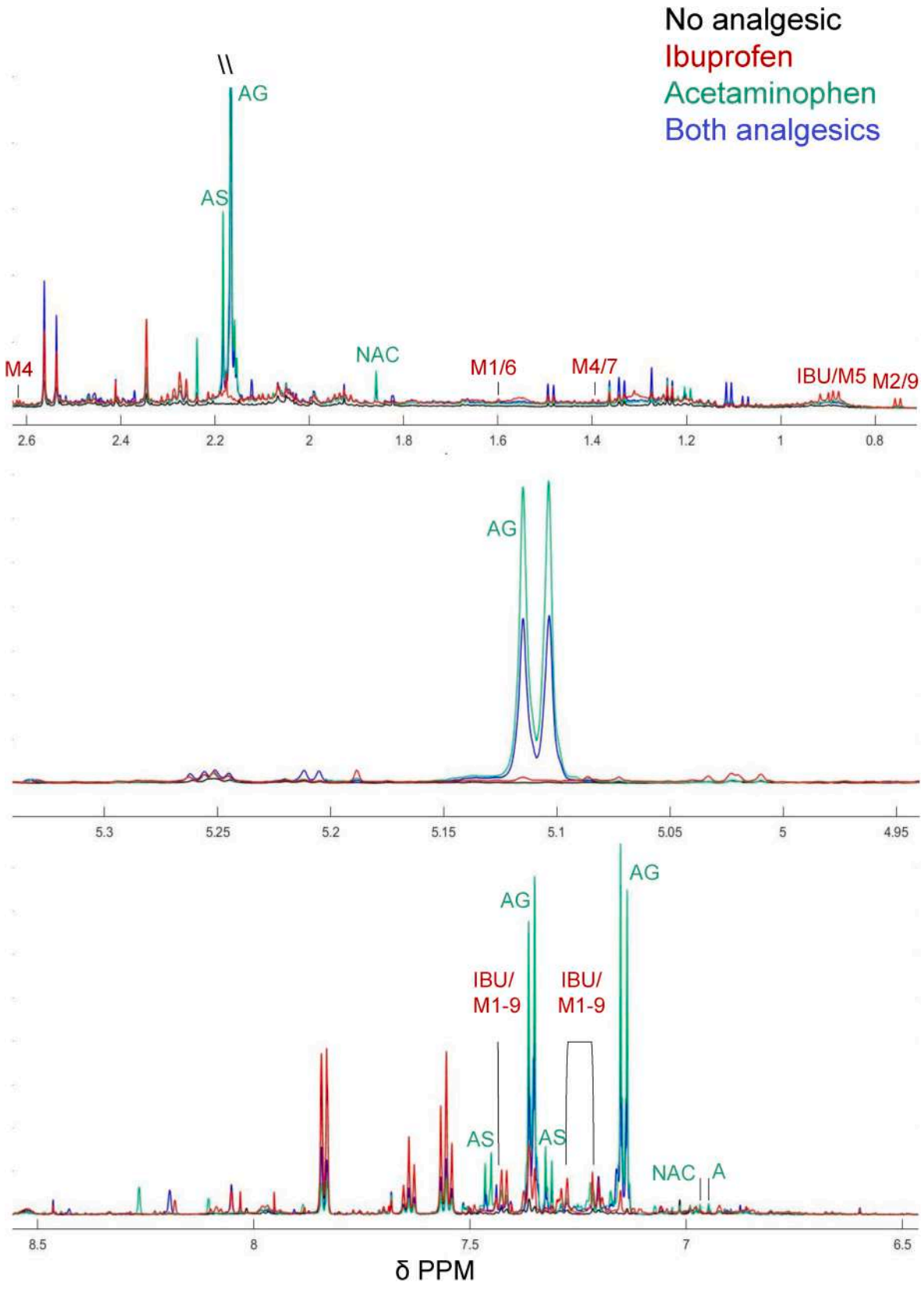

Fig. 3. Comparison of selected analgesic peak regions in the urinary ^{1}H NMR spectra of study participants prior to airport exposure. As labelled, these regions display peaks relating to the presence of parent compounds or metabolites of acetaminophen or ibuprofen. Included spectra are representative of those that contain no analgesic peaks (black spectrum), ibuprofen related peaks (red spectrum), acetaminophen related peaks (green spectrum) or peaks relating to both analgesics (blue spectrum). Acetaminophen (A), acetaminophen sulfate (AS), acetaminophen glucuronide (AG), N-acetylcysteine (NAC), Ibuprofen (IBU), 1-hydroxy ibuprofen (Ibuprofen metabolite (M) 2), carboxy ibuprofen (M4), 2-hydroxy ibuprofen glucuronide (M6), carboxy ibuprofen glucuronide (M7), 1-hydroxy ibuprofen glucuronide (M9).

glutamine, threonine, dimethylamine, alanine, TMAO, pyruvate, glutamate, lysine and N-acetylglutamate concentrations were significantly increased with ibuprofen uptake (Table 1). As such, urinary concentrations of acetaminophen and ibuprofen were input as confounding variables during modelling of emissions-related metabolomic change.

3.3. Exposure to airport-derived particulates causes significant alterations to the endogenous urinary metabolome

Preliminary analysis determined that total PNC exposure was associated with significant reductions in urinary taurine and dimethylamine concentrations (−0.263 arbitrary units (AU, as a ratio with internal creatinine signal), 95% CI: −0.507 to −0.020 and −0.232 AU, 95% CI: −0.396 to −0.670, respectively). Size apportioned PNCs confirmed that these changes associated with exposure to PNC <20 nm but not PNC >50 nm. The strength of association between dimethylamine concentration and PNC <20 nm was of equal size to the relationship between dimethylamine concentration and total PNC, while a 0.035 AU increase in coefficient size was seen for the association between taurine concentration and PNC <20 nm exposure when compared with total PM exposure (Table 2). These observations indicate that PNC <20 nm, which associate with airplane emissions, were responsible for the changes. No other changes to the metabolome associated significantly with PNC <20 nm or PNC >50 nm or with carbon black exposure specifically (Table 2) but exposure to combustion-associated pollutant gases displayed small but significant associations with changes to the urinary metabolome. NO_2 exposure related to small reductions in urinary 3-hydroxyisovalerate content (−0.005 AU, 95% CI: −0.009 to −0.001) as well as 3-hydroxyisobutyrate (−0.007 AU, 95% CI: −0.013 to −0.001). Exposure to CO also associated with reductions in 3-hydroxyisobutyrate concentration (−0.009 AU, 95% CI: −0.017 to −0.001) and increases in concentrations of N-acetylglutamine and an unassigned metabolite (0.020 AU, 95% CI: 0.002–0.038 and 0.006 AU, 95% CI: 0.001–0.010, respectively). Accounting for co-exposures to CO or NO_2 had minimal impact on the strength of association between total PNC, PNC <20 nm and taurine or dimethylamine. No novel associations between exposure and metabolomic change were identified following the correction (Table S3).

3.4. Exposure to UFP from different airport-related sources induces distinct alterations to the endogenous urinary metabolome

The PMF model established that airport activities accounted for 79.3% of total PNC (46.1, 26.7 and 6.5% from aircraft departures, aircraft arrivals and ground service equipment (GSE)/local airport

Table 1
Associations between Δ endogenous metabolites and xenobiotic metabolite concentrations.

Metabolite	Ibuprofen	Acetaminophen
	Coef. (95% CI)	Coef. (95% CI)
Citrate	**4.55 (2.30 – 6.90)**	0.17 (−1.63 – 2.03)
Glutamine	**1.69 (1.02 – 2.40)**	**0.866 (0.33 – 1.41)**
Threonine	**1.48 (1.09 – 1.88)**	0.34 (−0.04 – 0.72)
Dimethylamine	**1.48 (0.52 – 2.43)**	0.04 (−0.81 – 0.90)
Alanine	**1.42 (1.04 – 1.80)**	0.19 (−0.19 – 0.16)
TMAO	**1.39 (0.04 – 2.82)**	**1.19 (0.19 – 2.23)**
Unassigned at 2.33 ppm	**0.81 (0.36 – 1.44)**	0.32 (−0.04 – 0.67)
Pyroglutamate	**0.74 (0.45 – 1.05)**	0.33 (0.11 – 0.56)
Acetate/Phenylacetylglutamine	**0.54 (0.30 – 0.78)**	0.11 (−0.09 – 0.11)
3-Aminoisobutyrate	0.72 (−0.34 – 1.84)	**1.12 (0.32 – 1.95)**

Data are presented as coefficients (coef.) of the relationship between exposure and Δ in metabolite concentration (post-pre) with 95% confidence intervals (CI) (expressed as the range between lower and upper values). All coefficients are adjusted for room temperature and humidity. Numbers in bold represent significant relationships ($p \leq 0.05$).

Table 2
Single pollutant models for associations between Δ urinary metabolites and major air pollutants at Schiphol Airport.

Metabolite	Total PNC (5-95p = 120,280 #/cm^3)	PNC <20 nm (5-95p = 51,160 #/cm^3)	PNC >50 nm (5-95p = 3,900 #/cm^3)	Black carbon (5-95p = 1.4 μg/cm^3)	NO_2 (5-95p = 33.2 μg/cm^3)	CO (5-95p = 250 μg/cm^3)
	Coef. (95% CI)	**Coef. (95% CI)**	**Coef. (95% CI)**	**Coef. (95% CI)**	**Coef. (95% CI)**	**Coef. (95% CI)**
Taurine	**−0.263 (−0.507 – −0.020)**	**−0.298 (−0.550 – −4.709)**	−0.044 (−0.396 – 0.307)	−0.029 (−0.349 – 0.290)	−0.096 (−0.351 – 0.158)	−0.113 (−0.436 – 0.211)
Dimethylamine	**−0.023 (−0.040 – −0.067)**	**−0.023 (−0.040 – −0.067)**	0.006 (−0.018 – 0.029)	0.003 (−0.018 – 0.024)	−0.001 (−0.018 – 0.016)	−0.003 (−0.025 – 0.019)
Unassigned at 2.85 ppm	0.000 (−0.002 – 0.002)	−0.001 (−0.002 – 0.001)	0.000 (−0.002 – 0.003)	0.000 (−0.002 – 0.002)	0.001 (−0.003 – 0.002)	0.000 (−0.002 – 0.002)
3-Hydroxyisovalerate	−0.001 (−0.005 – 0.004)	0.000 (0.000 – 0.000)	−0.002 (−0.008 – 0.004)	−0.004 (−0.009 – 0.001)	**−0.005 (−0.009 – −0.001)**	−0.005 (−0.010 – 0.001)
3-Hydroxyisobutyrate	−0.002 (−0.005 – 0.001)	−0.002 (−0.005 – 0.002)	−0.002 (−0.006 – 0.002)	−0.001 (−0.005 – 0.003)	**−0.007 (−0.013 – −0.001)**	**−0.009 (−0.017 – −0.001)**
N-Acetylglutamine	0.000 (−0.015 – 0.015)	0.002 (−0.014 – 0.017)	0.001 (−0.022 – 0.023)	−0.007 (−0.029 – 0.013)	0.000 (−0.001 – 0.000)	**0.020 (0.002 – 0.038)**
Unassigned at 1.99 ppm	−0.001 (−0.004 – 0.002)	−0.001 (−0.004 – 0.003)	0.003 (−0.002 – 0.008)	0.003 (−0.001 – 0.000)	0.001 (−0.002 – 0.005)	**0.006 (0.001 – 0.010)**

Data are presented as coefficients (coef.) of the relationship between exposure and Δ in metabolite concentration (post-pre) with 95% confidence intervals (CI) (expressed as the range between lower and upper values). All coefficients are adjusted for urinary ibuprofen and paracetamol markers, room temperature and humidity. Total PNC refers to particles smaller than 2.5 μm in diameter, with a lower limit of 4 nm, as measured by a condensation particle counter and SMPS. Numbers in bold represent significant relationships ($p \leq 0.05$).

traffic respectively) while road traffic and urban background sources contributed 18% and 2.7% respectively (Pirhadi et al., 2020). Using this data, PNCs were assigned to three general emissions sources at Amsterdam Airport Schiphol; total aviation, airport traffic (from GSE and road traffic within the airport) and non-airport traffic (from the nearby highways and urban background). Consistent with the results above, the largest changes to the urinary metabolome of exposed participants were induced by total aviation PNC (5-95p = 73,485 particles/cm^3). Here, exposure associated significantly with a 0.26 AU decrease in urinary taurine (95% confidence interval (CI): −0.503 to −0.023) as well as smaller but statistically significant decreases in dimethylamine (- 0.021 AU, 95% CI: −0.037 to −0.005) and pyroglutamate concentration (0.005 AU, 95% CI: −0.01- <0.00). Neither PNC produced by airport traffic or non-airport traffic associated with changes in these metabolite concentrations but PNC relating to airport traffic (5-95p = 5077 particles/cm^3) did associate with significant yet small increases in urinary concentrations of methylguanidine (0.001 AU, 95% CI: >0.000–0.002) and decreases in 3-aminoisobutyrate (- 0.010 AU, 95% CI: −0.019 - - 0.001). In contrast, exposure to PNC produced by non-airport traffic (5-95p = 15290 particles/cm$^{3)}$ associated with significant increases in urinary 3-aminoisobutyrate concentration (0.010 AU, 95% CI: 0.002–0.017) as well as small increases in carnosine/arginine (0.005 AU, 95% CI: 0.001–0.008) and ethanolamine/isethionate concentrations (0.005 AU, 95% CI: 0.001–0.010) and a reduction in isocitrate concentration (−0.003, 95% CI: −0.005 to −0.001) (Table 3). Adjustment of the single pollutant models to account for co-exposure to UFP from the remaining key sources (total aviation, airport traffic, non-airport traffic, as appropriate), did not cause noteworthy changes to the strength of associations with metabolite features (Table 4). This indicates that the changes to the metabolome that associate with each key feature were unlikely to have been contributed to by co-exposure to the others.

3.5. Landing and take-off- related UFP both contribute to changes in the urinary metabolome

Using the PMF source apportionment model, it was possible to explore relationships between urinary metabolomic changes and individual aircraft behaviours. Single pollutant models demonstrated that total PNCs for UFPs produced during take- off (5-95p = 56,130 particles/cm^3) and landing (5-95p = 31,200 particles/cm^3) could be associated with the significant changes in urinary dimethylamine concentration (−0.019 AU, 95% CI: −0.037 to −0.001 for take-off and −0.031 AU, 95% CI: −0.012 to −0.001 for landing). Compared to the relationship with total aviation PNC, reductions in urinary taurine concentrations were larger when associated with landing PNC specifically (−0.413 AU, 95% CI: −0.689 to −0.136). While not statistically significant, due to variation in response levels, reductions in urinary taurine concentration were also present overall, following association with take-off PNC (−0.224 AU, 95% CI -0.495 – 0.047). Similarly, reductions in urinary pyroglutamate associated significantly with take-off PNC (−0.006 AU, 95% CI: −0.012 to −0.001) but their association with landing PNC displayed too much variability to be considered statistically significant (−0.004 AU, 95% CI: −0.001 – 0.003) (Table 5). These associations remained robust following adjustment of the models for co-exposure to airport or non-airport traffic UFP (Table S4).

3.6. Features of the metabolomic response to aviation UFP exposure may contribute to common biological processes

In order to assign mechanistic meaning to metabolomic change, it is necessary to explore relationships between feature metabolites. With the three metabolites that associate with aviation UFP exposure, there was not sufficient input data to perform an appropriately powered pathway analysis. As such, Pearson's correlation analyses were performed to identify metabolites that could contribute to or be products of common biological processes. This method identified a strong, positive correlation ($r = 0.88$, $p \leq 0.001$) between Δ dimethylamine and Δ taurine concentrations in post- and pre-exposure samples (Fig. 4). No strong nor significant correlations were found between Δ in taurine and Δ pyroglutamate concentration ($r = 0.02$) or between Δ dimethylamine and Δ pyroglutamate concentration ($r = 0.02$) (data not shown).

4. Discussion

Combining our cross-over intervention study of 21 healthy young adults with source apportionment modelling, we identified acute changes to the urinary metabolome that associate with exposure to UFP from distinct emission sources at Amsterdam Airport Schiphol. Metabolic signatures associating with aviation emissions dominated the response to total PNC and were characterised by significant reductions in urinary taurine, dimethylamine and pyroglutamate concentrations, consistent with increased utilisation or decreased synthesis of these metabolites.

Previously, exposure to airport UFPs has been associated with pulmonary and systemic inflammation (Bendtsen et al., 2019; Habre et al., 2018), oxidative stress (He et al., 2018) and reductions in cardiopulmonary function (Lammers et al., 2020). To our knowledge, this study is the first to assess responses to airport emissions at a global, biochemical level. Consistent with observations that airport UFPs have oxidative potential and induce reactive oxygen species (ROS) synthesis *in vitro* (He et al., 2018), several of the metabolites that associated with exposure to aviation UFPs in this study have been related to antioxidant responses to the imposition of oxidative stress.

The most pronounced of these changes was the reduction in urinary taurine which associated with UFP produced during aircraft landing and possibly take-off. The β-amino acid taurine, which is abundant in the cytosol of inflammatory and metabolically active cells, has been proposed to act as an indirect antioxidant via enhancement of classical antioxidant concentrations (Tabassum et al., 2006) and modulation of

Table 3
Single pollutant models for associations between Δ urinary metabolites and UFP from airport-related sources.

Metabolite	Total aviation PNC (5-95p = 73485 #/cm^3)	Airport traffic PNC (5-95p = 5077 #/cm^3)	Non-airport traffic PNC (5-95p = 15290 #/cm^3)
	Coef. (95% CI)	**Coef. (95% CI)**	**Coef. (95% CI)**
Taurine	**−0.263 (−0.503 – −0.023)**	0.035 (−0.271 – 0.342)	−0.029 (−0.269 – 0.211)
Dimethylamine	**−0.021 (−0.037 – −0.005)**	0.010 (−0.011 – 0.030)	−0.002 (−0.018 – 0.014)
Pyroglutamate	**−0.005 (−0.010 – < 0.000)**	−0.003 (−0.009 – 0.004)	0.003 (−0.002 – 0.008)
3-aminoisobutyrate	0.002 (−0.006 – 0.010)	**−0.010 (−0.019 – −0.001)**	**0.010 (0.002 – 0.017)**
Methylguanidine	−0.001 (−0.001 – < 0.000)	**0.001 (> 0.000 – 0.002)**	−0.001 (−0.001 – 0.000)
Isocitrate	0.001 (−0.001 – 0.003)	0.002 (<0.000 – 0.004)	**−0.003 (−0.005 – −0.001)**
Carnosine/arginine	0.002 (−0.002 – 0.006)	0.001 (−0.004 – 0.006)	**0.005 (0.001 – 0.008)**
Ethanolamine/isethionate	0.002 (−0.003 – 0.007)	0.002 (−0.004 – 0.008)	**0.005 (0.001 – 0.010)**

Data are presented as coefficients (coef.) of the relationship between exposure and Δ in metabolite concentration (post-pre) with 95% confidence intervals (CI). All coefficients are adjusted for urinary ibuprofen and paracetamol markers, room temperature and humidity. Numbers in bold represent significant relationships ($p \leq 0.05$).

Table 4
Two pollutant models for associations between Δ urinary metabolites and UFP from key sources, accounting for co-exposures to UFP from the remaining key sources.

PNC source of interest:	Total aviation (5-95p = 73485 #/cm^3)		Airport traffic (5-95p = 5077 #/cm^3)		Non-airport traffic (5-95p = 15290 #/cm^3)	
Co-exposing PNC source accounted for:	Airport traffic	Non-airport traffic	Total aviation	Non-airport traffic	Total aviation	Airport traffic
Metabolite	**Coef. (95% CI)**	**Coef. (95% CI)**	**Coef. (95% CI)**	**Coef. (95% CI)**	**Coef. (95% CI)**	**Coef. (95% CI)**
Taurine	**−0.262 (−0.502 – −0.022)**	**−0.270 (−0.516 – −0.024)**	0.021 (−0.276 – 0.319)	0.026 (−0.291 – 0.343)	0.023 (−0.195 – 0.241)	−0.027 (−0.253 – 0.198)
Dimethylamine	**−0.021 (−0.037 – −0.005)**	**−0.022 (−0.038 – −0.006)**	0.008 (−0.012 – 0.028)	0.008 (−0.012 – 0.030)	0.001 (−0.014 – 0.015)	−0.002 (−0.017 – 0.013)
Pyroglutamate	**−0.005 (−0.010 – < 0.000)**	**−0.006 (−0.011 – −0.001)**	−0.003 (−0.009 – 0.003)	−0.002 (−0.008 – 0.004)	0.004 (<0.000 – 0.009)	0.003 (−0.002 – 0.007)
3-Aminoisobutyrate	0.002 (−0.006 – 0.009)	0.000 (−0.008 – 0.004)	**−0.015 (−0.030 – −0.001)**	**−0.015 (−0.030 – <−0.000)**	**0.010 (0.004 – −0.017)**	**0.009 (0.003 – 0.016)**
Methylguanidine	−0.001 (−0.001 – >0.000)	0.003 (−0.008 – 0.014)	**0.001 (>0.000 – 0.002)**	0.001 (<0.000 – 0.002)	<0.000 (−0.001 – >0.000)	<0.000 (−0.012 – >0.000)
Isocitrate	0.001 (−0.001 – 0.003)	0.002 (0.000 – 0.004)	0.002 (<0.000 – 0.004)	0.001 (−0.001 – 0.003)	**−0.003 (−0.005 – −0.002)**	**−0.003 (−0.004 – −0.001)**
Carnosine/arginine	0.002 (−0.002 – 0.006)	0.001 (−0.003 – 0.005)	0.001 (−0.004 – 0.006)	0.002 (−0.003 – 0.007)	**0.004 (>0.000 – 0.008)**	**0.004 (>0.000 – 0.009)**
Ethanolamine/isethionate	0.002 (−0.003 – 0.007)	0.001 (−0.004 – 0.006)	0.002 (−0.004 – 0.008	0.004 (−0.003 – 0.010)	**0.004 (>0.000 – 0.008)**	**0.005 (>0.000 – 0.009)**

Data are presented as coefficients (coef.) of the relationship between exposure to key PNC sources and Δ in metabolite concentration (post-pre) following adjustment of the model for co-exposure to the remaining two key sources of PNC at the airport. Data are presented with 95% confidence intervals (CI). All coefficients are adjusted for urinary ibuprofen and paracetamol markers, room temperature and humidity. Numbers in bold represent significant relationships ($p \leq 0.05$).

Table 5
Single pollutant models for associations between Δ urinary metabolites and PNC produced through take-off and landing.

Metabolite	Take-off PNC (5-95p = 56130 #/cm^3)	Landing PNC (5-95p = 31200 #/cm^3)
	Coef. (95% CI)	**Coef. (95% CI)**
Taurine	−0.224 (−0.495 – 0.047)	**−0.413 (-0.689 – −0.136)**
Dimethylamine	**−0.019 (-0.037 – −0.001)**	**−0.031 (-0.049 – −0.013)**
Pyroglutamate	**−0.006 (-0.012 – −0.001)**	−0.004 (−0.010 – 0.002)

Data are presented as coefficients (coef.) of the relationship between exposure and Δ in metabolite concentration (post-pre) with 95% confidence intervals (CI). All coefficients are adjusted for urinary ibuprofen and paracetamol markers, room temperature and humidity. Numbers in bold represent significant relationships ($p \leq 0.05$).

mitochondrial ROS generation (Ju et al., 1007). It also acts as an anti-inflammatory agent through its capacity to react with neutrophil-derived hypochlorous acid (HOCl) to form taurine chloramine (Kim and Cha, 2014). When taken up into cells at sites of inflammation, taurine chloramine promotes a broad spectrum xenobiotic and antioxidant response through activation of the Nuclear factor erythroid 2-related factor 2, (Nrf2) transcription factor (Kim and Cha, 2014).

Decreased taurine concentrations have been measured in the BALF of rats following ZnO inhalation, reflecting enhanced antioxidant activity within the pulmonary tissue. Supporting the suggestion that landing UFPs also triggered this protective response, taurine has been shown to alleviate oxidative stress, pro-inflammatory cytokine secretion, inflammatory cell recruitment, mitochondrial dysregulation, autophagy and emphysema in mouse lung following exposure to DEP or 1-nitropyrene (Kim et al.; Li et al., 1073). It is difficult to hypothesise why the observed change in taurine concentration associated more robustly with landing UFP. To date, no considerable differences have been reported in the composition of emissions produced during take-off and landing (Shirmohammadi et al., 2017). Although landing particles did account for the majority of UFP <20 nm at our sampling site (Pirhadi et al., 2020), their concentrations were strongly correlated with those of take-off particles (r = 0.76), creating the possibility that the observed differences in effect size and significance were artefacts of collinearity within the model. While individuals are unlikely to only be exposed to landing UFP at an airport, confirming this observation and understanding its cause, could have bearing on future aviation engineering.

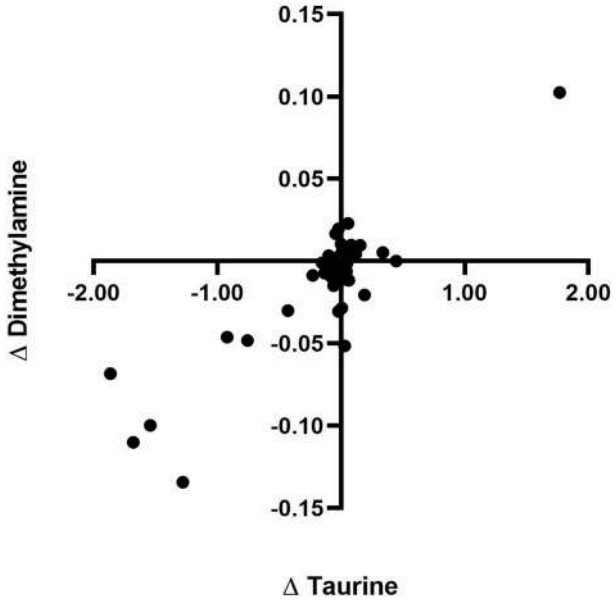

Fig. 4. Pearson correlation between Δ urinary concentrations of taurine and Δ urinary concentrations of dimethylamine following total aviation UFP exposure. Analysis was performed using post- exposure minus pre- exposure values with each data point representing the outcome of one visit for individual participants.

Exposure to both landing and take-off related UFPs induced reductions in urinary pyroglutamate. Pyroglutamate is produced in the γ-glutamyl cycle as a precursor to glutathione (GSH) (Lord and Bralley, 2008). A decrease in urinary pyroglutamate is therefore consistent with increased cellular GSH synthesis as an adaptive response to the imposition of oxidative stress. This aligns with a potential role for taurine chloramine in promoting GSH synthesis through Nrf2-mediated up-regulation of glutathione synthase expression (Steele et al., 2013) (Illustrated in Fig. 5). This hypothesis does require experimental confirmation, but in the context of the previous literature demonstrating the capacity for UFP to initially deplete antioxidants (Walker et al.,

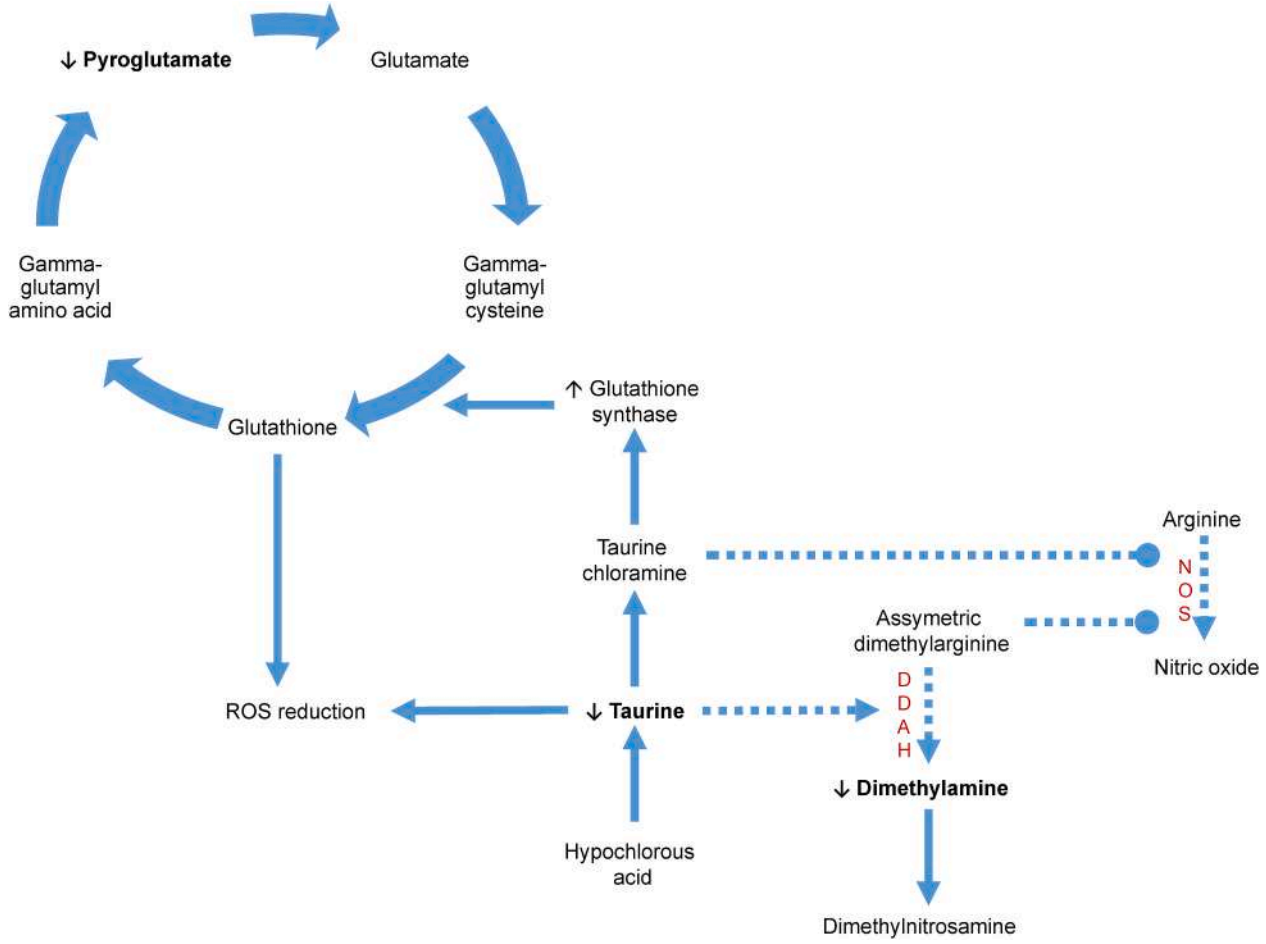

Fig. 5. Hypothesised interplay of altered pathway activity following exposure to aviation UFPs. Reductions in urinary pyroglutamate reflect increased demand for glutathione synthesis which is contributed to by conversion of taurine to taurine chloramine with downstream, Nrf2-mediated induction of glutathione synthase expression. Taurine availability also diminishes due to the role of taurine as an inhibitor of ROS generation, leading to decreased DDAH agonism and dimethylamine synthesis. Resultant accumulations of ADMA, combined with increased availability of taurine chloramine inhibit NOS activity, resulting in reduced nitric oxide synthesis. Increased conversion of dimethylamine to nitrosdimethylamine may also contribute to reductions in dimethylamine concentration. Dotted and solid arrows represent reduction and enhancement of reactions (respectively). Dimethylarginine dimethylaminohydrolase (DDAH), nitric oxide synthase (NOS).

2019) and subsequently induce protective, adaptive responses (Li et al., 2004), the observed relationships do illustrate the utility of metabolomics in generating novel, testable hypotheses to explore causal links between pollutants and adverse responses. It is important to note however, that our results reflect only responses to short term exposures in healthy individuals and there remains a need to understand the impact of recurrent or longer exposures in relation to chronic disease development and exacerbation (Downward et al., 2018).

As well as pyroglutamate, UFPs produced during take-off and landing were also associated with reductions in urinary dimethylamine. In humans, dimethylamine is produced endogenously by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) during hydrolysis of asymmetric dimethylarginine (ADMA) (Tsikas, 2020) and through microbial catabolism of dietary choline (Zeisel et al., 1985). In health, dimethylamine is excreted via the urine in the upper μM range (Tsikas, 2020), with a small fraction converted to dimethylnitrosamine (DMNA). Reduced urinary dimethylamine concentrations may therefore reflect enhanced DMNA synthesis or reduced DDAH activity. As DMNA exerts genotoxicity in mammalian cell lines and rodents and is hypothesised to act similarly in humans (International Agency for, 1978; Liteplo and Meek, 2001), this warrants further investigation.

As ADMA is an inhibitor of nitric oxide (NO) synthases (NOS), reduced conversion to dimethylamine could also result in decreased NO synthesis. Supporting this hypothesis, increased ADMA and NO precursors have been measured in the plasma of individuals exposed to highway UFPs (Walker et al., 2019) and reduced NO synthesis has been observed in human aortic endothelial cells following exposure to urban UFPs (Du et al., 2013). As well as impacting vascular tone, airway responsiveness and inflammatory cell function, reduced NO availability results in QT interval prolongation and reduced lung function (23,24). While cardiopulmonary function was not measured at the same time as urine sampling for this study, reductions in FVC and QT interval prolongation did associate with aviation UFP exposure in our cohort 4h post-exposure (Lammers et al., 2020). Strong positive associations ($r = 0.88$) were observed between the reductions in dimethylamine concentrations and reductions in taurine concentrations, suggesting some degree of interaction between the implicated metabolic pathways (Fig. 5). As DDAH is agonised by taurine (Pasaoglu et al., 2014; Tan et al., 2007) one explanation could be that the particle-induced decrease in taurine availability led to reduced DDAH activity via less agonism (Fig. 5). Like ADMA, taurine chloramine inhibits NOS activity during inflammation (Kim and Cha, 2014; Barua et al., 2001), supporting the plausibility of interactions between the two pathways following UFP exposure (Fig. 5).

Ibuprofen and acetaminophen metabolites were present in approximately 50% of spectra, making correction for their use, a necessity for our model. Like airport UFPs, these metabolites associated with changed urinary concentrations of dimethylamine, pyroglutamate, 3-

aminoisobutyrate and mitochondrial metabolism markers, indicating their potential to mask our responses of interest. While many studies prohibit use of analgesics, this was not feasible for our six-month study period. As a result of correcting for their use post-exposure, we add to our outcomes, a preliminary characterisation of how therapeutic doses of ibuprofen and acetaminophen impact the human urinary metabolome. Until now, study of the impacts that these pharmaceuticals have on the human metabolome has been limited to the contexts of overdose and hepatotoxicity (30–34))

It must be noted that the participants of this study were predominantly female (81%) and were all young individuals with 'healthy' BMI and cardiopulmonary function who live in areas without high levels of traffic pollution. As such, we cannot presume that the hypothesised mechanisms of aviation UFP toxicity reflect the responses of individuals who do not fit these criteria. As examples, metabolomic responses to traffic-related PM exposure are shown to be influenced by asthmatic status (arginine-related pathways) (Liang et al., 2019) and by sex and obesity (non-esterified fatty acid metabolism) (Chen et al., 2019). It is therefore important that the hypothesised impacts of aviation UFP on the urinary metabolome are validated in larger, more diverse cohorts, especially those that are inclusive of established vulnerable groups.

5. Conclusions

In this study, we have for the first time, demonstrated a clear distinction between the urinary metabolomic signatures that accompany exposure to aviation UFPs and those that associate with other UFP sources at a major airport. From the metabolic features identified, the direction of their relationship with the exposure estimates and pre-existing knowledge base on UFP toxicity, we have elaborated a series of potential testable hypotheses based on the (A) increased utilisation of taurine and induction of an adaptive antioxidant response, including increased synthesis of GSH and (B) modulation of nitric oxide production via enhanced dimethylarginine dimethylaminohydrolase activity.

There remain outstanding questions as to whether the hypothesised responses are informative to an understanding of longer-term or repeat exposures, especially within established vulnerable groups. Considering however, that these responses are consistent with effects induced by road-side levels of traffic particulates (Oeder et al., 2015; Walker et al., 2019; Törnqvist et al., 2007), which have established links with adverse health (Khan and Strand, 2018; Sinharay et al., 2018; Adar et al., 2007; Zhou et al., 2015; Kan et al., 2007), and that airport particulates have been found to induce similar acute phase, inflammatory and genotoxic responses to DEP in mice (Bendtsen et al., 2019), we believe that the hypotheses merit further exploration. Additionally, our findings, just as previous studies of UFP exposure, emphasise the importance of UFP monitoring networks for a comprehensive examination of long-term UFP exposures and adverse health outcomes, especially in near-source environments such as major airports, to determine threshold levels and support UFP regulations (Baldauf et al., 2016).

Ethics

The study protocol was reviewed and approved by the Medical Ethical Committee (METC) of the Amsterdam Medical Centre (Amsterdam, the Netherlands) and was registered at the Dutch Trial Register (identifier NTR 6955, www.trialregister.nl/). All participants provided informed consent.

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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.2021.113803.

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Association of ambient air pollution with depressive and anxiety symptoms in pregnant women: A prospective cohort study

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ABSTRACT

Background: Air pollution is associated with depressive and anxiety symptoms in the general population. However, this relationship among pregnant women remains largely unknown.
Objective: To evaluate the association between pregnancy air pollution exposure and maternal depressive and anxiety symptoms during the third trimester assessed using the Center for Epidemiologic Studies-Depression and State-Trait Anxiety Inventory scales, respectively.
Methods: We analyzed 1481 pregnant women from a cohort study in Seoul. Maternal exposure to particulate matter with an aerodynamic diameter <2.5 μm ($PM_{2.5}$) and <10 μm (PM_{10}), as well as to nitrogen dioxide (NO_2) and ozone (O_3) for each trimester and the entire pregnancy was assessed at participant's residential address by land use regression models. We estimated the relative risk (RR) and corresponding confidence interval (CI) of the depressive and anxiety symptoms associated with an interquartile range (IQR) increase in $PM_{2.5}$, PM_{10}, NO_2, and O_3 using modified Poisson regression.
Results: In single-pollutant models, an IQR increase in $PM_{2.5}$, PM_{10}, and NO_2 during the second trimester was associated with an increased risk of depressive symptoms ($PM_{2.5}$ RR = 1.15, 95% CI: 1.04, 1.27; PM_{10} RR = 1.13, 95% CI: 1.04, 1.23; NO_2 RR = 1.15, 95% CI: 1.03, 1.29) after adjusting for relevant covariates. Similarly, an IQR increase in O_3 during the third trimester was associated with an increased risk of depressive symptoms (RR = 1.09, 95% CI: 1.01, 1.18), while the IQR increase in O_3 during the first trimester was associated with a decreased risk (RR = 0.89, 95% CI: 0.82, 0.96). Exposure to $PM_{2.5}$, PM_{10}, and NO_2 during the second trimester was significantly associated with anxiety symptoms. The associations with $PM_{2.5}$ and O_3 in single-and multi-pollutant models were consistent.
Conclusions: Our findings indicate that increased levels of particulate matter, NO_2, and O_3 during pregnancy may elevate the risk of depression or anxiety in pregnant women.

1. Introduction

Depression and anxiety are the most common mental disorders (Lim

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et al., 2018; Steel et al., 2014) and are significant contributors to the global disease burden, accounting for 43.0 million and 27.1 million years lived with disability, respectively, in 2017 (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018). Mental disorder rates are substantial during pregnancy, with 12–18% of women are reported to experience prenatal depression and 13–21% experience prenatal anxiety (Toscano et al., 2021; Woody et al., 2017). Mental disorders during pregnancy could have profound consequences for mother and child in terms of adverse pregnancy outcomes (Accortt et al., 2015; Mannisto et al., 2016) and neurodevelopment in infants, including cognitive deficits and changes in temperamental traits (Kinsella and Monk, 2009). Therefore, to reduce these health burdens, it is crucial to identify modifiable risk factors for prenatal depression and anxiety.

Abbreviations

BMI	body mass index
CES-D	center for epidemiologic studies-depression
CI	confidence interval
COCOA	cohort for childhood origin of asthma and allergic disease
EPDS	Edinburgh postnatal depression scale
ICD	international classification of diseases
IQR	interquartile range
LOOCV	leave-one-out cross-validation
LUR	land use regression
NO_2	nitrogen dioxide
O_3	ozone
$PM_{2.5}$	particulate matter with an aerodynamic diameter <2.5 μm
PM_{10}	particulate matter with an aerodynamic diameter <10 μm
ppb	parts per billion
RR	relative risk
STAI	State-Trait Anxiety Inventory

The development of depression and anxiety can be attributed to genetic (Lawrence et al., 2019; Kang et al., 2020), socio-demographic (Dulaney et al., 2018; Gur et al., 2019; Park et al., 2015), and physical environmental factors (Pun et al., 2017; van den Bosch and Meyer-Lindenberg, 2019). Specifically, exposure to ambient air pollution is hypothesized to induce depression and anxiety (Altuğ et al., 2020; Pun et al., 2017; Zhao et al., 2020). Recent meta-analyses of observational studies are consistent with their findings of a positive association of depression with particulate matter of <2.5 μm ($PM_{2.5}$) (Braithwaite et al., 2019; Gu et al., 2019; Zeng et al., 2019). Further, particulate matter <10 μm (PM_{10}) and nitrogen dioxide (NO_2) are associated with depression in the meta-analysis reported by Zeng et al. (2019). However, the latest systematic review and meta-analysis with the sophisticated inverse variance heterogeneity model reported that exposure to $PM_{2.5}$, PM_{10}, and ozone (O_3) is not associated with depression (Fan et al., 2020). Therefore, current evidence for the impact of air pollution on depression is inconclusive. In addition, only the small number of studies explored the relationship between air pollution and anxiety (Pun et al., 2017; Shin et al., 2018; Vert et al., 2017; Zhao et al., 2020), which warrants further research.

Although pregnancy has been identified as a vulnerable window for the detrimental impacts of air pollution (Hannam et al., 2013), the majority of previous studies focused on non-pregnant populations, and increased exposure to air pollution had been linked to depressive and anxiety symptoms among adults in South Korea (Shin et al., 2018), general population in Germany (Zhao et al., 2020), elderly population in the United States (Pun et al., 2017), and in women in the Nurses' Health Study in the United States (Kioumourtzoglou et al., 2017; Power et al., 2015). Pregnant women may be especially vulnerable to air pollution due to their increased ventilation rate for the higher oxygen requirements of the developing fetus and a decreased oxygen-binding capability (Kannan et al., 2006). However, few studies have investigated the association between air pollution and mental disorders in pregnant women (Ahlers and Weiss, 2021; Kanner et al., 2021; Lin et al., 2017); no studies have examined the effect of air pollution on prenatal anxiety. Further, for ambient ozone, an air pollutant and potent oxidant, the relationship between exposure to O_3 in different trimesters of pregnancy and prenatal depression or anxiety has not been examined.

This study aimed to investigate the effect of prenatal exposure to air pollution during different trimesters on maternal depressive and anxiety symptoms, which were evaluated at 36th week of pregnancy using the Center for Epidemiologic Studies-Depression (CES-D) and State-Trait Anxiety Inventory (STAI) scales, respectively, hypothesizing that increased level of air pollution would be associated with higher risk for depression and anxiety in our sample of pregnant women.

2. Materials and methods

2.1. Study population

This study was conducted as part of the Cohort for Childhood Origin of Asthma and Allergic disease (COCOA), a prospective hospital-based birth cohort study conducted in South Korea. Details of the COCOA study have been previously published (Shin et al., 2013; Yang et al., 2014). In this study, pregnant women before 26 weeks of gestational age were recruited at five medical centers and eight public health centers in the Seoul metropolitan area between 2008 and 2015. For this analysis, subjects were limited to those with maternal depression and anxiety data. Of the 3102 women recruited, 870 women without depression and anxiety data and 83 with multiple records were excluded. Women with missing information on residential address, preterm births, and chronic diseases (thyroid diseases, cardiovascular disease, cancer, and tuberculosis) were not included in the study. Those with missing covariate information were also excluded. Supplementary material (Fig. S1) provides further details of the exclusion criteria. The final study population consisted of 1481 pregnant women. In general, there were no significant differences in general characteristics of pregnant women between the included and excluded participants, except for gestational age (Table S1). Prior to enrollment, written informed consent was obtained from all study subjects. The study protocol was approved by the institutional review boards (IRBs) of Asan Medical Center (IRB No. 2008-0616), Samsung Medical Center (IRB No. 2009- 02–021), Yonsei University (IRB No. 4-2008-0588), the CHA Medical Center (IRB No. 2010-010), and Seoul National University (IRB No. 1401-086-550).

2.2. Exposure assessment

We obtained hourly air pollution data from the Korean Ministry of Environment (http://www.airkorea.or.kr/web) measured at a maximum of 40 regulatory air pollution monitoring sites in Seoul from 2007 to 2015. Monthly exposures to particulate matter ($PM_{2.5}$ and PM_{10}), NO_2, and O_3 at residential addresses were estimated using a land use regression (LUR) model, as previously described (Lee et al., 2012; Yang et al., 2020). The LUR model contains several geographical variables, such as traffic indicators, surrounding-land use, topography, and spatial trends, and the final LUR model includes lengths of roads, traffic intensities on nearest roads, total heavy-duty traffic on all roads, and a variable representing spatial trends. Model performance was assessed using leave-one-out cross-validation (LOOCV). The models explained 66–81% of the variability in the measured $PM_{2.5}$, PM_{10}, NO_2, and O_3 levels, and the predicted values fitted well with the measured values, as reported elsewhere (Lamichhane et al., 2017). The model-adjusted R^2

and LOOCV R^2 for $PM_{2.5}$, PM_{10}, NO_2, and O_3 were 0.66 and 0.56, 0.69 and 0.60, 0.79 and 0.73, and 0.81 and 0.77, respectively. We calculated average particulate matter exposures over pregnancy using clinically defined trimesters (1st trimester: 1–13 weeks, 2nd trimester: 14–27 weeks, 3rd trimester: 28 weeks until birth) using gestational age, calculated from the last menstrual period, and birth dates.

2.3. Outcomes assessment

Maternal depressive symptoms were evaluated during the third trimester of pregnancy using the CES-D-10 scale, which is a brief screening tool for depressive symptoms (Andersen et al., 1994). The CES-D-10 has been shown to be acceptable for screening depression during pregnancy (Chang et al., 2016). Items were rated using a 4-point Likert scale (0, 1, 2, 3). A sample question from the scale is as follows: "I was bothered by the things that usually do not bother me." We calculated the sum of all question-specific scores as the CES-D-10 score to indicate the general depression status of each subject. The score ranges from 0 to 30, with a higher score indicating a higher severity of depression. Maternal depressive symptoms were defined as a dichotomous measure using an optimal threshold of CES-D-10 score (CES-D-10 $\geq$ 12), as this cutoff generated most balance sensitivity and specificity for the detection of depressive symptoms (Baron et al., 2017). In the current study, the internal consistency of CES-D-10 indicated excellent reliability (Cronbach's alpha = 0.882).

We assessed the level of anxiety of pregnant women by using the Korean version of STAI (K-STAI) (Kim and Shin, 1978; Spielberger, 1972). COCOA participants were asked to complete a 20-item trait anxiety subscale of K-STAI to indicate a general tendency to perceive situations as threatening (Yang et al., 2014). Each item of subscale scored on a 4-point Likert scale ("not at all", "somewhat", "moderately so" and "very much so"), and total possible scores range from 20 to 80 points, with higher scores indicating more anxiety. We used a cutoff score of $\geq$40 to identify clinically significant symptoms of anxiety, which was validated in pregnant women and was reported as a predictor of postpartum anxiety and mood states (Grant et al., 2009; Kimmel et al., 2021). In the current study, the Cronbach's alpha of the trait anxiety subscale of K-STAI was 0.937.

2.4. Other variables

Potential confounding variables, including mother's age ($\geq$35 vs. <35 years), maternal education, occupation during pregnancy (yes vs. no), pre-pregnancy body mass index (BMI) (kg/m^2: $\geq$25 vs. <25), birth order (first-born vs. second or later-born), smoking history (ever vs. never), family income, and drinking during pregnancy (yes vs. no), were ascertained at baseline. Mother's education was categorized into three levels: secondary school, college or university, and graduate school. Family income was dichotomized as high ($\geq$4 million Korean won per month) or low (<4 million Korean won per month) (Cho et al., 2018). The data on gestational age in weeks was obtained from medical records at delivery. We used children's birth season to indicate a seasonal variation in the depressive and anxiety symptoms. The birth season was defined according to weather patterns in Korea as warm (April to September) and cold (October to March). Residential-related variable included pets keeping during pregnancy and the past years. Previous studies indicated that these variables were related to mental disorders and may affect the estimated association between exposure to air pollution and depressive and anxiety symptoms (Biggai et al., 2016; Brooks et al., 2018; Lee et al., 2007).

2.5. Statistical analysis

Descriptive statistics are presented as mean, standard deviation (SD), interquartile range (IQR), and frequency (%). Modified Poisson regression with robust error variance approach of Zou (2004), which provides estimates of relative risk (RR), was used to investigate the association between air pollutants averaged over the full gestational period and depressive and anxiety symptoms. RR was estimated by an interquartile range (IQR) increases in pollutant concentrations averaged over the pregnancy. To facilitate comparison of results, we were consistent in the use of a whole-pregnancy IQR across the analyses. A p-value of less than 0.05 was considered statistically significant.

We adjusted for a priori-specified factors that could potentially confound the association between air pollution and depression and anxiety. Models were adjusted for maternal age, pre-pregnancy BMI, birth order, history of smoking, drinking during pregnancy, maternal education, maternal occupation, gestational age, birth season, family income, and pet ownership. The potential nonlinear relationship of air pollutants with the depressive symptoms was examined by utilizing restricted cubic splines with knots at the fifth, 35th, 65th, and 95th percentiles of the distribution of air pollution concentrations (Desquilbet and Mariotti, 2010). A test for nonlinearity was conducted by testing the regression coefficient of nonlinear term, with p for nonlinearity <0.05 indicating a non-linear association. The p-value for overall association indicated that the regression coefficients of both linear and non-linear terms of the factor were equal to zero. We also conducted stratified analyses to assess potential effect modification by selected maternal characteristics. Stratum-specific RRs were obtained by modified Poisson regression. We evaluated the significance of effect modification on the multiplicative scale by including an interaction (product) term between air pollution exposure and each characteristic. Statistical analyses were conducted using STATA (version 16.0; Stata Corporation).

To confirm the robustness of our findings, several sensitivity analyses were performed. First, we ran multi-pollutant models for pregnancy exposure to all pollutants except for PM_{10} due to its high correlation with $PM_{2.5}$ (Table S1). Second, we considered different cutoffs for depressive (CES-D-10 $\geq$ 10, CES-D-10 $\geq$ 11, and CES-D-10 $\geq$ 13) and anxiety (K-STAI $\geq$ 39, K-STAI $\geq$ 41, and K-STAI $\geq$ 44) symptoms. Third, we considered depression and anxiety as a continuous rather than binary measure. Negative binomial regression was used to examine the association between the air pollutants and continuous scale of CES-D-10 and K-STAI scores due to evidence of overdispersion. Fourth, we reanalyzed the models using multiple imputation technique. Fifth, the associations between air pollution and depressive and anxiety symptoms were investigated for trimester-specific exposure, where exposure during the three trimesters were simultaneously included (multi-trimester model). Finally, we calculated E-values to determine the degree to which potential unmeasured confounding could explain away associations between air pollution and depressive and anxiety symptoms (VanderWeele and Ding, 2017).

3. Results

Table 1 presents the summary statistics of the study population. Among the 1481 pregnant women, 221 (14.9%) and 827 (55.8%) had depressive (CES-D-10 $\geq$ 12) and anxiety (K-STAI $\geq$ 40) symptoms, respectively: none of them were being medically treated by physicians or used any antidepressants. The mean age was 32.9 years and the mean BMI was 20.7 kg/m^2. Most participants had high income (62.9%), with the majority of women reported to be never smokers (92.7%).

The mean trimester-specific air pollutant concentrations for the study period are reported in Table 2. The average concentrations of exposure during pregnancy were 26.9 μg/m^3, 49.8 μg/m^3, 34.6 ppb, and 43.2 ppb for $PM_{2.5}$, PM_{10}, NO_2, and O_3, respectively. The correlation of pollutants is provided in supplementary material (Table S2). The trimester-specific mean concentrations of pollutants in subjects showed very weak to strong correlations (Pearson's correlation coefficient r = −0.17 to 0.89), with the highest Pearson's correlation coefficient between $PM_{2.5}$ and PM_{10} at the first trimester. PM_{10} showed a moderate positive correlation with NO_2 (r = 0.46 to 0.51), whereas NO_2 showed a moderate negative correlation with O_3 (r = −0.48 to −0.53).

Table 1
Descriptive statistics for the study population (n = 1481).

Characteristics	n (%) or mean (SD)
Age (years)	32.9 (3.6)
Age group	
<35	1033 (69.7)
≥35	448 (30.3)
Pre-pregnancy BMI (kg/m^2)	20.7 (2.5)
BMI group	
<25	1387 (93.6)
≥25	94(6.4
Birth order	
First-born	984 (66.4)
Second or later-born	497 (33.6)
History of smoking	
Never	1373 (92.7)
Ever	108 (7.3)
Drinking during pregnancy	
No	1382 (93.3)
Yes	99 (6.7)
Occupation	
No	498 (33.6)
Yes	983 (66.4)
Education	
Secondary school	109 (7.4)
College or university	1039 (70.2)
Graduate school	333 (22.5)
Gestational age (weeks)	39.3 (1.1)
Family income	
High (≥4 million per month)	932 (62.9)
Low (<4 million per month)	549 (37.1)
Pet ownership	
No	1393 (94.1)
Yes	88 (5.9)
Birth season	
Warm	635 (42.9)
Cold	846 (57.1)
CES-D-10 score	6.6 (4.7)
CES-D-10 ≥ 12	221 (14.9)
K-STAI score	40.6 (9.0)
K-STAI ≥ 40	827 (55.8)

BMI, body mass index; CES-D-10, center for epidemiologic studies depression scale-10; K-STAI, Korean version of state-trait anxiety inventory. Numbers in the table are mean (standard deviation) or n (%).

Table 2
Mean air pollution exposures during pregnancy.

Pollutant and trimester	Mean	IQR	Min - Max
$PM_{2.5}$(μg/m^3)			
First	26.9	11.3	11.4–57.5
Second	26.6	11.2	12.2–62.0
Third	27.2	10.8	11.7–57.9
Pregnancy	26.9	6.9	14.8–52.5
PM_{10} (μg/m^3)			
First	49.8	19.6	24.3–77.9
Second	49.2	19.6	25.6–81.5
Third	50.4	19.1	24.5–85.6
Pregnancy	49.8	8.5	34.6–68.9
NO_2 (ppb)			
First	34.5	12.0	2.0–74.0
Second	34.4	12.0	2.0–70.0
Third	35.0	12.0	3.0–81.0
Pregnancy	34.6	9.0	2.0–65.0
O_3 (ppb)			
First	44.6	26.0	5.0–79.0
Second	43.1	25.0	9.0–83.0
Third	41.9	25.0	8.0–85.0
Pregnancy	43.2	10.0	9.0–69.0

IQR, interquartile range; PM_{10}, particulate matter with aerodynamic diameters ≤ 2.5 μm; $PM_{2.5}$, particulate matter with aerodynamic diameters ≤ 2.5 μm; NO_2, nitrogen dioxide; O_3, ozone.

Table 3
Single-pollutant model of associations between air pollution and depressive and anxiety symptoms among pregnant women.[a].

Air pollutants	Trimester	Depression (CES-D-10 ≥ 12)		Anxiety (K-STAI ≥ 40)	
		Unadjusted model	Adjusted model[b]	Unadjusted model	Adjusted model[b]
		RR (95% CI)	RR (95% CI)	RR (95% CI)	RR (95% CI)
$PM_{2.5}$ (IQR: 6.9 μg/m^3)	First	1.01 (0.91, 1.12)	1.03 (0.91, 1.17)	1.05 (1.01, 1.08) **	1.04 (0.99, 1.09) $^+$
	Second	1.13 (1.02, 1.24) *	1.15 (1.04, 1.27) **	1.04 (1.00, 1.07) *	1.07 (1.02, 1.12) **
	Third	1.00 (0.89, 1.13)	1.00 (0.88, 1.13)	1.01 (0.97, 1.04)	1.03 (0.98, 1.08)
	Pregnancy	1.09 (0.95, 1.26)	1.12 (0.97, 1.28)	1.07 (1.01, 1.11) *	1.07 (1.01, 1.13) *
PM_{10} (IQR: 8.5 μg/m^3)	First	0.99 (0.92, 1.08)	1.00 (0.90, 1.12)	1.03 (1.00, 1.06) *	1.03 (0.98, 1.07)
	Second	1.11 (1.02, 1.21) **	1.13 (1.04, 1.23) **	1.02 (0.99, 1.05)	1.07 (1.02, 1.12) **
	Third	0.98 (0.89, 1.08)	0.96 (0.87, 1.07)	0.99 (0.97, 1.02)	1.02 (0.97, 1.06)
	Pregnancy	1.11 (0.95, 1.30)	1.13 (0.96, 1.31)	1.05 (0.99, 1.11)	1.08 (1.01, 1.16) *
NO_2 (IQR: 9.0 ppb)	First	1.05 (0.94, 1.17)	1.14 (1.01, 1.28) *	1.02 (0.99, 1.06)	1.05 (1.00, 1.10) *
	Second	1.08 (0.96, 1.20)	1.15 (1.03, 1.29) *	1.02 (0.98, 1.06)	1.07 (1.02, 1.12) **
	Third	0.91 (0.81, 1.03)	0.95 (0.85, 1.07)	0.99 (0.95, 1.03)	1.01 (0.96, 1.06)
	Pregnancy	1.03 (0.90, 1.17)	1.11 (0.97, 1.27)	1.02 (0.97, 1.06)	1.05 (1.00, 1.11) *
O_3 (IQR: 10.0 ppb)	First	0.91 (0.84, 0.99) *	0.89 (0.82, 0.96) **	0.99 (0.97, 1.02)	0.97 (0.93, 1.01)
	Second	0.97 (0.89, 1.05)	0.95 (0.86, 1.06)	1.01 (0.98, 1.04)	0.97 (0.93, 1.02)
	Third	1.10 (1.02, 1.19) *	1.09 (1.01, 1.18) *	1.01 (0.98, 1.03)	1.04 (0.99, 1.09) $^+$
	Pregnancy	0.95 (0.82, 1.10)	0.91 (0.78, 1.06)	1.01 (0.96, 1.06)	0.98 (0.92, 1.05)

$^+p < 0.10$, $^*p < 0.05$, $^{**}p < 0.01$.

[a] Relative risk (RR) estimated from modified Poisson regression models with robust error variance, representing RRs for prenatal depression and anxiety as dichotomous outcomes.

[b] Models adjusted for maternal age, history of smoking, drinking during pregnancy, pre-pregnancy BMI, maternal education, gestational age, birth order, family income, and pet ownership.

Table 3 shows results from modified Poisson regression represented as RRs and 95% CIs in the single-pollutant models. The results from fully adjusted models showed significant positive associations between an IQR increase of $PM_{2.5}$ (RR = 1.15, 95% CI: 1.04, 1.27), PM_{10} (RR = 1.13, 95% CI: 1.04, 1.23), and NO_2 (RR = 1.15, 95% CI: 1.03, 1.29) during the second trimester and maternal depressive symptoms. A significant

association of NO_2 was also seen for the first-trimester exposure. For O_3, a significant positive association was observed for exposure during the third trimester (RR = 1.09, 95% CI: 1.01, 1.18); however, the exposure in the first trimester was associated with a decreased risk of depressive symptoms (RR = 0.89, 95% CI: 0.82, 0.96). Similarly, in the adjusted models, an IQR increase in exposure to $PM_{2.5}$ and PM_{10} during the second trimester was significantly associated with anxiety symptoms. Exposure to NO_2 during the first and second trimesters was significantly associated with anxiety symptoms, with a larger magnitude of RR for the second-trimester exposure (RR = 1.07, 95% CI: 1.02, 1.12). A positive association was found between the exposure to O_3 during the third trimester and anxiety symptoms, but the association was only marginally significant ($p < 0.10$). Patterns of associations from adjusted models were generally consistent with those from unadjusted models.

Spline regression analysis showed that the overall associations of $PM_{2.5}$ and PM_{10} during the second trimester with depressive symptoms were significant ($p = 0.049$; $p = 0.014$, respectively) (Fig. 1A and B): NO_2 during the second trimester and O_3 during the third trimester were only marginal level of significance for the overall association ($p = 0.101$; $p = 0.085$, respectively) (Fig. 1C and D). The p value testing for nonlinearity was marginally significant for PM_{10} ($p = 0.051$) and for other pollutants, p values were insignificant ($PM_{2.5}$, $p_{nonlinearity} = 0.982$; NO_2, $p_{nonlinearity} = 0.916$; and O_3, $p_{nonlinearity} = 0.172$), indicating a linear exposure-response function as shown in Fig. 1. The spline curves for exposure to $PM_{2.5}$, PM_{10}, and NO_2 during the first and third trimesters and exposure to O_3 during the first and second trimesters did not have significant positive overall associations and did not significantly deviate from linearity (Fig. S2). As with depression, the overall associations between exposure to $PM_{2.5}$ and PM_{10} during the second trimester and anxiety symptoms were significant (Figs. S3B and F). In addition, exposure to NO_2 during the second trimester had a significant overall association ($p = 0.038$) (Fig. S3J). The tests of nonlinearity in the associations between exposure to $PM_{2.5}$, PM_{10}, NO_2, and O_3 and anxiety symptoms were insignificant at the 0.05 level (Fig. S3), which were consistent with depression.

Effect modifications of the association between maternal depressive and anxiety symptoms and exposures to $PM_{2.5}$, PM_{10}, and NO_2 during the second trimester and O_3 during the third trimester, the exposure windows that show generally significant associations, are presented in Fig. 2 and supplementary material (Tables S3 and S4). We found no significant effect modification by the eight risk factors for the association between PM_{10} and depressive symptoms (Fig. 2). The results for the other three pollutants examined were also consistent with the findings for PM_{10} (Table S3). We observed stronger associations of $PM_{2.5}$ and PM_{10} with anxiety symptoms for individuals with a history of smoking ($PM_{2.5}$, p-interaction = 0.021 and PM_{10}, p-interaction = 0.049) (Table S4). In addition, family income was found to be an effect modifier in the association of NO_2 exposure with anxiety symptoms (p-interaction = 0.025), with stronger association in participants with lower income (Table S4).

In the multi-pollutant models (Table 4), the association of $PM_{2.5}$ during the second trimester with maternal depressive symptoms was slightly attenuated (RR = 1.12, 95% CI: 1.01, 1.25), but remained statistically significant after adjusting for NO_2 and O_3. The multi-pollutant model results were similar to the single-pollutant model for O_3. The multi-pollutant model for NO_2 remained positive but was not significant after adjusting for $PM_{2.5}$ and O_3 concentrations. For anxiety, the associations were consistent across single- and multi-pollutant models

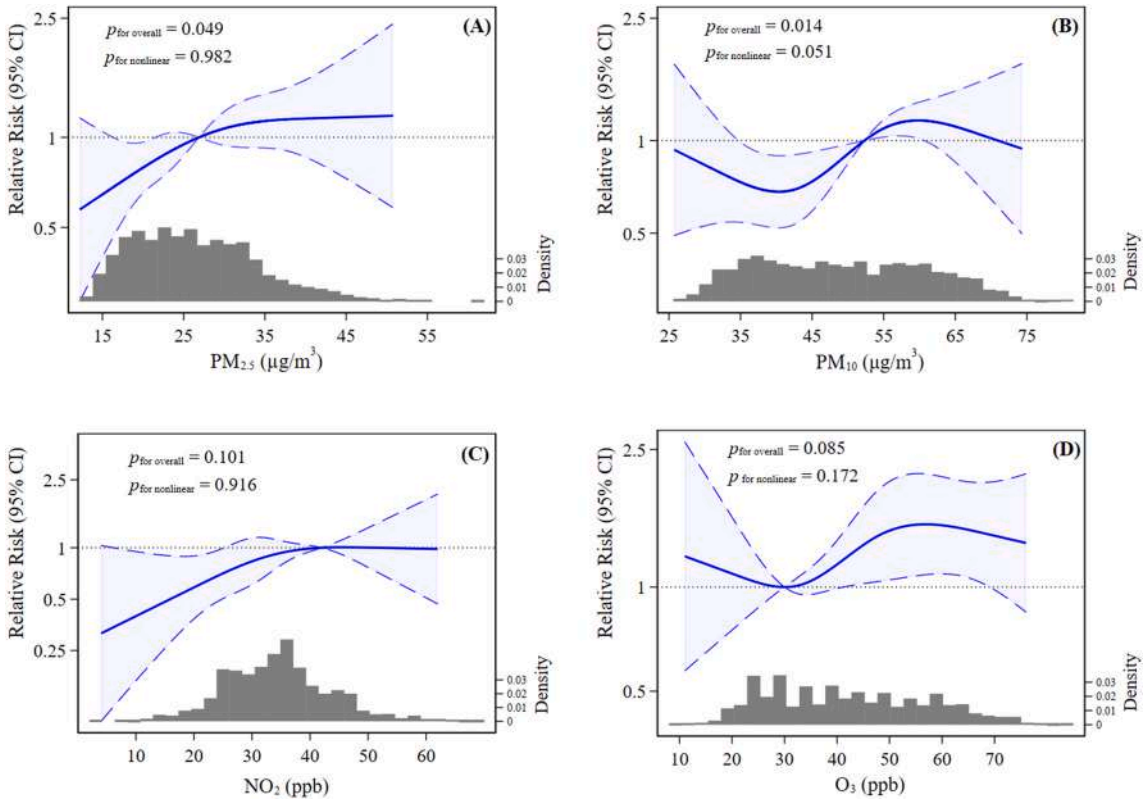

Fig. 1. Nonlinear effects of $PM_{2.5}$ (A), PM_{10} (B), NO_2 (C), and O_3 (D) concentrations on maternal depressive symptoms (CES-D-10 ≥ 12). This dose-response curve was calculated using restricted cubic splines with knots at the 5th, 35th, 65th, and 95th percentile of the distribution of $PM_{2.5}$, PM_{10}, and NO_2 concentrations during the second trimester and O_3 concentration during the third trimester. The solid line represents the relative risks, and the dashed lines represent the confidence intervals. The reference $PM_{2.5}$, PM_{10}, NO_2, and O_3 for these plots (with RR fixed as 1.0) was 26.9 μg/m^3, 52.3 μg/m^3, 42.0 ppb, and 30.0 ppb, respectively. The relative risks were adjusted for maternal age, history of smoking, drinking during pregnancy, pre-pregnancy BMI, maternal education, gestational age, birth order, family income, and pet ownership. The histograms show the distribution of $PM_{2.5}$ (A), PM_{10} (B), NO_2 (C), and O_3 (D) exposures.

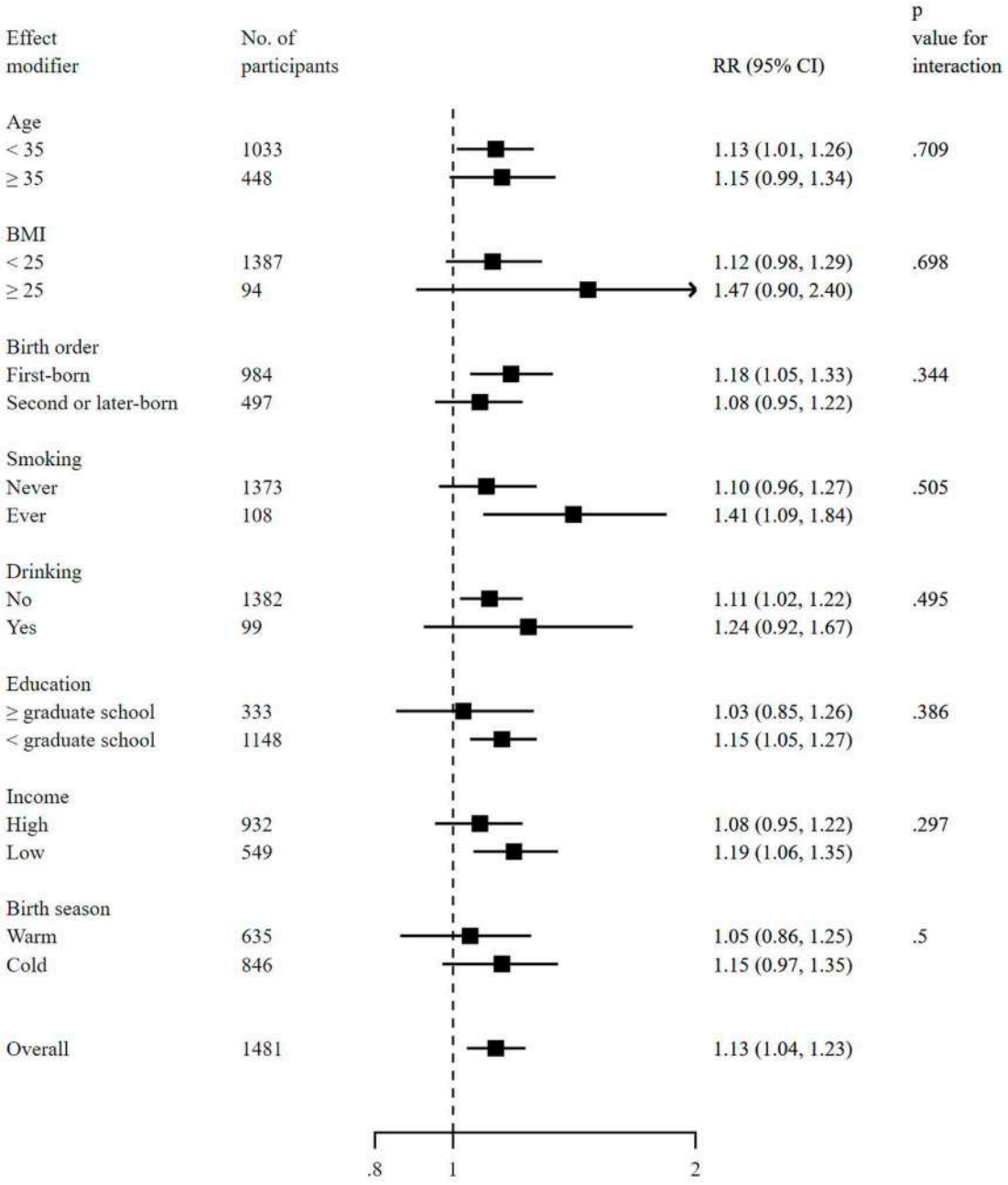

Fig. 2. Relative risks (95% confidence intervals) for maternal depressive symptoms (CES-D-10 ≥ 12) associated with an IQR increase in PM_{10} concentration during the second trimester, stratified by a modifier. Analyses were adjusted for maternal age, history of smoking, drinking during pregnancy, pre-pregnancy BMI, maternal education, gestational age, birth order, family income, and pet ownership. Education was categorized as graduate level or above and less than graduate level, including no education, elementary school, middle school, high school, technical college, and undergraduate school.

(Table S5). When we evaluated the associations of air pollutants with depressive and anxiety symptoms by changing the depression and anxiety cutoff scores, the associations were robust for depression (Table S6). For anxiety, significant associations for $PM_{2.5}$, PM_{10}, and NO_2 during the second trimester were observed at a cutoff ≥39, and a higher cutoff score (≥44) was found to be significant only for $PM_{2.5}$ and O_3 (Table S7). The associations of air pollution with depressive and anxiety symptoms were consistent with regard to direction of association and critical windows of susceptibility to air pollution across binary and continuous outcomes (Table S8). Our findings were also similar when examining the association using the multiple imputation technique (Table S9). We found that positive associations of exposure to $PM_{2.5}$, PM_{10}, and NO_2 during the second trimester and O_3 during the third trimester with depressive and anxiety symptoms were consistently significant in the trimester model (Table S10). This finding suggested that the second and third trimesters are the sensitive windows for maternal exposure to $PM_{2.5}$, PM_{10}, NO_2, and O_3 and development of prenatal depressive and anxiety symptoms. Further, sensitivity analyses to unmeasured confounding showed that our results were robust to unmeasured confounding bias (Table S11). For example, the E-value for the observed association estimates of exposure to PM_{10} during the second trimester and depressive symptoms was 1.51 (1.24 for the lower confidence interval) (Table S11). This indicates that if the observed RR of 1.13 for PM_{10} in the second trimester would be completely due to unmeasured confounder, a 1.51-fold association between unmeasured confounder and depressive symptoms would be required.

Table 4
Multi-pollutant models of associations between exposure to air pollutants during pregnancy and depressive symptoms (CES-D-10 ≥ 12) among pregnant women.[a].

Trimester	+ $PM_{2.5}$	+ NO_2	+ O_3	+ $PM_{2.5}$ + NO_2 + O_3
	RR (95% CI)[b]	RR (95% CI)[b]	RR (95% CI)[b]	RR (95% CI)[b]
First				
$PM_{2.5}$	–	1.000.87, 1.15)	1.01 (0.90, 1.15)	1.00 (0.88, 1.14)
NO_2	1.13 (0.99, 1.28) +	–	1.05 (0.92, 1.20)	1.05 (0.91, 1.21)
O_3	0.89 (0.82, 0.97) **	0.90 (0.82, 0.99) *	–	0.90 (0.82, 0.99) *
Second				
$PM_{2.5}$	–	1.12 (1.01, 1.25) *	1.15 (1.03, 1.27) **	1.12 (1.01, 1.25) *
NO_2	1.08 (0.96, 1.22)	–	1.14 (1.00, 1.30) *	1.09 (0.95, 1.25)
O_3	0.98 (0.89, 1.09)	1.01 (0.90, 1.13)	–	1.01 (0.90, 1.13)
Third				
$PM_{2.5}$	–	1.02 (0.89, 1.16)	1.01 (0.89, 1.15)	1.01 (0.89, 1.15)
NO_2	0.94 (0.83, 1.06)	–	1.01 (0.88, 1.15)	1.00 (0.87, 1.15)
O_3	1.09 (1.01, 1.18) *	1.09 (1.00, 1.19) *	–	1.09 (1.00, 1.19) *
Pregnancy				
$PM_{2.5}$	–	1.10 (0.95, 1.28)	1.11 (0.96, 1.28)	1.10 (0.95, 1.27)
NO_2	1.07 (0.94, 1.22)	–	1.07 (0.92, 1.25)	1.05 (0.90, 1.23)
O_3	0.93 (0.79, 1.10)	0.95 (0.78, 1.15)	–	0.96 (0.79, 1.16)

$^{+}p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$.

[a] Relative risk (RR) estimated from modified Poisson regression models with robust error variance, representing RRs for depressive symptoms as a dichotomous outcome. RR (95% CIs) were estimated for per IQR increase in $PM_{2.5}$, NO_2, and O_3.

[b] Multi-pollutant model was further adjusted for the effects of other air pollutants in the same time window on the adjusted single-pollutant model in Table 3.

4. Discussion

This prospective cohort study showed significant positive associations between higher concentrations of air pollutants and maternal depressive (CES-D-10 ≥ 12) and anxiety (K-STAI ≥ 40) symptoms during pregnancy. An IQR increase in exposure to $PM_{2.5}$, PM_{10}, and NO_2 during the second trimester and O_3 during the third trimester were associated with increased risks of depressive symptoms during the third trimester, 15% for $PM_{2.5}$, 13% for PM_{10}, 15% for NO_2, and 9% for O_3. We also found increased risks of anxiety symptoms associated with an IQR increase in exposure to $PM_{2.5}$, PM_{10}, and NO_2 during the second trimester. Exposure to O_3 during the third trimester was significantly associated with a higher cutoff score of anxiety (≥44). Estimates were mostly closer to the null for other trimesters. The findings were robust across different sensitivity analyses. Our findings suggest that exposure to particulate matter, NO_2, and O_3 during mid to late pregnancy may be associated with mental disorders during pregnancy. No evidence of effect modification was found for the associations between four pollutants examined in this study and depressive symptoms. However, we observed enhanced associations for some pollutants with anxiety symptoms among women who were of low income or had history of smoking.

To our knowledge, this is the first study to investigate the effect of air pollution exposure during a particular period of gestation on depressive and anxiety symptoms among pregnant women. To date, only three epidemiologic studies have examined the association of air pollution with mental health risk among pregnant women (Ahlers and Weiss, 2021; Kanner et al., 2021; Lin et al., 2017). In line with our findings for depression, a recent study in the United States evaluated the relationship between maternal depression and air pollution exposure during 3-month preconception, first trimester, and whole pregnancy, and found that an IQR increase in PM_{10}, $PM_{2.5}$, and NO_2 exposure during the whole pregnancy was associated with 11%–21% increased risk of maternal depression, as defined by International Classification of Diseases (ICD) codes (Kanner et al., 2021). However, this study identified maternal depression based on delivery admission medical records and was unable to provide the timing of diagnosis. Furthermore, they were unable to provide symptoms of depression and may have missed a high proportion of subjects who did not realize that they had depressive symptoms (Bell et al., 2011). Based on data from 50 pregnant women, Ahlers and Weiss (2021) found significant positive associations between prenatal exposure to $PM_{2.5}$ and depressive symptoms in the third trimester, which was assessed by the 9-item Patient Health Questionnaire. A study in China examined the association between air pollution and maternal stress during pregnancy, reporting that exposure to $PM_{2.5}$ and NO_2 was associated with higher scores on Global-Severity-Indices, indicating higher levels of emotional stress (Lin et al., 2017). However, they did not find significant associations specifically with depressive symptoms. We found mid-to-late pregnancy as a vulnerable window for the detrimental impact of air pollution on maternal depressive symptoms, which is consistent with a study in the United States that showed association of $PM_{2.5}$ exposure in mid-pregnancy with postpartum depression based on the Edinburgh Postnatal Depression Scale (EPDS) (Sheffield et al., 2018). Likewise, a recent study in Mexico reported that higher $PM_{2.5}$ in pregnancy was associated with increased maternal postpartum depression, as measured by EPDS score (Niedzwiecki et al., 2020).

To the best of our knowledge, no study has examined the association between ambient air pollution, particularly O_3, and anxiety symptoms during pregnancy. Nevertheless, two studies (Lin et al. (2017; Niedzwiecki et al., 2020) partially investigated the association of our interest. Lin et al. (2017) explored the lag effect of PM_{10} and NO_2 on anxiety subscale of the Symptom Checklist-90-Revised Scale during pregnancy and found no significant associations. Similarly, Niedzwiecki et al. (2020) found no significant association of $PM_{2.5}$ exposure during pregnancy with anxiety subscale of EPDS at 6 months postpartum.

Previous studies in non-pregnant populations reported significant positive associations of short or long-term air pollutant concentrations with depression and anxiety (Kioumourtzoglou et al., 2017; Lim et al., 2012; Pun et al., 2017; Zhang et al., 2019; Zhao et al., 2020). A recent prospective study in Korea (n = 123045) found that long-term exposure to PM_{10} is associated with an increased risk of developing depression, assessed using the CES-D scale, during follow-up (mean follow-up 2.5 years) in middle-aged women (Zhang et al., 2019). A panel study including 560 elderly population in Korea reported that increase in short-term PM_{10}, NO_2, and O_3 was significantly associated with depressive symptoms based on the Geriatric Depression scale (Lim et al., 2012). A cohort study among middle-aged and older women in the United States (n = 41844) found an association between higher $PM_{2.5}$ and O_3 exposure in the past year and an increased risk of depression diagnosis and antidepressant use (Kioumourtzoglou et al., 2017). A study in the general population, conducted in Germany, found that increased levels of PM_{10} and O_3 were associated with increased risks of diagnoses of depression and anxiety, the diagnoses were done according to ICD codes (Zhao et al., 2020). A cohort study among elderly subjects in the United States (n = 4008) identified significant positive associations of $PM_{2.5}$ exposure with depressive and anxiety symptoms based on the CES-D scale (11-items) and Hospital Anxiety and Depression Scale, respectively, particularly among individuals with lower socioeconomic status (Pun et al., 2017). Our findings of significant associations between exposure to particulate matter, NO_2, and O_3 during pregnancy and prenatal depressive or anxiety symptoms are supported by these studies conducted in non-pregnant populations. Nevertheless, some studies found no significant association of air pollution with depression and

anxiety (Lin et al., 2017; Wang et al., 2014; Zijlema et al., 2016). The inconsistencies in studies may have arisen from the differences in study areas and populations, exposure levels, exposure assessment methods, measured pollutants, study design, use of depression and anxiety scales, sample size, and covariates included in models (Pun et al., 2017).

In our study, we observed similar patterns of risk among pollutants averaged over the second trimester for particulate matter and NO_2, reporting increased risks of depression and anxiety for these pollutants. Average O_3 exposure during the third trimester showed a significant positive association with depressive symptoms. On the other hand, the findings for O_3 averaged over the first trimester are in contrast to the findings for the third trimester. We reported that O_3 exposure during the first trimester was associated with a modest decrease in the risk of depression (11%), which is consistent with the finding that O_3 during pregnancy is associated with a decreased odd of any depression (Kanner et al., 2021). Ozone is a secondary pollutant and is produced by photochemical reactions involving primary pollutants (Cho et al., 2014). This may explain the reason why it is associated with lower risk in some time frames. Ozone was negatively correlated with all other pollutants investigated in our study, most strongly with PM_{10} ($r = -0.71$), which corroborated the finding that O_3 levels are often inversely related to particulate matter (Jia et al., 2017).

Different pathways may explain the biological mechanisms linking air pollution to mental disorders. The pathways include oxidative stress or inflammation (Black et al., 2015; Han et al., 2016), the dysregulation of the endocrine system or metabolic processes (Miller et al., 2016; Thomson et al., 2018), and the disturbance of neurotransmitters (Gonzalez-Pina and Paz, 1997). Air pollutants, such as particulate matter, nitrogen oxides, and O_3 are potent oxidants and they may reach the brain and affect the brain by oxidative stress and neuroinflammation (Fonken et al., 2011; Genc et al., 2012). Mice models demonstrated that exposure to dim light at night and $PM_{2.5}$ may result in neuroinflammation, alter the hippocampal structure, and induce a depressive-like response (Hogan et al., 2015). Chronic inflammation in the brain leads to the formation of reactive oxygen species and oxidative stress, which may disrupt the blood-brain barrier and alter the immune response, and thereby affect normal brain function (Calderon-Garciduenas et al., 2015). Furthermore, oxidative stress may also lead to anxiety-depression-like behaviors in rats (Patki et al., 2013)

The present study has several limitations that warrant consideration. First, exposure to air pollution was estimated using the LUR model based on maternal residential address, and this model did not account for exposure when women were away from their homes (e.g., at places of employment or during transportation), which may have resulted in some degree of exposure misclassification. Researchers have reported that maternal mobility during pregnancy is usually limited and generally restricted to residential areas (Bell and Belanger, 2012). Thus, we believe this shortcoming had little effect on our exposure estimates and was unlikely to have changed our findings. Second, depression and anxiety were measured using self-reports and were assessed only once during pregnancy. Third, although we adjusted for a number of important variables considered to potentially confound the association between air pollution and mental disorders, we cannot dismiss the possibility that other potential confounders such as noise pollution, meteorologic conditions, sleep deficit, history of previous delivery, and maternal occupational exposure may have confounded the identified associations. Furthermore, stressful life events, such as serious parental illness, accidents, or marital dissatisfaction, may have played important roles in the onset of mental disorders among pregnant women (Lancaster et al., 2010; Park et al., 2014). However, we evaluated E-values to see the strength of the relationship a hypothetical unmeasured confounder would have with the air pollution and mental disorders to fully account for our results, suggesting that unmeasured confounders might not have a major influence on the association. Finally, while the homogenous makeup of the COCOA study in the Korean population provided excellent internal validity, its generalizability in other populations may be limited.

5. Conclusions

In the present study, average concentrations of $PM_{2.5}$, PM_{10}, and NO_2 assigned to the residential address of each pregnant woman were positively associated with maternal depressive and anxiety symptoms. The results for O_3 and depression varied depending on the time of exposure; a significant positive association during third-trimester exposure, while it was negative during first-trimester exposure. Our findings add further evidence to recent data suggesting that air pollution is responsible for neuropsychological dysfunction in pregnant women and further reduction of air pollution might provide an effective avenue for reducing the burden of mental disorders. Future research on the influence of air pollution on maternal depression and anxiety may further the understanding of how individual psychological characteristics affect depressive and anxiety symptoms during pregnancy.

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Declaration of competing interest

The authors have no conflict of interest to declare.

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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.2021.113823.

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Associations between rice consumption, arsenic metabolism, and insulin resistance in adults without diabetes

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ABSTRACT

Rice consumption is an important source of arsenic exposure. Little has known about the impact of rice consumption on arsenic metabolism, which is related to insulin resistance. In this study, we examined the associations between rice consumption and arsenic metabolism, and between arsenic metabolism and insulin resistance in non-diabetic U.S adults who participated in the National Health and Nutrition Examination Survey (NHANES) 2003–2016. Rice consumer was defined as ≥0.25 cups of cooked rice/day. HOMA2-IR was calculated using HOMA2 Calculator software based on participant's fasting glucose and insulin values. Urinary arsenic concentrations below limits of detection were imputed first, and then arsenic metabolism (the proportions of inorganic arsenic (iAs), monomethylarsonate (MMA), and dimethylarsinate (DMA) to their sum) were calculated (expressed as iAs%, MMA%, and DMA%). Using the leave-one-out approach, rice consumers compared with non-consumers had a 1.71% (95% CI: 1.12%, 2.29%) higher DMA% and lower MMA% when iAs% fixed; a 1.55% (95% CI: 0.45%, 2.66%) higher DMA% and lower iAs% when MMA% fixed; and a 1.62% (95% CI: 0.95%, 2.28%) higher iAs% and lower MMA% when DMA% fixed, in multivariable adjustment models. With every 10% decrease in MMA%, the geometric mean ratio of HOMA2-IR was 1.06 (95% CI: 1.03,1.08) and 1.05 (95% CI: 1.02, 1.09) when DMA% and iAs% was fixed, respectively; however, the associations were attenuated after adjusting for body mass index. In stratified analysis, we found that lower MMA% was associated with higher HOMA2-IR in participants with obesity: a 10% increase in iAs% with a 10% decrease in MMA% was associated with higher HOMA2-IR with the geometric mean ratio of 1.05 (95% CI: 1.01, 1.09). Our findings suggest that rice consumption may contribute to lower MMA% that was further associated with higher insulin resistance, especially in individuals with obesity. Future prospective studies are needed to confirm our results in different populations.

1. Introduction

Inorganic arsenic is common in ground water and certain foods (e.g. rice, grains), and it is a toxicant that has been related to several acute, subacute, and chronic diseases (Nurchi et al., 2020; Wang et al., 2020; Xue et al., 2010). After absorption in human bodies, inorganic arsenic is methylated to form monomethylarsonate (MMA) (~10–20%) and dimethylarsinate (DMA) (~60–70%), which are excreted in urine together with unmethylated inorganic arsenic (~10–30%) (Thomas et al., 2007; Vahter, 2000). Inter-individual differences in methylation capacity are responsible for various arsenic metabolic profiles, which may be further linked to the differences in risks of arsenic-induced diseases. For example, a relatively higher proportion of MMA (MMA%) and a lower proportion of DMA (DMA%) in urine have been associated with cardiovascular disease (Chen et al., 2013; Huang et al., 2007, 2008) and several cancers (Chen et al., 2003; Huang et al., 2008; Steinmaus et al., 2006, 2010; Yu et al., 2000). On the contrary, a decreased urinary MMA % has been linked to higher body mass index (BMI) (Gribble et al., 2013), type 2 diabetes mellitus (Kuo et al., 2015; Mendez et al., 2016; Navas-Acien et al., 2008; Nizam et al., 2013), insulin resistance (Grau-Perez et al., 2017), and metabolic syndrome (MetS) (Chen et al., 2012). These findings highlight the possibility that methylated arsenic compounds may exert unique toxic effects and an evaluation of the arsenic metabolisms in addition to arsenic exposures is critical to better understand arsenic toxicity and associated health risks.

Several studies have examined the association between arsenic and insulin resistance, though the results are mixed. For example, a small cross-sectional study of non-diabetic Amish adults reported a positive

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association between urinary total arsenic and insulin resistance (Park et al., 2016). In a prospective cohort study of American Indian adults, lower MMA% due to an increase in either inorganic arsenic% (iAs) or DMA% was associated with greater insulin resistance (Grau-Perez et al., 2017). In contrast, other studies found no significant associations between arsenic and insulin resistance (Wang et al., 2020). Other lab evidence pointed out that arsenite (As III), the more toxic oxidation state of inorganic arsenic, and/or its methylated trivalent metabolites cause insulin resistance in adipocytes by inhibiting insulin signaling and insulin-activated glucose uptake. This inorganic arsenic species can also interfere with the formation of insulin-sensitive adipocytes and myotubes by inhibiting adipogenic and myogenic differentiation (Salazard et al., 2004; Trouba et al., 2000; Walton et al., 2004; Yen et al., 2010).

Rice consumption is one of the major sources of inorganic arsenic in the U.S., especially in populations exposed to relatively low arsenic in drinking water (Kurzius-Spencer et al., 2014). Rice consumption as a contributor to higher arsenic exposure has been established in several U. S. adult populations (Wang et al., 2020; Wei et al., 2014). The high dietary glycemic index and glycemic load after eating rice may lead to excess postprandial variations in blood glucose and insulin concentrations, leading to insulin resistance has been found in previous studies, primarily carried out in Asian populations (Zuñiga et al., 2014). However, whether arsenic exposure and its metabolisms involve in the relationship between rice consumption and insulin resistance are largely unknown.

In this study, we examined rice as a source of arsenic exposure and evaluated the association of arsenic metabolism calculated using urinary concentrations of arsenic species with insulin resistance in non-diabetic U.S. adults using data from the National Health and Nutrition Examination Survey (NHANES). Specifically, we examined (1) the associations between rice consumption and arsenic metabolism biomarkers, and (2) the associations between arsenic metabolism biomarkers and insulin resistance.

2. Methods

2.1. Study population

Participants included were from 7 subsequent cycles of NHANES from 2003 to 2004 to 2015–2016, which used a complex, multi-stage, probability sampling design, to obtain a representative sample of the civilian, noninstitutionalized U.S. population. All NHANES protocols were approved by the National Center for Health Statistics (NCHS) Ethics Review Board, and all participants gave written informed consent (Zipf et al., 2013).

The participants included 5469 adults aged 20 years and older (age threshold was set in accordance with NHANES questionnaires and questionnaire strategies for adults and to meet our goal of evaluating arsenic and insulin resistance in adults) who had their arsenic data and fasting glucose and insulin data. While the NHANES does not distinguish type 1 from type 2 diabetes, an estimated of 90%–95% persons are with type 2 diabetes (Menke et al., 2015). The overall term "Diabetes" is used through the present study. We excluded 928 participants with prevalent diabetes (defined as fasting glucose $\geq$ 126 mg/dL, self-reported use of insulin or oral medications for diabetes, or self-reported physician diagnosis of diabetes), and 811 participants who had missing information on rice consumption and other key covariates, leaving a final analytical sample of 3730 participants. An overview of our sampling procedures is illustrated in Fig. 1.

2.2. Fasting glucose, insulin and HOMA2-IR

Participants who had glucose and insulin levels measured in a morning examination were asked to fast overnight. Fasting serum glucose level was determined using the enzyme hexokinase method (Roche Diagnostics, Indianapolis, IN) (Selvin et al., 2014). Serum insulin was measured using a two-site immunoenzymometric assay (TOSOH Bioscience Inc., South San Francisco, CA) (Park et al., 2016). HOMA2-IR (homeostasis model assessment for insulin resistance) was used as the indicator of insulin resistance and calculated from fasting glucose and insulin levels with the computed solved model, using the University of Oxford Diabetes Trails Unit HOMA Calculator software (Diabetes Trials Unit & University of Oxford, 2019). A higher HOMA2-IR indicates greater insulin resistance.

2.3. Arsenic measurements and urine creatinine

Urinary concentrations of total arsenic and arsenic species including arsenous acid (AsIII), arsenic acid (AsV), MMA, DMA, and arsenobetaine were measured using high performance liquid chromatography coupled to Multi-Element Inductively Coupled Plasma-Mass Spectrometry (National Health and Nutrition Examination Survey, 2020). The detection rates of arsenic concentrations are summarized in Table S2. Given the low detection rates of AsIII and AsV, iAs was calculated using the following formula: iAs = Total arsenic − MMA − DMA − arsenobetaine. Concentrations of total arsenic, MMA, DMA, and arsenobetaine below the LODs were imputed using multiple imputation by chained equation (Azur et al., 2011). Details of multiple imputation

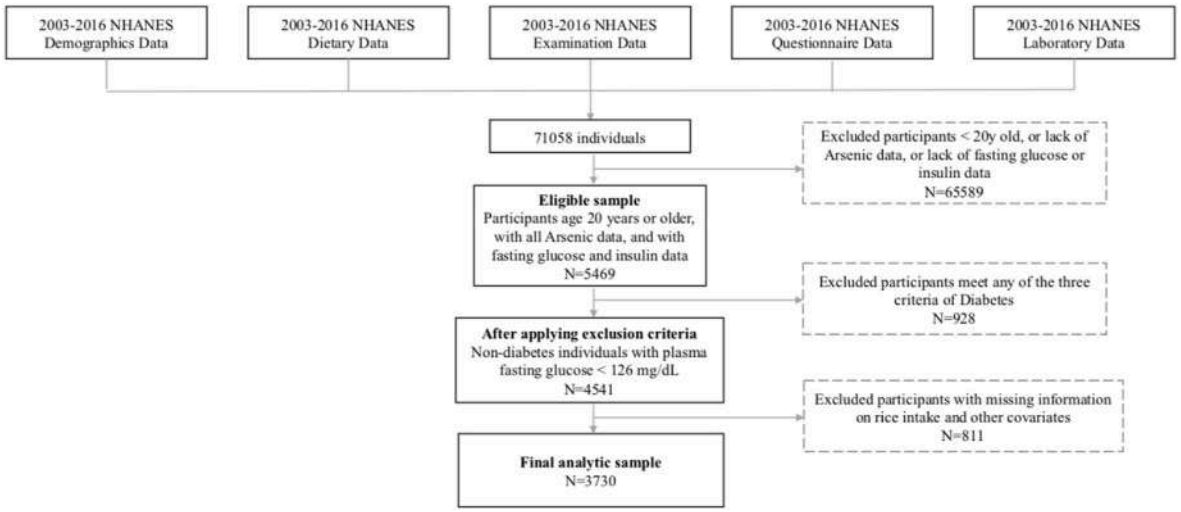

Fig. 1. Schematic diagram of analytic sample.

are described in supplementary methods. Urinary creatinine adjusted concentrations of total arsenic and its metabolites were calculated to account for urine dilution. Based on the urinary creatinine adjusted forms, the percentage of iAs, MMA, and DMA were calculated for the present study by using the individual concentration divided by the sum of arsenic (ΣAs = iAs + MMA + DMA).

2.4. Rice consumption

Information on rice consumption was collected through an interviewer-administered questionnaire that included a validated 24-hr dietary recall instrument on two nonconsecutive survey days (Alanna J et al., 2008). Dietary intake of each food item including rice as component, for example, "Spanish rice", was measured by recorded "as consumed" or not. This food intake was then converted to the amount of rice consumed by linking with the Food Commodity Intake Database (FCID) created by the U.S. Environmental Protection Agency (U.S. EPA) and U.S. Department of Agriculture (USDA) (Gilbert-Diamond et al., 2011; A. E. Nigra et al., 2017), which provides information on individual food ingredients of each food item recorded. For example, FCID food commodities in 100g of "Spanish rice" included 15.57g of white rice. The most recent FCID (FCID 2005–2010) includes all food items collected in NHANES 1999–2010 survey cycles but not those added to the survey after 2011. We considered food items in the current FCID that most closely represent those after 2011. There are eight categories of rice in the FCID codes, including white, brown, flour, bran, and their baby food types. We omitted baby food types since we focused on the adult population.

We computed the sum of rice consumption included all types of rice consumed because urinary inorganic arsenic concentrations were not different between people who primarily consumed white rice versus those who ate brown rice evidenced in a previous NHANES study (Wu et al., 2015). We defined "rice consumer" in consistence with previous studies where individuals reported at least 14.1g dry weight per day (0.25 cup of cooked rice) (Davis et al., 2012; Fulgoni et al., 2010).

2.5. Other covariates

Information on age, sex, race/ethnicity, education, poverty income ratio (PIR), smoking status, alcohol drinking was collected using self-administered questionnaires. We categorized age groups as 20–39, 40–59, 60–69, and 70 years and older. Race/ethnicity was classified as "Mexican American", "Other Hispanic", "Non-Hispanic White", "Non-Hispanic Black", and "Others". Education was categorized as "<high school", "Some high school", and "High school or more". PIR was classified as "<1" or "≥1" where a value below 1 indicates that the family is living below the poverty threshold. Smoking status was categorized into "never smoker", "former smoker", and "current smoker". Alcohol drinking was calculated using the response to questions of whether had at least 12 drinks of any type of alcoholic beverage in entire life, the frequency of drinking in the past 12 months, and the average alcohol drinks per day in the past 12 months, then, was categorized into tertiles. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters and was further categorized into "underweight (BMI<18.5)", "normal weight (18.5 ≤BMI< 25)", "overweight (25 ≤BMI< 30)" and "obese (BMI≥ 30). Physical activity was measured as metabolic equivalent (MET) scores and categorized into tertiles. Dietary fish consumption and blood mercury as indicators of fish consumption were included because fish consumption is a well-known source of DMA (DeCastro et al., 2014). Total energy intake-adjusted fish consumption was calculated using all types of fish consumption in the past 24 h and categorized into tertiles. Additionally, individuals reported any fish consumption were defined as "fish consumer" in the present study. Blood mercury concentration was measured using inductively coupled-plasma dynamic reaction cell-mass spectrometry and categorized into tertiles.

2.6. Statistical analysis

Distributions of HOMA2-IR and urinary arsenic concentrations were compared by participant characteristics using Wilcoxon Rank Sum Test or Kruskal-Wallis Test. Urinary arsenic concentrations and HOMA2-IR were log-transformed to normalize the distributions for subsequent statistical analyses. Pairwise Spearman correlation coefficients were calculated between urinary arsenic concentrations, arsenic metabolism biomarkers, rice consumption, and presented via a correlation-matrix plot.

Linear regression models were used to compare arsenic metabolism (iAs%, MMA%, and DMA%) between rice consumers and non-consumers. Rather than having one arsenic metabolism biomarker in the model at a time, we followed "leave-one-out approach" by (Kuo et al., 2015), that helps addressing the difficult interpretation of the traditional "conventional approach" given that arsenic metabolism markers are proportions of their sum and therefore, a change in one metabolism marker yields changes in one or two of the other metabolism markers. By entering two arsenic metabolism biomarkers in the same model (variable of interest as outcome and the other as covariate), we were able to obtain the impact of rice consumption on the mean changes of arsenic metabolism of interest, when fixing the second biomarker constant, meaning that the third biomarker we left out of the model changed the same amount in the opposite direction. Two sequentially adjusted models were fitted. Model 1 included age, sex, race/ethnicity, smoking status, alcohol drinking, education level, PIR, physical activity (tertiles), fish consumption, blood mercury (tertiles), and the sum of arsenic (ΣAs, log-transformed). Model 2 additionally adjusted for BMI because BMI as a measure of adiposity is a risk factor for insulin resistance and can be affected by arsenic exposure (Gomez-Rubio et al., 2011; Grashow et al., 2014; Tseng et al., 2000). We also examined individual arsenic concentrations (iAs, MMA, and DMA) as the outcomes. Geometric mean ratios (GMR) of individual arsenic concentrations was compared given the outcome was log-transformed in the linear regression models.

Arsenic exposure was evaluated based on the urinary concentration of total arsenic or the sum of inorganic arsenic (iAs+DMA+MMA). We first evaluated the insulin resistance effect of urinary total arsenic and the sum of inorganic arsenic in separate models. Then the "leave-one-out approach" was used to examine the associations between HOMA2-IR and arsenic metabolism. By entering two arsenic metabolism biomarkers in the same model (variable of interest as the predictor and the other as the covariate), we were able to evaluate the association between one specific arsenic biomarker of interest on HOMA2-IR when fixing the second biomarker constant, meaning that the third biomarker we left out of the model changed the same amount in the opposite direction. GMRs (and 95% CIs) of HOMA2-IR for a 10% increase in each arsenic biomarker was computed with adjustment for the same covariates above.

Additionally, we examined the possible effect modifications by obesity on the association between arsenic and HOMA2-IR by stratifying the study population into BMI< 30 and BMI≥ 30.

Finally, we conducted additional sensitivity analyses to evaluate the robustness of primary analyses. First, because rice consumption is also considered to increase the risk of insulin resistance through high glycemic index and glycemic load after meals (Villegas et al., 2007), we therefore further adjusted rice consumption in the association of arsenic metabolism and insulin resistance with the existing covariates. Second, to examine any different effect by consuming different types of rice, we categorized rice consumption in the present study into subtypes – white, brown, or both. Then we evaluated the difference of urinary arsenic concentrations by rice consumption category using white rice as the reference level. Last, rice category was included as a potential effect modifier into the association between arsenic metabolism and insulin resistance.

The NHANES applied a complex sampling method that makes it

Table 1
Urinary arsenic concentrations, HOMA2-IR by sociodemographic variables (Median (IQR)).

Variables	No (%)	iAs (μg/g creatinine)	MMA (μg/g creatinine)	DMA (μg/g creatinine)	HOMA2-IR
All	3730 (100.00)	0.63 (2.50)	1.21 (0.74)	3.98 (3.82)	1.02 (1.00)
Urine creatinine calibrated	3730 (100.00)	0.54 (1.89)	0.93 (0.97)	4.05 (4.01)	–
Age group (y)					
20–39	1487 (39.87)	0.54 (1.87)	0.84 (0.87)	3.76 (3.58)	1.01 (1.04)
40–59	1197 (32.09)	0.55 (1.83)	0.96 (0.97)	4.17 (4.34)	1.02 (1.00)
60–69	502 (13.46)	0.56 (2.22)	1.07 (1.11)	4.65 (4.98)	1.16 (1.02)
≥70	544 (14.58)	0.50 (2.12)	1.04 (1.02)	4.17 (3.88)	0.95 (0.84)
P-value		0.9931	<.0001	<.0001	0.0009
Sex					
Male	1854 (49.71)	0.63 (1.89)	0.84 (0.77)	3.61 (3.46)	1.04 (1.03)
Female	1876 (50.29)	0.43 (1.90)	1.05 (1.16)	4.57 (4.47)	1.01 (0.98)
P-value		0.0353	<.0001	<.0001	0.5019
Race/Ethnicity					
Mexican American	600 (16.09)	0.55 (1.79)	1.03 (0.98)	4.24 (3.48)	1.19 (1.00)
Other Hispanic	327 (8.77)	0.73 (2.20)	1.11 (1.08)	5.13 (4.37)	1.16 (1.03)
Non-Hispanic White	1810 (48.53)	0.40 (1.71)	0.97 (1.01)	3.95 (3.79)	0.93 (0.96)
Non-Hispanic Black	706 (18.93)	0.74 (1.97)	0.68 (0.60)	3.07 (2.84)	1.09 (1.08)
Others	287 (7.69)	0.91 (4.07)	1.18 (1.38)	7.55 (8.48)	0.98 (0.94)
P-value		<.0001	<.0001	<.0001	<.0001
Body Mass Index (BMI, kg/m^2)					
Under weight	71 (1.90)	0.52 (2.64)	1.24 (1.94)	4.74 (4.43)	0.51 (0.47)
Normal weight	1157 (31.02)	0.61 (2.11)	1.06 (1.17)	4.58 (3.42)	0.66 (0.51)
Over weight	1324 (35.50)	0.55 (1.95)	0.96 (0.90)	4.11 (3.94)	1.02 (0.78)
Obese	1178 (31.58)	0.48 (1.63)	0.78 (0.82)	3.59 (3.89)	1.67 (1.35)
P-value		0.1042	<.0001	<.0001	<.0001
Education level					
Less than high school	343 (9.20)	0.52 (1.83)	1.15 (1.00)	4.39 (4.13)	1.10 (0.99)
Some high school	527 (14.13)	0.78 (2.01)	0.88 (0.87)	3.91 (3.75)	1.01 (1.16)
High school or more	2860 (76.68)	0.51 (1.89)	0.91 (0.97)	4.03 (4.02)	1.01 (0.98)
P-value		0.0879	<.0001	0.0353	0.2451
Smoking status					
Never	2035 (54.56)	0.51 (1.95)	0.94 (0.99)	4.16 (4.22)	1.05 (0.96)
Former	915 (24.53)	0.64 (2.03)	1.00 (1.03)	4.15 (3.83)	1.05 (1.03)
Current	780 (20.91)	0.51 (1.73)	0.83 (0.86)	3.60 (3.47)	0.92 (1.09)
P-value		0.7276	0.0004	<.0001	<.0001
Alcohol drinking tertiles (drinks/d)					
T1 (= 0)	1353 (36.27)	0.42 (1.74)	0.92 (0.99)	4.00 (4.13)	1.09 (1.08)
T2 (0–0.14)	888 (23.81)	0.40 (1.60)	0.91 (1.08)	3.96 (4.02)	1.11 (1.03)
T3 (>0.14)	1489 (39.92)	0.77 (2.14)	0.95 (0.89)	4.14 (3.86)	0.93 (0.90)
P-value		<.0001	0.4874	0.3131	<.0001
Physical activity (MET) Z score tertiles*					
T1	1201 (32.20)	0.56 (1.92)	0.92 (0.97)	3.97 (4.00)	1.08 (1.00)
T2	1295 (34.72)	0.55 (1.96)	0.98 (0.98)	4.35 (4.15)	1.03 (1.02)
T3	1234 (33.08)	0.51 (1.83)	0.90 (0.94)	3.85 (3.77)	0.95 (0.96)
P-value		0.8575	0.1024	0.0015	<.0001
Poverty income ratio (PIR)					
<1	712 (19.09)	0.39 (1.46)	0.97 (1.03)	3.72 (3.70)	1.02 (1.03)
≥1	3018 (80.91)	0.59 (1.97)	0.92 (0.96)	4.13 (4.08)	1.02 (1.00)
P-value		<.0001	0.7132	0.0092	0.2189
Blood mercury tertiles ($\mu mol/L$)					
T1 (<2.90)	1224 (32.82)	0.15 (1.01)	0.83 (0.89)	3.33 (2.96)	1.09 (1.05)
T2 (2.90–6.50)	1264 (33.89)	0.53 (1.69)	0.88 (0.91)	3.93 (3.33)	1.04 (0.99)
T3 (>6.50)	1242 (33.30)	1.31 (3.92)	1.10 (1.11)	5.32 (5.75)	0.93 (0.97)
P-value		<.0001	<.0001	<.0001	<.0001
Rice consumed over 24-h					
Non-consumer (<14.1g)	2651 (71.07)	0.38 (1.58)	0.89 (0.99)	3.67 (3.64)	1.04 (1.00)
Rice consumer (≥14.1 g)	1079 (28.93)	1.13 (2.74)	1.03 (0.92)	5.04 (4.62)	0.97 (0.99)
P-value		<.0001	<.0001	<.0001	0.0020
Fish consumed over 24-h					
No	2972 (79.68)	0.37 (1.40)	0.89 (0.93)	3.69 (3.38)	1.03 (1.00)
Yes	758 (20.32)	2.15 (5.97)	1.14 (1.25)	6.11 (6.81)	0.98 (1.01)
P-value		<.0001	<.0001	<.0001	0.0836

Medians (IQRs) of arsenic species across covariates were urine creatinine-corrected.
P-values obtained from Wilcoxon Rank Sum Test if comparing two groups, and obtained from Kruskal-Wallis Test if comparing more than two groups.
*, Z scores were calculated as $(X - \mu)/\sigma$

possible to derive national estimates from survey data. However, arsenic biomarkers and HOMA2-IR (derived from fasting glucose and insulin) were measured in two separate subsamples that only partially overlapped. For this reason, we did not use sampling weights in all of analyses per recommendation of NHANES (Trasande et al., 2013; Peng et al. 2015). All data analyses were performed using the imputed arsenic data. Multiple imputations and correlation matrix plot in this study were performed with R software (version 3.6.3). All other analyses were performed with SAS 9.4 (SAS Institute Inc., Cary, NC).

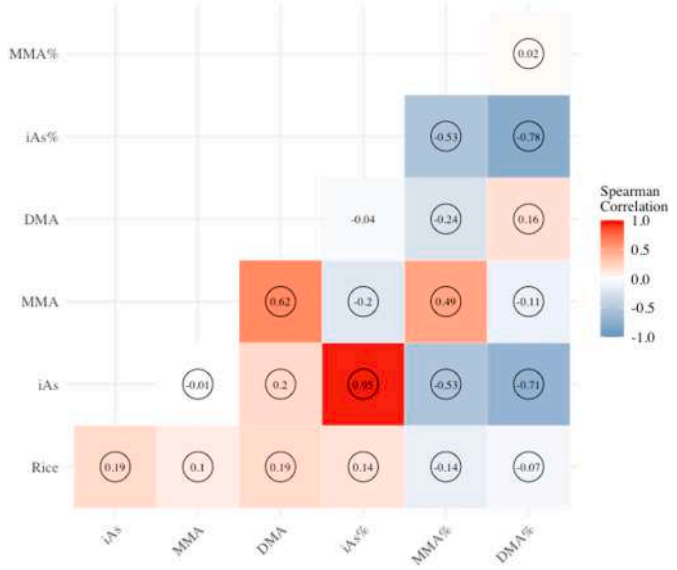

Fig. 2. Spearman correlation matrix of arsenic concentrations, arsenic metabolites, and rice consumption ("iAs", "MMA", and "DMA" are urinary creatinine calibrated and log-transformed; "Rice" represents rice consumption, continuous variable). Numbers shown in matrix represent correlation coefficients; circles highlight the significance at 0.05 level.

Table 2
The mean difference (β (95%CI)) in As metabolism biomarkers by rice consumption (Rice consumer versus non-consumer) using the leave-one-out approach.

Outcome	Covariate (fixed)	Model 1[a] (N = 3730)		Model 2[b] (N = 3730)	
		β_1 (95% CI)	p-value	β_1 (95% CI)	p-value
iAs%	MMA%	−1.55% (−2.66%, −0.45%)	0.006	−1.54% (−2.65%, −0.43%)	0.007
	DMA%	1.62% (0.95%, 2.28%)	<.0001	1.64% (0.97%, 2.30%)	<.0001
MMA%	iAs%	−1.71% (−2.29%, −1.12%)	<.0001	−1.72% (−2.31%, −1.13%)	<.0001
	DMA%	−1.62% (−2.28%, −0.95%)	<.0001	−1.64% (−2.30%, −0.97%)	<.0001
DMA%	iAs%	1.71% (1.12%, 2.29%)	<.0001	1.72% (1.13%, 2.31%)	<.0001
	MMA%	1.55% (0.45%, 2.66%)	0.006	1.54% (0.43%, 2.65%)	0.007

[a] Model1: Adjusted for Age group (20–39, 40–49, 50–69, 70 and older), Sex (Male, Female), Race/ethnicity (Mexican America, Other Hispanic, Non-Hispanic White, Non-Hispanic Black, Others), Smoking Status (Never, Former, Current), Alcohol drinking tertiles, Education level (Less than high school, Some high school, High school or more), Physical activity Z score tertiles, PIR (PIR<1 vs. PIR> = 1), Fish consumption (Yes vs. No), Blood mercury tertiles.

[b] Model2: Further adjusted for BMI (Underweight, Normal weight, Overweight, Obese).

3. Results

The median (IQR) age was 45.0 (29.0) years. The median (IQR) HOMA2-IR was 1.02 (1.00). Median (IQR) concentrations of ΣAs, iAs, MMA, and DMA in urine were 5.96 (6.44) μg/L, 0.63 (2.50) μg/L, 1.21 (0.74) μg/L, and 3.98 (3.82) μg/L, and corresponding creatinine-corrected concentrations were 6.23 (6.32) μg/g, 0.54 (1.89) μg/g, 0.93 (0.97) μg/g, and 4.05 (4.01) μg/g, respectively. The median (IQR) rice consumption over 24 h was 0.01 (18.69) g and 28.9% of the participants consumed at least 14.1g rice over the past 24 h (i.e., rice consumers).

Table 3
The geometric mean ratio (GMR) of HOMA2-IR associated with per doubling increase in total arsenic or sum of inorganic arsenic (iAs + DMA + MMA).

Per doubling increase	GMR of HOMA2-IR[a] (95% CI)	p-value
Total Arsenic[b]		
Model 1[c]	0.97 (0.95, 0.99)	0.0005
Model 2[d]	0.99 (0.97, 1.00)	0.1235
Sum of Inorganic Arsenic[b]		
Model 1[c]	0.94 (0.92, 0.97)	<.0001
Model 2[d]	0.99 (0.97, 1.01)	0.1447

[a] HOMA2-IR is log-transformed.

[b] Total arsenic and Sum of Inorgainc Arsenic are urine creatinine corrected, and logged to the base 2.

[c] Model1: Adjusted for Age group (20–39, 40–49, 50–69, 70 and older), Sex (Male, Female), Race/ethnicity (Mexican America, Other Hispanic, Non-Hispanic White, Non-Hispanic Black, Others), Smoking Status (Never, Former, Current), Alcohol drinking tertiles, Education level (Less than high school, Some high school, High school or more), Physical activity tertiles, PIR (PIR<1 vs. PIR> = 1), Fish consumption (Yes vs. No), Blood mercury tertiles.

[d] Model 2: Further adjusted for BMI (Underweight, Normal weight, Overweight, Obese).

Arsenic concentrations (iAs, MMA, and DMA), and HOMA2-IR by characteristics were presented in Table 1. Rice consumers and fish consumers had higher level of arsenic concentration (iAs, MMA and DMA) but lower level of HOMA2-IR. Participants with older age, female, "Others" race/ethnicity, and lower BMI showed higher concentrations of MMA and DMA. Males, individuals in the highest alcohol drinking tertile, and those had PIR≥ 1 showed higher concentration of iAs. HOMA2-IR were different by age and race/ethnicity and were higher in participants who had higher BMI and less physical activity.

Rice consumption was positively correlated with arsenic concentrations (iAs, MMA, and DMA), with the correlation coefficients 0.19, 0.10, and 0.19, respectively (Fig. 2). Rice consumption was positively correlated with iAs% ($\rho = 0.14$), but negatively correlated with MMA% ($\rho = -0.14$) and DMA% ($\rho = -0.07$). Within arsenic concentration-metabolism pairs, iAs showed strong correlations with iAs% ($\rho = 0.95$), and negatively correlated with either MMA% ($\rho = -0.53$) or DMA% ($\rho = -0.71$). MMA also had moderate correlation with MMA% ($\rho = 0.49$). DMA was positively correlated with DMA% and negatively correlated with MMA%.

Using the "leave-one-out" approach, compared with non-consumers, rice consumers had a 1.71% (95% CI: 1.12%, 2.29%) and a 1.55% (95% CI: 0.45%, 2.66%) higher DMA% when fixing iAs% and MMA%, respectively, after adjusting for age, sex, race/ethnicity, education, PIR, smoking, alcohol drinking, physical activity, fish consumption, blood mercury concentration, and log-transformed sum of arsenic (Table 2). In contrast, rice consumers were observed to have a 1.71% (95% CI: −2.29%, −1.12%) and a 1.62% (95% CI: −2.28%, −0.95%) lower MMA %s, when fixing iAs% and DMA% respectively, adjusting for the same covariates. The rice consumers also had a lower iAs% when MMA% was fixed but a higher iAs% when DMA% was fixed. Robust findings were observed after further adjustment for BMI (Table 2, Model 2). Similar associations were observed between rice consumption and urinary concentrations of iAs, MMA, and DMA (Table S3).

Arsenic exposure, assessed as total arsenic or sum of inorganic arsenic in urine, was not associated with insulin resistance (measured as HOMA2-IR) in the fully-adjusted model (Table 3, Model 2).

For every 10% increase in MMA%, the GMR of HOMA2-IR was 0.95 (95% CI: 0.92, 0.97) and 0.95 (95% CI: 0.92, 0.98) when fixing DMA% and iAs% respectively, after adjusting for age, sex, race/ethnicity, education, PIR, smoking, alcohol drinking, physical activity, fish consumption, blood mercury concentration, and log-transformed sum of

Table 4
Geometric Mean Ratios (GMR) (95% CIs) of HOMA2-IR by Arsenic Metabolism Biomarkers using the leave-one-out approach.

As metabolism biomarker			Model 1 (N = 3730)		Model 2 (N = 3730)	
10% increase	10% decrease	Covariate (fixed)	GMR (95% CI)	p-value	GMR (95% CI)	p-value
iAs%	MMA%	DMA%	1.06 (1.03,1.08)	<.0001	1.02 (1.00,1.04)	0.12
	DMA%	MMA%	1.00 (0.99,1.02)	0.77	1.00 (0.98,1.01)	0.75
MMA%	iAs%	DMA%	0.95 (0.92,0.97)	<.0001	0.98 (0.96,1.00)	0.12
	DMA%	iAs%	0.95 (0.92,0.98)	0.0003	0.98 (0.96,1.01)	0.12
DMA%	iAs%	MMA%	1.00 (0.98,1.01)	0.77	1.00 (0.99,1.02)	0.75
	MMA%	iAs%	1.05 (1.02,1.09)	0.0003	1.02 (0.99,1.04)	0.12

[a] Model1: Adjusted for Age group (20–39, 40–49, 50–69, 70 and older), Sex (Male, Female), Race/ethnicity (Mexican America, Other Hispanic, Non-Hispanic White, Non-Hispanic Black, Others), Smoking Status (Never, Former, Current), Alcohol drinking tertiles, Education level (Less than high school, Some high school, High school or more), Physical activity tertiles, PIR (PIR<1 vs. PIR> = 1), Fish consumer (Yes vs. No), Blood mercury tertiles, and log-transformed sum of arsenic (sum of iAs, MMA and DMA, μg/g creatinine).
[b] Model 2: Further adjusted for BMI (Underweight, Normal weight, Overweight, Obese).

Table 5
Geometric Mean Ratios (GMR) (95% CIs) of HOMA2-IR by Arsenic Metabolism Biomarkers using the leave-one-out approach by obesity.

As metabolism biomarker			Non-obese[a] (N = 2552)		Obese (N = 1178)	
10% increase	10% decrease	Covariate (fixed)	GMR[b] (95% CI)	p-value	GMR (95% CI)	p-value
iAs%	MMA%	DMA%	1.02 (0.99,1.05)	0.21	1.05 (1.01,1.09)	0.02
	DMA%	MMA%	0.99 (0.98,1.01)	0.52	1.01 (0.99,1.03)	0.40
MMA%	iAs%	DMA%	0.98 (0.95,1.01)	0.21	0.96 (0.92,0.99)	0.02
	DMA%	iAs%	0.98 (0.95,1.01)	0.14	0.96 (0.92,1.01)	0.09
DMA%	iAs%	MMA%	1.01 (0.99,1.02)	0.52	0.99 (0.97,1.01)	0.40
	MMA%	iAs%	1.02 (0.99,1.05)	0.14	1.04 (0.99,1.08)	0.09

[a] Obese was defined as *BMI* ≥ 30.
[b] All models were adjusted for Age group (20–39, 40–49, 50–69, 70 and older), Sex (Male, Female), Race/ethnicity (Mexican America, Other Hispanic, Non-Hispanic White, Non-Hispanic Black, Others), Smoking Status (Never, Former, Current), Alcohol drinking tertiles, Education level (Less than high school, Some high school, High school or more), Physical activity tertiles, PIR (PIR<1 vs. PIR> = 1), Fish consumer (Yes vs. No), Blood mercury tertiles, and log-transformed sum of arsenic (sum of iAs, MMA and DMA, μg/g creatinine).

arsenic (Table 4, Model 1). Inverse associations were observed between MMA% and HOMA2-IR when either iAs% or DMA% were fixed. However, no significant associations between arsenic metabolism and HOMA2-IR were observed after further adjustment for BMI (Table 4, Model 2).

Table 5 showed that MMA% was inversely associated with HOMA2-IR and iAs% was positively associated with HOMA2-IR only in participants with obesity. After multiple adjustments, a 10% increase in iAs% with a 10% decrease in MMA% was associated with 1.05 (95% CI: 1.01, 1.09) geometric GMR of HOMA2-IR. The GMR of HOMA2-IR was 0.96 (95% CI: 0.92,0.99) for a 10% increase in MMA% increases with a 10% decrease in iAs% decreases.

In sensitivity analyses further adjusting for rice consumption in the association of arsenic metabolism and insulin resistance, the results showed that the GMRs, 95%CIs, and the significance remain robust (Table S5). Additionally, we did not observe differences in arsenic concentration when comparing either brown rice consumption only, or both white and brown consumption to white consumption only (Table S6, Table S7). Consuming different types of rice did not modify the associations between arsenic metabolism and insulin resistance in our data (Table S8).

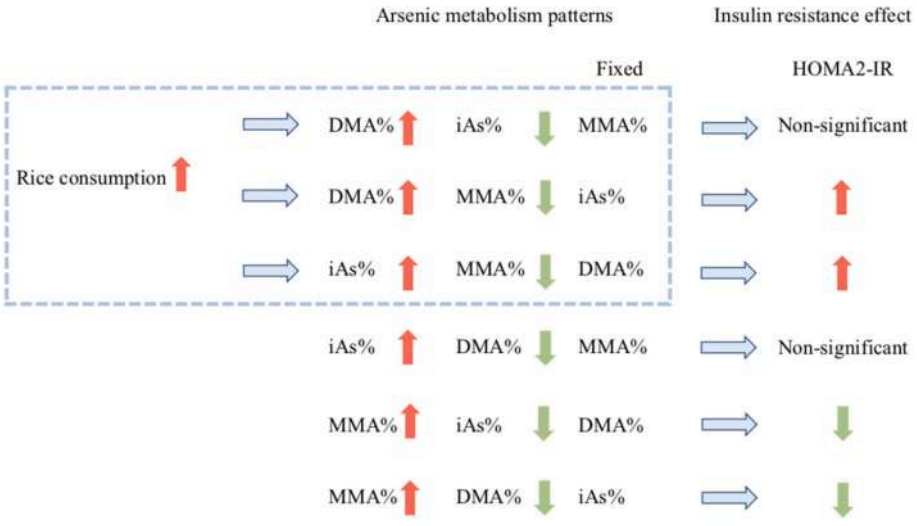

Fig. 3. Summary of the associations between rice consumption, arsenic metabolisms, and insulin resistance. Three scenarios of arsenic metabolism effects resulted from rice consumption that observed in present study are highlighted in the dashed box.

4. Discussion

In this sample of non-diabetes U.S. adults, we found rice consumption was associated with higher DMA% and lower MMA%. Lower MMA %, due to either higher iAs% or higher DMA%, was further associated with higher HOMA2-IR level after adjustment for sociodemographic factors, lifestyle factors, and fish consumption (Fig. 3). However, the associations attenuated after additional adjustment for BMI. In stratified analyses by obesity, lower MMA% due to higher iAs% was associated with higher HOMA2-IR only in participants with obesity.

The arsenic metabolism pattern, in particular lower MMA% with either higher iAs% or DMA%, was found to be associated with insulin resistance. Our findings were consistent with a previous study conducted in adults from American Indian communities (Grau-Perez et al., 2017), where lower MMA% was associated with greater insulin resistance and risk of diabetes. The biological plausibility for arsenic metabolism in the pathogenesis of insulin resistance is supported by evidence from laboratory studies (Douillet et al., 2017). MMA is a middle stage product of methylation, and high MMA% was considered insufficiency of methylation to DMA. Due to a relatively shorter half-life and rapid excretion through urine than iAs, higher DMA% is considered a more efficient arsenic metabolism profile and protective against arsenic toxicity (Spratlen et al., 2018). In contrast, the full methylation of MMA to DMA, possibly its toxic trivalent form (DMA^{III}), was also found to enhance the diabetic effects of iAs exposure (Del Razo et al., 2011) by inhibitory effects on adipogenesis (Hou et al., 2013). Impairment of triglyceride storage in white adipose tissue also results in reduced insulin sensitivity (Vigouroux et al., 2011).

Our study detected associations between arsenic metabolism and insulin resistance in participants with obesity. Additionally, Table S3 reflected the distributions of arsenic metabolites changed across BMI levels. Specifically, higher mean of iAs% and DMA% but lower MMA% as BMI increased though the trend of significance only observed in MMA %. Previous studies have provided similar findings that lower MMA% and higher DMA% were observed in people with higher BMI compared to those with lower (Gribble et al., 2013; Hall et al., 2009; Pace et al., 2018; Tseng, 2009). Alternatively, the observed association may be related to arsenic induce dysfunction of adipocytes and dysregulation of differentiation (Klei et al., 2013; Salazard et al., 2004), which further associated with insulin resistance (Hou et al., 2013). More investigations into the potential role of obesity in the association between arsenic and insulin resistance are needed in future studies.

The present study found that rice consumption was associated with higher DMA% and lower MMA%. Evidence from both Asian populations (Bahadoran et al., 2014; Zuñiga et al., 2014) and Western populations (Cascio et al., 2011; Gilbert-Diamond et al., 2011; Wei et al., 2014) supports that rice consumption is a major source of inorganic arsenic exposure. The effect of rice consumption on arsenic metabolism identified in the present study was relatively small compared to the wide inter-individual variability in arsenic metabolism (Table S2). Further investigation in populations with a broader range in rice consumption is needed to determine the extent of rice consumption affecting arsenic metabolism where nutrition conditions (i.e. protein (LeCroy and Stevens, 2017), etc.) should also be accounted for.

Important strength of this study includes the use of multiple imputations enabled a less biased calculation of arsenic metabolism biomarkers comparing to the conventional method of replacing values with the LOD divided by the square root of two (A. Nigra et al., 2019). Our study also has several limitations. First, misclassification in self-reported dietary assessments would most likely nondifferential and therefore biased the associations toward null. Second, we were unable to disentangle urinary DMA concentrations from different sources, considering urinary DMA concentrations reflect the DMA methylated from iAs and those uptaken directly from rice (Molin et al., 2014; Signes-Pastor et al., 2016). Information on DMA from different sources would improve the assessment of the association between rice consumption and arsenic metabolism in future studies. Third, the cross-sectional nature of NHANES data precludes the ability to determine chronicity of rice consumption, arsenic exposure, and persistence of insulin resistance. Urinary inorganic arsenic and its metabolites have elimination half-lives of approximately 2–4 days (Centers for Disease Control and Prevention, Biomonitoring Summary), which may not reflect long-term exposure. Therefore reverse causation could be an explanation for our results since participants with insulin resistance may have adapted lifestyle changes, including diet (Torjesen et al., 1997). Fourth, despite of extensive adjustment for confounding factors, residual confounding due to unmeasured dietary factors and environmental competing risk factors, such as use of water (Rahman and Hasegawa, 2011) or heavy metal contamination (Wang et al., 2020) cannot be completely ruled out. Lastly, while we found associations between rice consumption and arsenic metabolism, arsenic metabolism and insulin resistance in the present study, we did not observe the association between rice consumption and insulin resistance in the fully adjusted model, which is one of the premises for a mediation analysis. This can be addressed in future studies where insulin resistance results from rice consumption is well-established with less reverse causality.

5. Conclusion

In conclusion, the present study provides evidence of the association between rice consumption and arsenic metabolism. In particular, the rice consumption was positively associated with DMA%, while inversely associated with MMA%. Lower MMA% due to either higher iAs% or higher DMA% were further associated with insulin resistance in participants with obesity. Future prospective cohort studies are needed to confirm our findings in different populations with a wider range of rice consumption and arsenic exposures.

Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113834.

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Atmospheric dispersion and transmission of *Legionella* from wastewater treatment plants: A 6-year case-control study

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ABSTRACT

Legionnaires Disease incidence has risen in the Netherlands in recent years. For the majority of the cases, the source of infection is never identified. Two Dutch wastewater treatment plants (WWTPs) have previously been identified as source of outbreaks of Legionnaires Disease (LD) among local residents. The objective of this study is to examine if LD patients in the Netherlands are more exposed to aerosols originating from WWTPs than controls.

Methods: An atmospheric dispersion model was used to generate nationwide exposure maps of aerosols from 776 WWTPs in the Netherlands. Municipal sewage treatment plants and industrial WWTPs were both included. Exposure of LD cases and controls at the residential address was compared, in a matched case-control design using a conditional logistic regression. Cases were notified LD cases with onset of disease in the period 2013–2018 in the Netherlands ($n = 1604$).

Results: Aerosols dispersed over a large part of the Netherlands, but modelled concentrations are estimated to be elevated in close proximity to WWTPs. A statistically significant association was found between LD and the calculated annual average aerosol concentrations originating from WWTPs (*odds*-ratio: 1.32 (1.06–1.63)). This association remained significant when the two outbreak-related WWTPs were removed from the analysis (*odds*-ratio: 1.28 (1.03–1.58)).

Conclusion: LD cases were more exposed to aerosols from WWTPs than controls. This indicates that exposure to aerosols dispersed from WWTPs caused Legionnaires Disease in residents living near WWTPs in the period 2013–2018. In order to investigate which characteristics of WWTPs are associated with an increased LD risk, the WWTP database should be updated and more data is needed on the presence and survival of aerosolized *Legionella* bacteria to improve the Legionella dispersion modelling. Furthermore, it is recommended to further investigate how aerosol dispersion of WWTPs can effectively be reduced in order to reduce the potential health risk.

1. Introduction

Legionnaires Disease (LD) is a severe pneumonia caused by the bacterium *Legionella*, of which the species *L. pneumophila* is responsible for most LD cases. *Legionella* bacteria can be found in soil and water systems, and may cause disease when aerosols containing pathogenic *Legionella* are inhaled. Sometimes, *Legionella* causes large disease outbreaks. For example, in the Netherlands in 1999, a whirl pool at a large flower exhibit caused an outbreak with 188 patients of which 17 died (Den Boer et al., 2002). Transmission of *Legionella* is also possible from environmental (outdoor) sources, such as natural soil and wet cooling towers, and transmission has been described over long distances up to 12 km (van Heijnsbergen et al., 2015; Walser et al., 2014).

The incidence of Legionnaires Disease has risen in the Netherlands in recent years (Reukers et al., 2019). The majority of the cases are not linked to an outbreak, and for these sporadic cases the source of infection is rarely found, although more and more possible sources have been identified over recent years (van Heijnsbergen et al., 2015). In 2017 and 2018 two biological wastewater treatment plants (WWTPs) could be linked to LD clusters in two towns, Boxtel and 'Son en Breugel' (hereafter: Son), located in the South of the Netherlands (Loenenbach et al., 2018; Reukers et al., 2018). Both WWTPs are industrial installations,

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one in food processing and the other for animal carcass disposal (i.e. rendering). Both WWTPs operate at elevated temperatures, between 30 and 35 °C, and contain nutrient-rich process water, which are favorable conditions for *Legionella* growth (Caicedo et al., 2019; Loenenbach et al., 2018).

Wastewater can contain high numbers of microorganisms (Korzeniewska, 2011; Uhrbrand et al., 2017). During the wastewater treatment process aerosols are formed, which may disperse bacteria originating from WWTPs over large distances and reach local residents in aerosols of inhalable size (Gangamma et al., 2011; Jahne et al., 2015; Korzeniewska, 2011; Uhrbrand et al., 2017). Aerosols are mainly formed during aeration processes of wastewater (Korzeniewska, 2011; Michalkiewicz and Kruszelnicka, 2018). *Legionella* has been found in wastewater in the Netherlands in concentrations up to 10^8 CFU per L (Loenenbach et al., 2018; Medema et al., 2004), and in Norway concentrations of $>10^{10}$ CFU/L in an aeration pond of a biological WWTP have been reported (Olsen et al., 2010). In various studies, *Legionella* has been detected with culture or molecular methods (PCR) in air samples at WWTPs (Blatny et al., 2008; Medema et al., 2004; Mirzaee et al., 2015; Pascual et al., 2001; Roll and Fujioka, 1995). Blatny et al. (2008) found concentrations of up to 3300 CFU/m^3 directly above aeration basins. In the aeration ponds of the WWTPs in Boxtel and Son high concentrations (10^8 CFU/L) of *Legionella pneumophila* were detected (Loenenbach et al., 2018). The bacteria were also identified in air samples taken directly above the aeration ponds, as well as downwind (Loenenbach et al., 2018). In the LD outbreak linked to the WWTP in Son, transmission occurred over a distance of at least 3 km, as one patient, with a clinical isolate identical to the sequence type of the *Legionella* found in the wastewater, had not been closer than 3 km to the WWTP. In the international literature, several other examples can be found where (industrial) WWTPs have caused LD or the milder disease Pontiac Fever among employees (Allestam et al., 2006; Gregersen et al., 1999; Isozumi et al., 2005; Kusnetsov et al., 2010) or local residents (Blatny et al., 2008; Maisa et al., 2015; Nguyen et al., 2006; Nogueira et al., 2016; Olsen et al., 2010).

To study aerosol dispersion from WWTPs, an atmospheric dispersion model can be used. Atmospheric dispersion modelling has been successfully applied to airborne infectious diseases, as reviewed by Van Leuken et al. (2016). The Operational Priority Substances (OPS) atmospheric dispersion model (Sauter et al., 2018) is applied in the Netherlands to produce year-averaged maps showing airborne concentrations of several substances. The OPS model has also been applied to Q fever (van Leuken et al., 2015), and to aerosol dispersion in a study on livestock farming and health of local residents (Hagenaars et al., 2017), but not yet for *Legionella*.

The objective of this study is to examine if LD patients in the Netherlands are more exposed to aerosols originating from WWTPs than controls. To examine this hypothesis we calculated exposure using the OPS model for atmospheric dispersion of aerosols from WWTPs in the Netherlands to generate nationwide exposure maps. We then compare exposure of LD patients and controls. Additional analyses were performed to further probe our modelling approach and outcomes.

2. Methods

2.1. Case-control study

In this study, a matched case-control design was chosen with controls matched by age group and sex. Cases were notified LD patients (EU case definition (European Centre for Disease Prevention and Control, 2017)) with onset of disease in the period 2013–2018 in the Netherlands (n = 2674). As the spatial analysis was done based on the residential postal code, only cases were selected who had stayed at home overnight during the incubation period (2–10 days) and for whom no probable source of infection was found. Thus, the following cases were excluded: 1) cases who had travelled with an overnight stay during the incubation period (n = 998), 2) cases who were potentially infected in a health care setting (n = 22), 3) cases without source finding information (n = 10), 4) cases for whom a probable source of infection was found, other than a WWTP (n = 39), and 5) cases with unknown residential postal code (n = 8). After this selection, 1604 cases remained and were included in the analysis (Table 1). Age and sex distribution showed the usual distribution for LD, with the majority (71.9 per cent) of patients being male and 92.8 per cent aged >45 years. Selected LD cases had residential addresses spread around the Netherlands (Fig. 1). For this analysis, cases were assigned *xy*-coordinates on the basis of the population-weighted centroid of their residential postal code. The 6-digit postal code was available for 83 % of cases. If a 6-digit postal code was not available, the 4-digit postal code was used. The average distance of the location of a residential address to the population-weighted centroid is 40 m for a 6-digit postal code and 640 m for a 4-digit postal code.

Controls (n = 16040) were sampled from the Dutch general population (per January 1st, 2016) (CBS, 2016), using a 100 × 100 m grid. At this resolution, the spatial precision of the controls was similar to that of the patients. This grid holds information on number of inhabitants (rounded to 5 persons) per age category (age category 0–14, 15–44, 45–64 and≥65 years) and per sex (male, female). To estimate the number of inhabitants for each age category and sex combination, iterative proportional fitting (Bacharach, 1965) was applied to each grid cell. Given the age and sex of each patient, controls were sampled from 10 randomly selected grid cells, where the chance of selecting a grid cell was proportional to the number of inhabitants of the respective age category - sex combination.

Ideally, smoking behavior would also be corrected for as this is a known risk factor for LD (Den Boer et al., 2006). However, information on smoking behavior was only available for cases, not controls. There are, however, data available on percentage of smokers at neighborhood level (RIVM, 2019; van de Kassteele et al., 2017). For an additional analysis including a correction for smoking, this percentage was linked to each case and control based on their location.

2.2. Wastewater treatment plant data

WWTPs in the Netherlands were inventoried by the Dutch Environmental Services (Omgevingsdiensten) and the Foundation for Applied Water Research STOWA (Bartels et al., 2019; Vermeulen et al., 2019).

Table 1
Notified LD cases in the Netherlands per year, shown as total cases (row 2), excluded cases (row 3) and included cases (row 4) and characteristics of the included cases (row 5–9).

Year of onset disease	2013	2014	2015	2016	2017	2018	Total
Number of notified cases[a]	308	348	419	454	561	584	2674
Number of excluded cases	148	157	173	156	213	223	1070
Number of cases in analysis (100 %)	160	191	246	298	348	361	1604
% < 45 year	6.9 %	6.8 %	9.3 %	8.7 %	7.2 %	5.0 %	7.2 %
% 45–64 year	41.9 %	45.5 %	47.6 %	43.0 %	42.5 %	42.7 %	43.7 %
% ≥ 65 year	51.3 %	47.6 %	43.1 %	48.3 %	50.3 %	52.4 %	49.1 %
% male	68.8 %	76.4 %	69.5 %	73.2 %	70.4 %	73.1 %	71.9 %
% complete postal code available[b]	11 %	79 %	91 %	94 %	91 %	95 %	83 %

[a] Notified cases according to EU case definition.
[b] Percentage of cases for whom the complete (6-digit) postal code was available. For the remaining cases the less spatially precise 4-digit postal code was available.

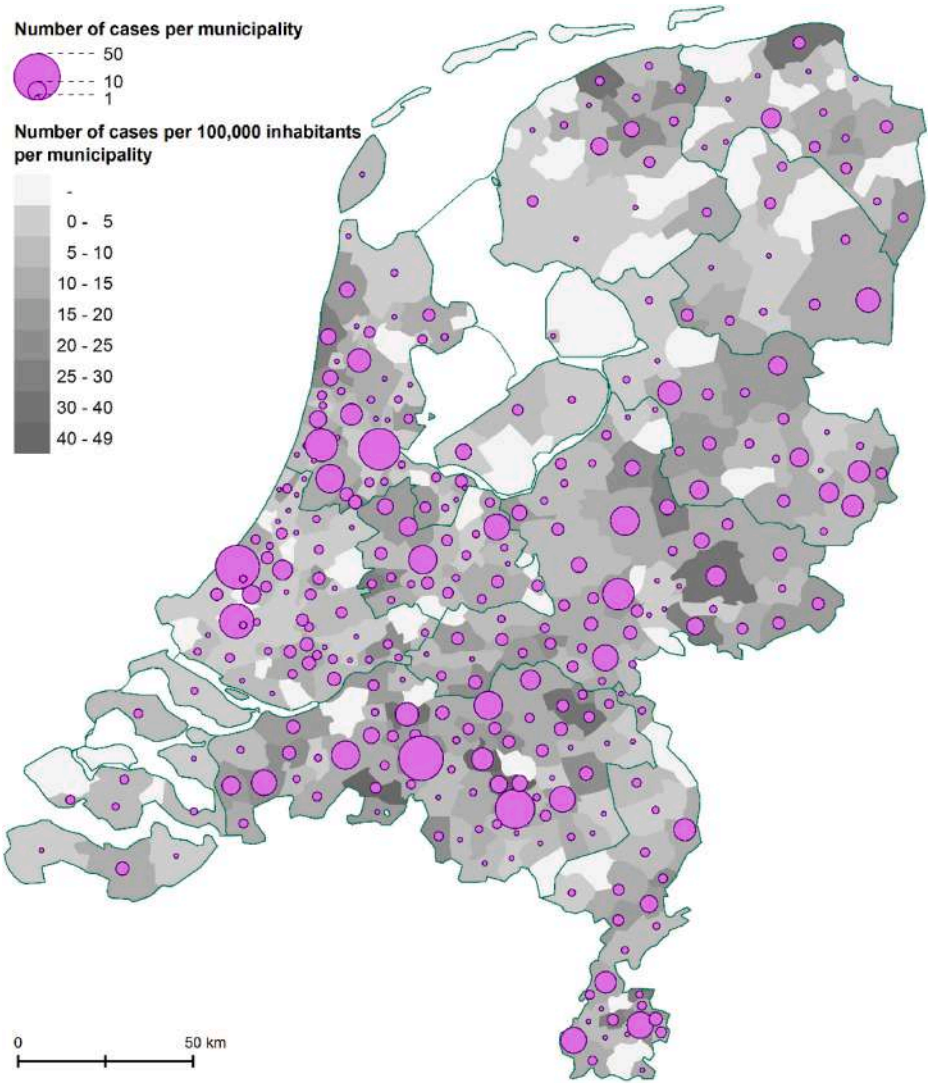

Fig. 1. Map of included LD cases per municipality from the period 2013–2018. The map shows both number of included cases and incidence (cases/100,000 inhabitants).

The resulting database of 776 WWTPs was used for this analysis. This database included both industrial WWTPs (n = 449) and municipal sewage treatment plants (n = 327). Information on the characteristics of these WWTPs was incomplete, but the installations comprise a variety of different treatment processes, both biological and physicochemical, aerated and non-aerated. The WWTPs were geographically positioned based on their full address (Kadaster, 2019). Fig. 2 shows that WWTPs are located all over the Netherlands, with higher densities in industrialized regions such as the port area in the West of the Netherlands.

2.3. Atmospheric dispersion modelling

For this study, the OPS atmospheric dispersion model (Operational Priority Substances model) was applied (Sauter et al., 2018). OPS can simulate the atmospheric dispersion of a variety of polluting substances such as particulate matter, ammonia and nitrogen oxides. It uses a Gaussian plume model in which the cross wind concentration follows a Gaussian distribution. This distribution depends on the meteorological conditions, such as the atmospheric stability. For dispersion over relatively longer distances, OPS uses trajectories to follow the path of an air parcel in a changing wind field. Furthermore, the removal processes of deposition and chemical conversion are included, of which the first is also relevant for particles. At a certain location, the contributions of all individual sources are added to obtain the total concentration at that site. The supplementary material provides more background information on aerosol formation and dispersion (S1) and more information on the OPS model (S2).

The OPS model was applied to calculate annual average aerosol concentrations originating from wastewater treatment plants for 2013–2018 on a 500 m × 500 m grid for the entire Netherlands. To apply the OPS model for *Legionella* the following assumptions were made.

First, it was assumed that *Legionella*-containing aerosols from WWTPs behave similar to primary particulate matter up to 10 μm (PM_{10}). This same assumption has also been made when OPS was applied to model *Coxiella burnettii* bacteria (Q fever) by van Leuken et al. (2015) and to model bio-aerosols in a livestock and residential health research project (Hagenaars et al., 2017) (the latter study also included coarser PM as a proxy for bio-aerosol dispersion).

Second, it was assumed that all WWTPs emit equal amounts of aerosols. The available heights and diameters of the aerations tanks were included in the model, as this influences aerosol dispersal. However many other factors were unknown and could not be taken into account. It was unknown, for example, which WWTPs, during which period in 2013–2018 emitted *Legionella*-containing aerosols, and how many *Legionella*-containing aerosols per time unit a positive installation

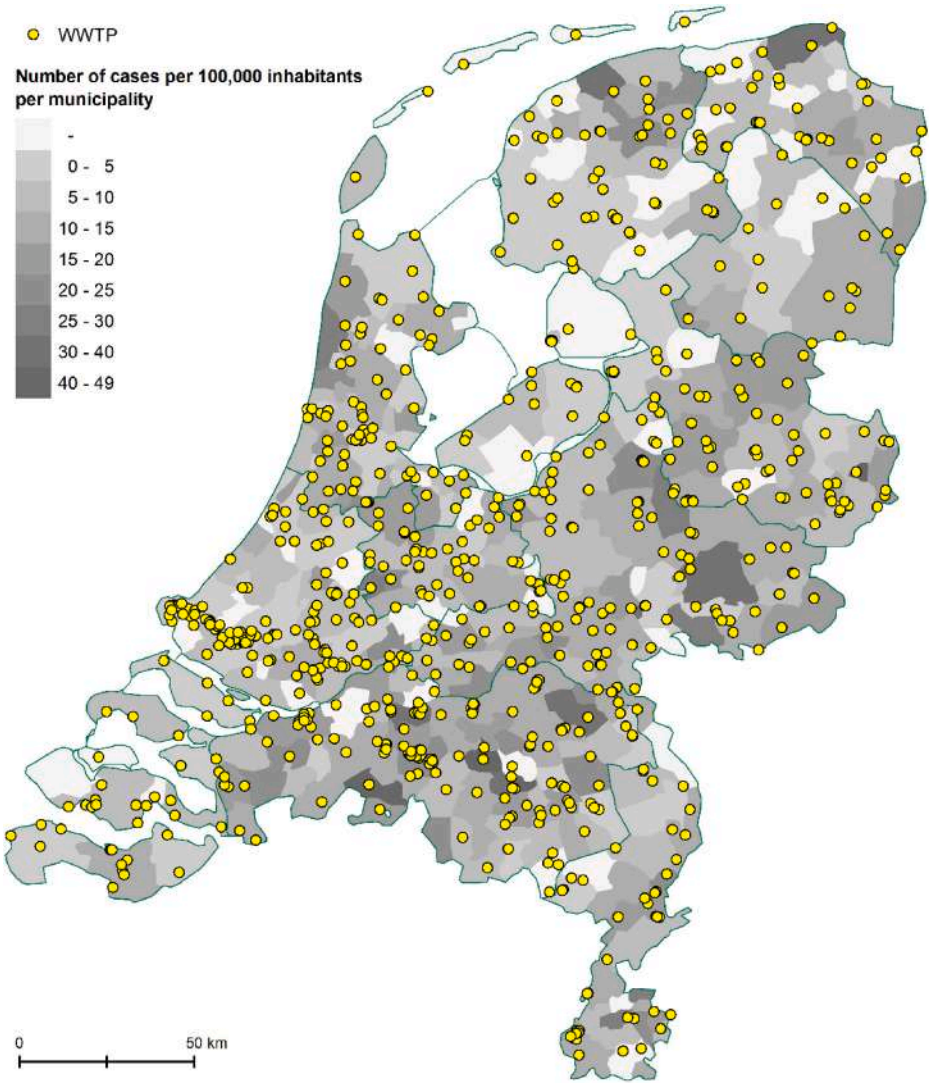

Fig. 2. Map of WWTPs in the Netherlands, with on the background LD incidence (included cases/100,000 inhabitants) per municipality from the period 2013–2018.

emits. As such, a fictitious emission strength was applied (a dummy value). This means that the annual average aerosol concentrations that OPS calculates cannot be interpreted as absolute values, but should only be interpreted as relative concentrations.

Third, it was assumed that the calculated average annual aerosol concentrations at the residential address of cases and controls can be used as proxy for exposure. In doing so, it was assumed that breathing volume is equal for everyone, meaning that the calculated annual average aerosol concentrations are directly proportional to exposure.

Fourth, the WWTPs were assumed to be continuous sources of *Legionella*-containing aerosols, which applies for open aeration tanks that are operated continuously. Potential measures to prevent spread via the air (such as covering of aeration tanks) were not taken into account, as these data were incomplete.

Lastly, it was assumed that no reduction of *Legionella* in air takes place, as suitable quantitative data on this process are lacking. The assumptions are further specified in Table S1 in the Supplementary Material.

The OPS model was applied for three scenarios:

1. Including only the two WWTPs (in Boxtel and Son) that have been confirmed as source of LD outbreaks in the Netherlands ($n = 2$)
2. Including all WWTPs identified in the inventory ($n = 776$)
3. Including all WWTPs identified in the inventory, excluding the outbreak-related WWTPs in Boxtel and Son ($n = 774$)

Scenario 1 was chosen to verify that the method works; a causal relation should be found between WWTPs and LD patients in this scenario as the two WWTPs are known to have caused LD patients. Scenario 2 was chosen to investigate whether a relation between WWTPs and LD patients can also be found nationwide. Scenario 3 was chosen to investigate whether an observed nationwide relation is solely due to the two known outbreak-related WWTPs in Boxtel and Son, or not.

2.4. Statistical analysis

A conditional logistic regression analysis was performed using the calculated average annual aerosol concentrations at the residential address of cases and controls as proxy for exposure. The resulting *odds*-ratios (*OR*s) are presented with 95 % confidence intervals and *p*-values. After visual inspection, the modelled aerosol concentrations were $\log_{10}$-transformed before analysis to correct for the positively skewed distribution. Consequently, the interpretation of the *OR* is as follows: an *OR* of 1.5 means that the number of people with a 10 times higher exposure is 1.5 times larger in the case group than in the control group. The analysis was repeated with a correction for smoking behavior at the neighborhood level (van de Kassteele et al., 2017). All statistical analysis

was performed in R (R Core Team, 2020). More details on the statistical analysis can be found in the supplementary material S3.

3. Results

3.1. Statistical analysis

The results of the conditional logistic regression for the three exposure scenarios are shown in Table 2. All three scenarios show a statistically significant association between LD and calculated annual average aerosol concentrations originating from WWTPs. The first scenario, including only the two outbreak-related WWTPs, found a significant association with an *odds*-ratio of 1.43 (1.32–1.54). When all WWTPs nationwide were included (scenario 2), a significant association was also found, with an *OR* of 1.32 (1.06–1.63). For the scenario where the two outbreak-related WWTPs of Boxtel and Son are excluded from the analysis (scenario 3), a smaller but still significant association was found. After adjustment for smoking at neighborhood level, minor changes are seen in the *OR*, although the association in the third scenario is no longer significant at the 5 % level with an *OR* of 1.23 (0.99–1.53).

3.2. Atmospheric dispersion of aerosols from WWTPs

Fig. 3 shows the logarithm of the aerosol concentrations originating from WWTPs, as calculated by the OPS model on a 500 m × 500 m grid for the Netherlands, averaged over the period 2013–2018. LD cases are projected as black dots. The different years only showed minor differences (data not shown).

To gain more insight in the pattern of modelled aerosol concentrations, a west-east cross-section was made around the WWTP in Son (Fig. 4). The location of this cross-section on a map is shown in Fig. 5, with on the background the modelled aerosol concentrations. Modelled aerosol concentrations are highest close to the source WWTP, and decrease rapidly with distance (Fig. 4). Little difference is seen between years of the average modelled aerosol concentrations (Fig. 4). Furthermore, the curves do not decrease fluently, but show local variations. These variations are rather small and likely due to differences in surface roughness related to land use (such as the presence of forests and urban areas). The OPS model takes into account the effect of roughness on atmospheric dispersion and deposition.

The WWTP in Boxtel (north-west of Son in Fig. 5) commenced operation in 2015 with the current treatment processes, and was therefore included from this year onwards. The model adds up the aerosol concentrations of both WWTPs, resulting in a slightly higher aerosol concentration in the area between Boxtel and Son from 2015 onwards (most visible on the left side of the curves in Fig. 4).

4. Discussion

4.1. Reflection on the results

In this study, for the first time an atmospheric dispersion model was applied for aerosols from WWTPs in the Netherlands and compared to Legionnaires' Disease occurrence. Application of the model for all WWTPs in the Netherlands in the period 2013–2018 (scenario 2) shows that LD patients were significantly more exposed to aerosols from WWTPs than controls. Since LD is a rare disease, an *odds*-ratio of 1.32 can be interpreted as a relative risk and indicates that a 10 times higher exposure increased the LD risk with 32 %, based on this data set and assumptions. Even without the Boxtel and Son WWTPs (scenario 3), the association between modelled aerosols from WWTPs and LD incidence remains significant. This indicates that *Legionella* transmission from other WWTPs has occurred in the period 2013–2018, causing disease in residents. It is plausible that the demonstrated association is a causal relationship: *Legionella* can be detected up to high concentrations in the water and in aerosols at various WWTPs in the Netherlands (Lodder et al., 2019; Loenenbach et al., 2018) and these aerosols can be of inhalable size and may contain viable organisms. As observational data on the occurrence of *Legionella* in WWTPs during 2013–2018 are lacking, it cannot be proven which WWTPs may have caused LD.

In large WWTP-related LD outbreaks, such as the outbreaks of Warstein (Maisa et al., 2015) and Pas-des-Calais (Nguyen et al., 2006), a secondary disseminator (a contaminated cooling tower) was found, spreading *Legionella* to the environment. In contrast to these large outbreaks, the temporal pattern of the two Dutch WWTP outbreaks were of a sporadic nature and the investigations found no secondary transmitters. Therefore direct transmission of *Legionella* from the aeration pond of the WWTP over a long distance was assumed. Possible direct dissemination from an aeration pond was also suggested in outbreaks in Sweden and Norway (Blatny et al., 2008) (personal communication, Andersson, S, Public Health Agency of Sweden). The exposure maps from the WWTPs found in this study support the assumption of direct transmission over a long distance from the aeration pond of a WWTP to the environment. The curves of the modelled concentrations (Fig. 4) start to level off at about 5–10 km distance from the source. This is in line with the transmission found in this outbreak. Transmission occurred over a distance of at least 3 km, as one patient, who had clinical isolate with an identical sequence type as the environmental isolate from the WWTP, was linked to this outbreak and had not been closer to the WWTP (Reukers et al., 2018). The WWTPs were geographically positioned based on their full address (Kadaster, 2019). However, this is usually based on the main entrance of a property. The exact location of aerated tanks could potentially be tens of meters away, which may lead to a small error.

4.2. Limitations

In this analysis, a relative measure for aerosol exposure was calculated based on dummy emission values; absolute exposure is unknown. To determine absolute values, measurements of *Legionella* in the air could be performed, as described in Lodder et al. (2019). Such data would be valuable for model input and validation. It is not possible to deduce from this analysis whether the association with LD concerns spatial clusters or sporadic (isolated) cases. In this study, various scenarios were calculated with the OPS model, whereby a specific group of WWTPs was always included. With this setup, however, only single regression analysis was possible. As a result, it was not possible to test the effect of various characteristics of WWTPs on LD occurrence, such as size and height of aeration basins, process water temperature or industry type. Furthermore no correction was possible for other environmental sources of *Legionella* that may potentially influence the analysis.

Table 2
Association between exposure to aerosols originating from WWTPs (as calculated using the OPS model) and Legionnaires' Disease (LD) in 2013–2018. The table shows the odds-ratio (OR) with 95 % confidence interval (CI) and the p-value. The WWTPs of Boxtel and Son are both confirmed as WWTPs linked to a community outbreak of LD. The three columns on the right show the results adjusted for smoking at neighborhood level.

Scenario	*OR*	95 % *CI*	*p*-value	Adjusted *OR*	Adjusted 95 % *CI*	Adjusted *p*-value
1. WWTPs Boxtel and Son	1.43	1.32–1.54	<0.0001	1.46	1.35–1.57	<0.0001
2. All WWTPs	1.32	1.06–1.63	0.0116	1.27	1.02–1.58	0.0290
3. All WWTPs without Boxtel and Son	1.28	1.03–1.58	0.0250	1.23	0.99–1.53	0.0590

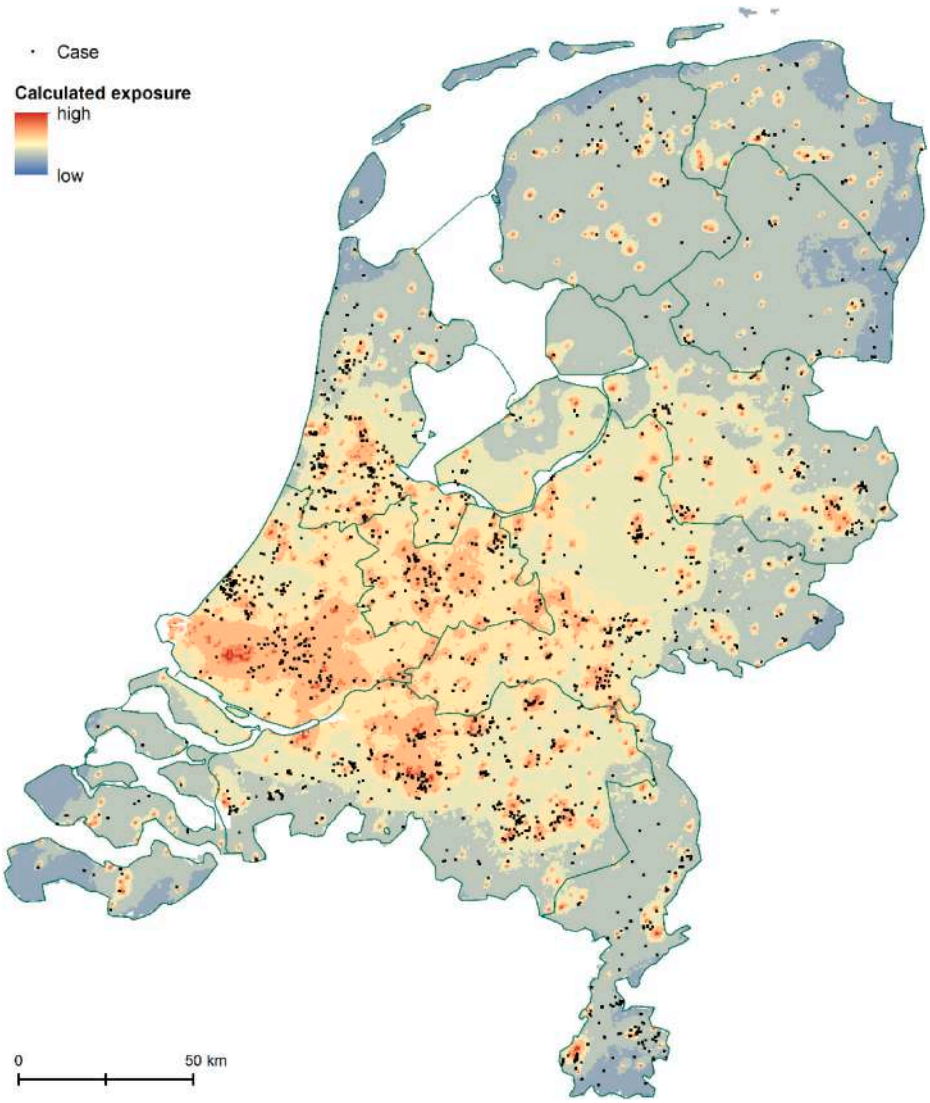

Fig. 3. Logarithm of the calculated aerosol concentrations to which patients are exposed, originating from WWTPs in the Netherlands, averaged over 2013–2018. The location of LD cases included in this study is shown as black dots. The relative aerosol concentrations are shown from high to low.

Examples include wet cooling towers, which are also often present in industrial areas and are a known source of LD outbreaks (Walser et al., 2014), and composting facilities (van Heijnsbergen et al., 2015). Complete registration of such other possible sources was unavailable. Furthermore, the WWTP dataset still had some shortcomings. Some WWTPs may have been missing from the dataset, and all known WWTPs were included for the entire study period, because detailed information on period of use was incomplete. Also WWTP characteristics were incomplete or sometimes unreliable. For example the data on water temperature was incomplete. Furthermore, WWTPs and LD patients near the border in neighboring countries were not included as no data were available, which might lead to an underestimate of the association.

Case and controls were matched for age group and gender. It was not possible to match for smoking behavior, while smoking is a known LD risk factor (Den Boer et al., 2006). Information about smoking was available for the patients but not for the controls. Smoking is more common among people with a lower socio-economic status (SES). It is possible that SES is associated with residential proximity to a WWTP, for example, it might be that people with a lower SES live closer to industrial areas where industrial water treatment plants are located. Yet WWTPs are not only located at industrial sites, since municipal sewage treatment plants are often located in more rural areas. Nevertheless, additional correction for smoking behavior at the neighborhood level was performed (RIVM, 2019; van de Kassteele et al., 2017). A limitation of this method is that data at the neighborhood level does not necessarily correspond to individual data, leading to additional uncertainty. Therefore, the results are presented both with and without the correction for smoking. Another possible confounder is underlying disease. Chronic pulmonary disease and cardiac disease are risk factors for LD (Cooley et al., 2019) and these conditions may also be associated with exposure to air pollution and industry (Anderson et al., 2012). It was not possible to adjust for underlying disease, because this information was not available for the controls.

4.3. *Assumptions in using the OPS model and data availability*

The OPS model is designed for modelling several gaseous substances and particulate matter. In this study, particulate matter served as a substitute for aerosols containing *Legionella* bacteria. Larger and heavier particles deposit more easily and smaller and lighter particles are more easily transported over larger distances. Therefore, the particle/aerosol size distribution as well as the relative distribution of *Legionella* bacteria over the particle size classes should be taken into account when determining the exposure. A uniform distribution of *Legionella* bacteria over

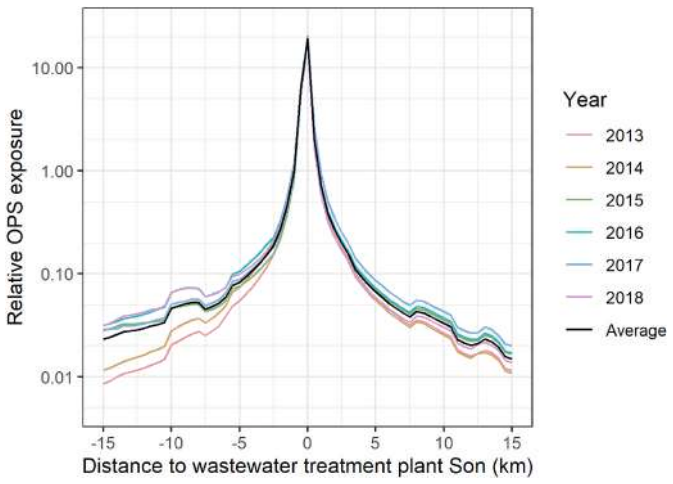

Fig. 4. Calculated annual average aerosol concentrations to which cases are exposed, as function of the distance to the WWTP in Son (km). To generate this figure, the OPS model was run for the scenario including only the WWTPs in Boxtel and Son. The colors show different years. The concentrations are shown on a log scale. The concentrations should only be interpreted relatively, as a dummy emission value was assigned to WWTPs (explained in section 2.3). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

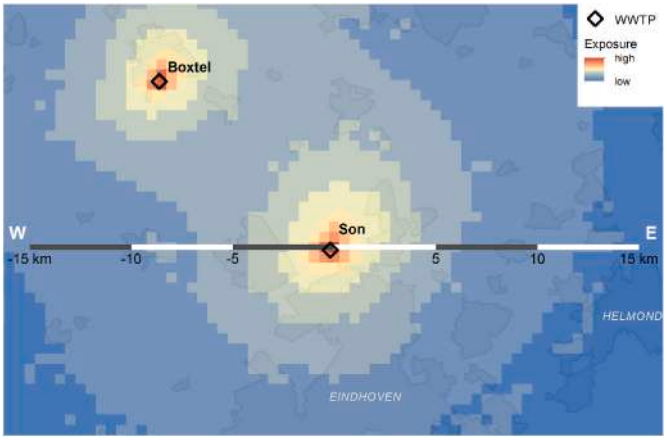

Fig. 5. Location of the cross-section in Fig. 4, with modelled aerosol concentrations to which patients are exposed in the background (average 2013–2018). The location of the black and white bar indicates the location of the cross-section, and gives the distance in km.

the particle size classes was assumed since the relative distribution of *Legionella* bacteria over the particle size classes is unknown. This may somewhat impact the resulting exposure map. Furthermore, aerosols from WWTPs consist mainly of water which may evaporate. In addition to the relative humidity, it has been suggested that the osmolarity of the WWTP water could play a role (National Academies of Sciences, 2019) by affecting evaporation, but quantitative data are unavailable. However, evaporation of aerosols is a process which is currently not accounted for in OPS. In any case, it is likely that the OPS model adequately describes the dispersion direction and the specific weather conditions that play an important role.

Legionella can be present in vesicles excreted by amoebae, which could also influence dispersion. Such vesicles are mostly of inhalable size and can contain hundreds of *Legionella* bacteria per vesicle (Shaheen and Ashbolt, 2018). Moreover, bacteria in vesicles are known to possess a thicker cell wall. The vesicles and thicker cell wall could offer some protection against dehydration and UV, possibly increasing survival in the air. Given the size of the vesicles, it is likely that their atmospheric dispersion characteristics correspond to those of other solid particles as modelled by the OPS model. The strong significant association that was found for the scenario in which only the WWTPs in Boxtel and Son were included also gives confidence for the application of particulate matter as a substitute for aerosols containing *Legionella*.

Since a full postal code was available for most patients, they could be located with an accuracy of 100 m. The simulations with the OPS model were performed on a 500 m × 500 m grid. Although this may have an influence on the calculated exposure, it is unlikely that this will lead to a systematic overestimate or a systematic underestimate of the effect. It was assumed that exposure took place at the residential address. Only the residential address of cases and controls was used as the location for which the exposure was calculated. Exposure may have taken place elsewhere, such as at a work address or while traveling. This may have underestimated the found association. On the other hand, the population at increased risk for LD may be less mobile than the average population and some LD patients in the outbreaks were known to be home bound. Moreover, the influence of outdoor activities may not be large; a Dutch study in which the movement pattern of patients with pneumonia was mapped out found that most of the time spent outdoors was around the residential home, while the time spent outside at other locations was very limited (Klous et al., 2018).

The height and diameter of the aeration basin were included in the OPS model as relevant source characteristics influencing aerosol dispersion. However, the extent to which a WWTP emits *Legionella* will also depend on the presence and concentration of *Legionella* in wastewater, and possibly also the composition and temperature of the wastewater. In addition, the type of aeration process may play a role in aerosol formation (Korzeniewska, 2011; Michalkiewicz and Kruszelnicka, 2018; Sanchez-Monedero et al., 2008). WWTPs were assumed to be a continuous source of aerosols, which is consistent with emissions from open aeration basins that are in continuous operation. However, other processes where aerosols could be formed include cleaning activities (such as high pressure spray cleaning) (Castor et al., 2005; Nguyen et al., 2006; Walser et al., 2014), sludge separation techniques (Szyłak-Szydłowski et al., 2016), the direct use of effluent (for example in cleaning, air washers or a cooling tower), or use of surface water where effluent contaminated with *Legionella* has been discharged (for example in cooling towers or irrigation) (Maisa et al., 2015). The use of elevated process water temperature is a risk for *Legionella* growth. To contribute to the circular economy, new developments in (industrial) wastewater systems increasingly use elevated water temperature to aid nutrient recovery. To develop sustainable installations, health should also be an aspect that is considered in cost-benefit assessments. Therefore, the reduction of aerosols should be included in the design of new WWTPs, especially for WWTPs that are located near populated areas.

The so-called OPS long-term model was used in this study, as six entire years were modelled. A short-term version of the OPS model exists as well (OPS-ST). OPS-ST uses hourly meteorological data so that hourly concentrations can be calculated. Use of OPS-ST may provide more insight into the specific weather conditions that play a role in the transmission of *Legionella*. LD incidence in the Netherlands is associated with warm, wet weather (Brandsema et al., 2014; Karagiannis et al., 2009). It is plausible that climate change will have an effect on *Legionella* dispersion and survival in the environment (Caicedo et al., 2019). In addition to WWTPs with intentional heating of the process water, prolonged periods of warm weather may also increase the water temperature of systems operating on ambient temperatures. The impact of these increased ambient temperatures on *Legionella* growth and dispersion from WWTPs is still unknown. The relation between the concentration of bacteria in wastewater and the concentration in the air is ambiguous (Bentham and Whiley, 2018; Crimi et al., 2006; Michalkiewicz and Kruszelnicka, 2018). Also the influence of the size and osmolarity of the aerosol on the distance of dispersion needs more research to improve atmospheric dispersion modelling. Similarly, little data is available on

persistence rates of *Legionella* in ambient air and how these are influenced by meteorological variables (temperature and UV radiation) (Prussin et al., 2017; Walser et al., 2014).

The OPS model and additional analyses could be applied to answer further questions on characteristics of WWTPs and *Legionella* dispersion, and the model could also be applied to investigate the relation between aerosol dispersion from WWTPs and other health outcomes. For LD, the use of the OPS model appears to be suitable and useful. Other hazards from WWTPs include emissions of H_2S (Godoi et al., 2018) and endotoxins from Gram-negative bacteria (Thorn and Kerekes, 2001). Applying an atmospheric dispersion model for such other hazards could be valuable. In addition, investigation of the association between exposure to aerosols from WWTPs and pneumonia in general would provide insight, as most LD pneumonia is not diagnosed as such and other forms of pneumonia might also be associated with exposure to WWTPs.

5. Conclusion

This study demonstrates that atmospheric dispersion modelling can be a useful tool to assess *Legionella* dispersion from WWTPs. This conclusion is supported by the similarity between the distance of transmission found in the outbreak of Son and the calculated dispersion map at this location. Comparing the calculated aerosol dispersion to LD in the Netherlands in the period 2013–2018 showed that cases were more exposed to aerosols from WWTPs than controls. This indicates that *Legionella* dispersion leading to disease from WWTPs, other than the two outbreak-related WWTPs, has occurred in the period 2013–2018. Exposure to WWTPs is thus a potential risk factor for LD. Our findings of dispersal of aerosols from WWTPs over a large distance and the association with LD indicate that WWTP's should be included in source finding investigations of LD cases. Furthermore, it is recommended to further investigate how aerosol dispersion of WWTPs can effectively be reduced in order to reduce the potential health risk.

To advance the field of *Legionella* dispersion modelling, more data are needed on 1) the WWTPs inventory which must be made as complete and up-to-date as possible, including information on measures taken, 2) numbers of WWTPs where *Legionella* was found in the process water, 3) the formation and emission of *Legionella*-containing aerosols in WWTPs including the aerosol size distribution, 4) the survival of *Legionella* in the air in different weather conditions, and 5) possible other sources, such as cooling towers and effluent dispersion of WWTPs via surface water. For other airborne infectious diseases, modelling such as applied here could also contribute to insight into possible sources in the living environment. In the future, climate change and (sustainable) developments in WWTP technology that increase water temperature could influence *Legionella* dispersion from WWTPs.

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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.2021.113811.

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Community design and hypertension: Walkability and park access relationships with cardiovascular health

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ABSTRACT

Background: There is an increased literature focusing on the role of the built and natural environments in preventing hypertension. However, very few studies have quantitively analyzed specific pathways through which urban form affects blood pressure levels.
Objectives: To examine how features of the built and natural environments relate to hypertension and the mediating role of transportation and leisure walking and body mass index in this relationship.
Methods: We examined the association between neighbourhood walkability and park availability with hypertension through generalized linear models in two independent population cohorts. One Cohort was 22,418 adults (My Health My Community) and the other cohort was 11,972 adults (BC Generations Project). We employed a path analysis modelling approach to explore the presence and significance of mediating factors that may contribute to any association between walkability or park availability and hypertension. This study intentionally employed walkability measures enforced through municipal zoning and subdivision regulations legally underpinned by health, safety, and welfare. All models were adjusted for socioeconomic and other characteristics where data were available.
Results: Our analysis of two population-based Canadian cohorts consistently found that higher levels of walkability and park accessibility were both associated with significantly lower odds of self-reported hypertension, especially for lower income individuals. Mediation analysis showed that obesity accounted for 50% and 52.9% of the total effect of walkability and park accessibility on hypertension, respectively.
Discussion: We suggest an integrated population health approach that considers multimorbidity as a result of exposure to car-dependent areas and the lack of green spaces. Longitudinal research is needed to document causal effects of built and natural environments on hypertension.

1. Introduction

High systolic blood pressure remains the leading risk factor for mortality and disability worldwide. In 2018, it accounted for 10.4 million deaths and 218 million years lost due to ill-health, disability or premature death (Stanaway et al., 2018). While hypertension and related cardiovascular disease (CVD) are global issues, a healthy lifestyle can prevent nearly 80% of this morbidity. Diet, physical activity, body mass index, and consumption patterns play a pivotal role in both the development and prevention of high blood pressure (BP) (Olsen et al., 2016).

Increased sprawl and poor connectivity in residential areas during the past decades have resulted in the creation of obesogenic environments in high-income nations, which in turn has increased the

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prevalence of CVD. More recent industrializers are now following a similar pattern; over 80% of CVD is expected to occur in low-and-middle-income countries in 2020 (Mills et al., 2020; Teo et al., 2009).

While many researchers have identified a linkage from built environment attributes to CVD risks and the emergence of several other chronic diseases, very few have investigated the impact of neighbourhood configuration on hypertension until recently. A systematic review published in 2016 (Malambo et al., 2016) identified only two of such studies, both looking at the availability of grocery shopping and restaurants, walkability, and their effect on BP. While walkability correlated with reduced BP, this effect was partially attenuated by the presence of fast-food chains in highly walkable neighbourhoods (Drewnowski et al., 2012; Wilson et al., 2011).

In a study from 2016, Chiu and colleagues found a significantly decreased risk of incident hypertension by moving to a neighbourhood with high walkability in Ontario, Canada (2016). Researchers replicated these findings in both Toronto and the U.S. (Lindsay M Braun et al., 2016a,b; Loo et al., 2017). The largest study on the subject to this date—nearly 430,000 people from a population-based cohort in the U.K.—also found that walkability was negatively correlated with hypertension risk, predominantly among females, people between 50 and 60 years of age, employed individuals, and residents of deprived neighbourhoods (Sarkar et al., 2018). The Canadian cohort study CANHEART revealed two interesting results: a dose-response association between walkability and systolic BP and effect modification in this relationship by the levels of traffic-related air pollution (nitrogen dioxide, NO_2). Higher levels of pollution in the more walkable areas diminished the difference in probability of hypertension between highly walkable and non-walkable neighbourhoods (Howell et al, 2019a, 2019b). Finally, a pooled meta-analysis of all longitudinal studies published before July 2016 found "very strong evidence … for the impact of walkability on hypertension" (Chandrabose et al., 2019, p. 6).

The association between parks and cardiovascular risk factors, including hypertension, has been examined using several methodologies. Some studies have demonstrated benefits of park-based interventions and activities (D'Agostino et al., 2018; Messiah et al., 2018, 2017) and further research has examined the effects of general exposure to greenspace on CVD and risk factors. Shen and Lung found a negative association between green spaces and CVD mortality, which they surmised was mediated by a reduced impact of air pollution and heat (2016). Other studies focusing on hypertension have come to similar conclusions (Dzhambov et al., 2018; Jia et al., 2018; Leng et al., 2020; Paquet et al., 2013) and among the many health outcomes that have been associated with exposure and access to green spaces, the reduced risk of CVD mortality seems to be one of the most consistent findings (Colom et al., 2018; Gascon et al., 2016; Richardson and Mitchell, 2010).

While both green space and walkability have respectively been associated with a decreased risk of hypertension, more studies are required to confirm these findings in a diverse set of geographies and contexts. Mechanisms and pathways behind associations also need to be further analyzed. The purpose of our study is to examine how features of the built and natural environments relate to hypertension directly and indirectly, by testing whether this relationship is mediated through physical activity or body mass index in two independent population cohorts.

2. Methods

2.1. Study area

The study is based in the "lower mainland" of British Columbia (BC), Canada, including Metro Vancouver and the Fraser Valley Regional District. Metro Vancouver is one of the most rapidly urbanizing regions of Canada. It includes 22 municipalities, one electoral district, and one treaty First Nation. The Vancouver region provides a wide array of built environments ranging from the fifth most densely populated city in North America to low-density sprawling suburban and exurban environments. One-third of the area is designated as urban (Metro Vancouver, 2018), and the population density is approximately 850 persons/km^2 (Statistics Canada, 2017), whereas the Fraser Valley Regional District primarily comprises suburban and rural communities, although it has also seen a rapid increase in density and urbanization during the last decade.

2.2. Design and population

Cross-sectional samples were obtained from two separate large cohorts: The My Health My Community Survey (MHMC) and The British Columbia Generations Project (BC Gen). MHMC targeted individuals 18 years and older who resided in areas served by Vancouver Coastal Health and Fraser Health (n = 22,418); it was conducted between June 2013 and July 2014. It used a non-probability sampling technique to recruit participants and used post-collection weighting by age, gender, education, and municipality of the population aged 18 and over to adjust for sampling bias. This type of sampling allows researchers to make their sample more representative, although it increases the variance of the final estimates. MHMC sampling strategy and other technical notes can be accessed on their website (My Health My Community, 2019).

BC Gen is a prospective population-based cohort (n = 29,850) that obtained health-related information and biological samples of approximately thirty thousand British Columbians between 35 and 69 years of age. Participants consented to link their medical records from 1986 and forward and to future use of their data. Details from the sampling method and other notes had been previously described (Dhalla et al., 2019). While the surveys cover the entire BC region, only the participants residing in the Metro Vancouver region and parts of Fraser Valley Regional District were included in the study (n = 11,972). Data on study participants were linked to environmental variables using the centroid of the 6-digit postal code. The 6-digit postal code is the most commonly used spatial unit in Canadian epidemiological studies and in urban areas; it typically corresponds to a single-block face (Khan et al., 2018).

The study was approved by the Behavioural Research Ethics Board of the University of British Columbia (H17-00648).

2.3. Walkability

The walkability index was calculated by adding the normalized values (z-scores) of four indicators: residential density, the commercial floor-to-area ratio (the number of storeys relative to the net area of the site), land-use mix, and intersection density. Further details on the methodology and weights for each component can be found elsewhere (Frank et al., 2010). The index has been widely used and validated in numerous studies across North America and overseas (Frank et al., 2010; Kligerman et al., 2007; Sallis et al., 2016). It uses 1 km pedestrian "walksheds" that map pedestrian-accessible roads around each postal code centroid. Each walkshed corresponds to approximately 10–15 min of walking time, a commonly used time frame to assess perceived proximity to amenities and services (Annerstedt van den Bosch et al., 2015). Finally, we divided the walkability index into quintiles that allowed us to compare the variance of other predictors, as done in most health geography studies (Fig. 1 and Fig. S1). The quintiles were slightly modified to match the median residential density for walkability quintiles using MHMC data. We labelled quintiles as car-dependent (Q1), somewhat car-dependent, somewhat walkable, moderately walkable, and walkable (Q5).

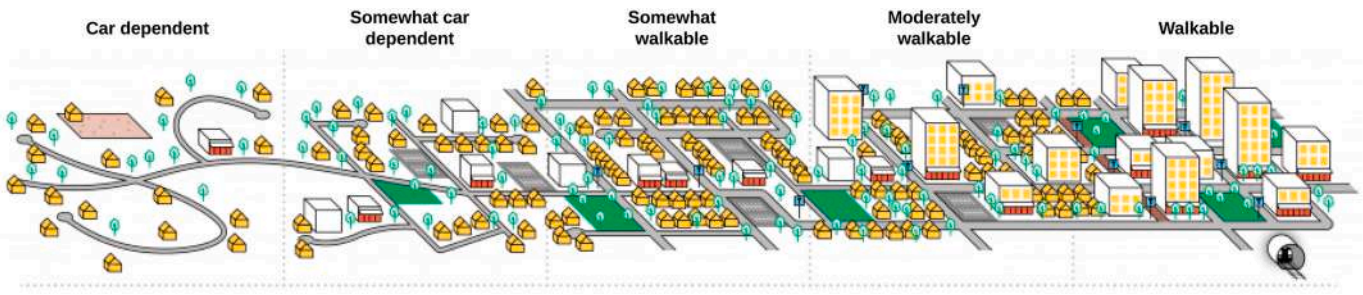

Fig. 1. Graphic representation of quintiles from car-dependent to walkable neighbourhoods.

2.4. Park availability

Using the CanMap® Parks and Recreation database (DMTI Spatial Inc., 2010), we created a proxy measure for park availability based on the number of parks within each walkshed as done elsewhere (Cohen et al., 2006; Schipperijn et al., 2017; Veitch et al., 2016). We also aggregated park availability into quintiles and modified them to match the median number of parks. There was no specific size definition of the parks, but we excluded a small proportion (around 5%) that charge an entry fee during the day (Fig. S2).

2.5. Primary outcome

The primary outcome of this study was having a reported diagnosis of high blood pressure by a doctor, included in both MHMC and BC Gen questionnaires, although only the latter asked for age of diagnosis. The response was coded into a binary outcome.

2.6. Mediating variables and confounders

Physical activity was measured using two indicators: transport walking and leisure walking. Participants were asked to report the time they spent walking as a part of a commute, doing errands or shopping and time spent on walking for recreation or leisure in the past seven days. The responses were averaged to create a daily mean value, which was then dichotomized using 30 min as the cut point, equivalent to the weekly 150 min of moderate to vigorous physical activity (MVPA) recommended by the WHO if we consider only weekdays. Body Mass Index (BMI) was calculated from self-reported height and weight. Obesity was coded as binary, using a cut-off value of 30, in accordance with the WHO definition (1995). Demographic variables assessed by the survey were age, gender, income, ethnicity, smoking status, and the number of years lived in the neighbourhood. Income was categorized into four groups. Since MHMC and BC Gen use slightly different income categories in their survey, we created two separate sets of income categories for our analyses. Ethnicity was categorized into five groups: (i) Caucasian, (ii) Aboriginal, (iii) Asian, (iv) South Asian, and (v) for MHMC and into two groups: (i) Caucasian and (ii) others for BC Gen. Smoking was coded as a binary variable (never versus past/active smoker). We accounted for missingness by adding missing values as an extra category when applicable (Vach and Blettner, 1991).

2.7. Statistical analyses

2.7.1. Exposure effect estimation

We assumed neighbourhood walkability and park availability affected the risk of hypertension through independent pathways (transportation and leisure walking, respectively). Hence, we created two separate generalized linear models (logit link) to examine the association of our exposures with hypertension considering there is substantial overlap between both measures. We adjusted our models using Pearls' backdoor criterion (Textor et al., 2016), which controls for variables that are predecessors of both exposure and outcome, except for mediators. We used two independent cohorts to further validate our study findings. MHMC and BC Gen are the main and supporting cohorts, respectively. We also performed stratified analyses to explore how income and gender modified the effect of walkability and park accessibility on self-reported hypertension. We defined statistical significance a priori with a two-tailed alpha of 0.05 for all analyses. E-values were calculated to estimate the necessary confounding to significantly affect the estimates (Van Der Weele and Ding, 2017)

2.8. Path analyses

We then employed a mediation analysis in the MHMC cohort; this regression-based procedure estimates the effect with and without a designated mediator (Robins and Greenland, 1992). The primary objective of the mediation analysis was to examine the effects of environmental variables on hypertension through several, possibly interlinked mediators. We developed two saturated models that included all available pathways, one for each exposure (Figs. 2 and 3). The indirect effects were assumed to be mediated—by obesity via walking, and walking (Frank et al., 2019)—whereas the direct effects were assumed to occurred directly—e.g., throw other non-specified pathways.

The total indirect effect was decomposed to specific mediating effects. We calculate the 95% confidence intervals of the path coefficients using bootstrapping techniques. We fitted weighted least square mean and variance adjusted (WLSMV) models in M-Plus 8, used in cases of categorical mediators and outcomes (Muthén et al., 2017).

Young people might cluster around certain neighbourhoods (e.g., nearby educational institutions) and are also less likely to develop hypertension. The same could be said about income, and ethnicity, and movers versus non-movers, and those with reduced accessibility. Hence, all models were adjusted for age, income, gender, ethnicity, smoking history, time lived in the neighbourhood, and regional accessibility because these are known confounders of the relationship between walkability and hypertension (Lindsay M. Braun et al., 2016a,b; Claudel et al., 2018; Kershaw et al., 2017; Van Dyck et al., 2011). Smoking history was added for improved precision because it is a well-known risk factor for elevated blood pressure. Regional accessibility measures the number of major destinations accessible within 45 min by transit during morning peak hours. We identified major destinations using Metro Vancouver's regional growth strategy and added two additional locations in the urbanizing area of the Fraser Valley. Finally, we conducted a sensitivity analysis controlling for the effects of the alternative main exposure on each path model.

3. Results

3.1. Descriptive analyses

Table 1 shows the demographic characteristics of participants for both MHMC and BC Gen datasets and the Metro Vancouver population for reference. Because the studies recruited participants using different age

Table 1
Sociodemographic characteristics of MHMC, BCGP participants and Metro Vancouver.

Variables	MHMC Sample[a]	BC Gen Sample[b]	Metro Vancouver[c]
	Percentage/ Mean (SD)	Percentage/ Mean (SD)	Percentage/ Mean (SD)
Age	45.58 (16.19)	55.09 (8.94)	46.63
Gender			
Male	45.11%	30.50%	48.23%
Female	54.28%	69.50%	51.77%
Missing	0.62%	–	–
Household Income			
Under $50,000 ($60,000 for MHMC)	33.29%	19.64%	47.42%
$50,000 ($60,000 for MHMC)-$100,000	20.42%	33.81%	24.16%
$100,000-$150,000 ($160,000 for MHMC)	17.80%	21.11%	16.17%
$150,000 ($160,000 for MHMC) and above	7.70%	18.52%	12.25%
Missing	20.79%	6.92%	–
Ethnicity			
Caucasians	59.03%	79.99%	51.98%
Aboriginal	2.97%	1.56%	2.85%
Asian	21.52%	10.17%	28.26%
South Asian	7.23%	1.88%	11.07%
Other	6.57%	1.56%	5.85%
Missing	2.69%	4.84%	–
Smoking			
No	61.1%	55.49%	–
Past or current	38.9%	43.2%	–
Missing		1.31%	–
Years lived in neighbourhood	10.68 (11.88)	13.64 (10.64)	–
Missing		1.08%	
Years since hypertension diagnosis		9.56 (8.91)	–
Missing		83.6%	–

[a] MHMC responses were weighted using the 2011 National Household Survey data by age, gender, education, and neighbourhood income. The total MHMC sample size used in this study was 22,418.
[b] The total BC Gen sample size used in this study was 11,792.
[c] The age and gender data for Metro Vancouver were derived from Census Canada 2011 using 18 years and above data only (n = 1,872,935); ethnicity (n = 2,280,690), and household income (n = 891,310) were taken from National Household Survey 2011. Census Canada and NHS have a wider age range and cover more participants.

limits, the average age of participants was 45.6 years & 55.1 years for MHMC and BC Gen, respectively. According to the 2011 Canadian Census, the average age for these two cut-off points in the general population were slightly lower (39.5 years and 51.1 years, respectively) (Statistics Canada, 2011a). Likewise, the average Metro Vancouver population ages were 46.6 years and 50.5 years, respectively (Table 1). Time lived in the neighbourhood averaged 10.7 years in MHMC and 13.6 years in BC Gen. Overall, female participation was higher than males in both datasets; BC Gen had a higher proportion of female participants compared to the MHMC participants. BC Gen data also had a higher percentage of female participants compared to the Metro Vancouver population (51.1%). Almost half of the total participants (MHMC = 53.7%; BC Gen = 53.5%) had household income less than $100,000 which was lower than the regional percentage (71.6%) assessed by the 2011 National Household Survey (NHS) (Statistics Canada, 2011b). Similarly, a larger percentage (80.0%) of the BC Gen participants were Caucasian compared to MHMC (59.3%) and Metro Vancouver region (52.0%).

The average walkability index value was 1.1 for both cohorts; MHMC had a wider range (−6.1072 to 16.3757) compared to BC Gen (−5.9437 to 15.8446) (Table 2). The mean number of parks available in the neighbourhood was 3.1 and 3.4 in MHMC and BC Gen respectively. Additionally, 15.9% and 19.0% of the total sample in MHMC and BC Gen respectively reported being diagnosed with hypertension. In the MHMC dataset, 19.7% were obese; 25.4% reported walking at least 30 min per day for transport; 29.6% reported walking at least 30 min daily for leisure. A detailed description by quintile for every cohort is provided in supplementary tables (Table S1-Table S4).

Table 2
Distribution of key outcome, predictors, and mediators.

Variables	MHMC	BC Gen
	Percentage/Mean (SD)	Percentage/Mean (SD)
	N = 22,418	N = 11,972
Outcome and Key Predictors		
Hypertension		
Yes	15.92%	18.99%
No	79.54%	79.90%
Missing	4.55%	1.11%
Walkability Index	1.097 (3.28)	1.1238 (3.319)
Park Availability	3.136 (2.869)	3.393 (3.033)
Mediators		
Transport Walking (≥30 min/ day)		NA
Yes	25.35%	
No	53.8%	
Missing	20.85%	
Leisure Walking (≥30 min/day)		
Yes	29.62%	
No	48.73%	
Missing	21.65%	
MVPA (≥150 min/week)		
Yes	40.36%	
No	51.46%	
Missing	8.18%	
Obesity		
Yes	19.67%	
No	69.7%	
Missing	10.62%	

MHMC responses were weighted using the 2011 National Household Survey data by age, gender, education, and neighbourhood income.

3.2. Exposure effect estimate

3.2.1. Walkability

Participants living in the most walkable neighbourhoods (quintile 5) were less likely to report a diagnosis of hypertension than those living in car-dependent neighbourhoods (quintile 1) for both datasets (Table 3). After controlling for income, age, gender, ethnicity, time in the neighbourhood, smoking, and regional accessibility; living in a walkable neighbourhood was associated with 0.29 lower odds of self-reported hypertension compared to living in a car-dependent neighbourhood in MHMC (OR = 0.71; 95% CI: 0.57, 0.88) and 0.18 lower odds in BC Gen (OR = 0.82; 95% CI: 0.68, 0.99). There were no significant differences in the odds of hypertension when comparing the most car dependent neighbourhoods (Q1) with quintiles 4 or lower in either dataset; significant differences were only observed at the margins. Missing data for the included variables ranged from 0.6% for gender to 20.8% for household income.

3.3. Park availability

Participants living in neighbourhoods with greater park availability (quintile 5) were less likely to report a past diagnosis of hypertension than those living in neighbourhoods with lower park availability (quintile 1) in both datasets (Table 4). Compared to neighbourhoods with low park availability (quintile 1), living in a neighbourhood with higher park availability (quintile 5) was associated with 0.35 lower odds of self-reported hypertension in MHMC (OR = 0.65; 95% CI: 0.54, 0.79) and 0.25 lower odds in BC Gen (OR = 0.75; 95% CI: 0.64, 0.88) in adjusted models. We also found a significant difference for quintile 4 compared to quintile 1 in both MHMC and BC Gen datasets (Table 4).

Table 3
Neighbourhood walkability and odds of hypertension.

	MHMC		BC-Gen	
	Unadjusted Model OR (95% CI)	Adjusted Model[a] OR (95% CI)	Unadjusted Model OR (95% CI)	Adjusted Model[a] OR (95% CI)
Walkability Index (Ref: Car-Dependent)				
Somewhat Car-Dependent	0.983 (0.832,1.16)	0.923 (0.765,1.119)	1.087 (0.958,1.233)	1.083 (0.939,1.250)
Somewhat Walkable	1.010 (0.853,1.197)	0.924 (0.759,1.124)	0.922 (0.798,1.064)	0.909 (0.770,1.072)
Moderately Walkable	0.939 (0.795,1.108)	0.853 (0.699,1.042)	0.892 (0.763,1.042)	0.873 (0.727,1.048)
Walkable	0.687 (0.578,0.816)	0.712 (0.571,0.887)	0.818 (0.705,0.947)	0.819 (0.681,0.985)

[a] Adjusted for age, income, gender, ethnicity, smoking, years lived in the current neighbourhood and regional accessibility.

Table 4
Park availability and odds of hypertension.

	MHMC		BC Gen	
	Unadjusted Model OR (95% CI)	Adjusted Model[a] OR (95% CI)	Unadjusted Model OR (95% CI)	Adjusted Model[a] OR (95% CI)
Park Availability (Ref: Quintile 1)				
Quintile 2	0.996 (0.839,1.183)	0.893 (0.735,1.086)	1.109 (0.96,1.279)	1.078 (0.914,1.270)
Quintile 3	0.958 (0.829,1.107)	0.847 (0.714,1.004)	0.906 (0.797,1.03)	0.887 (0.764,1.029)
Quintile 4	0.775 (0.651,0.924)	0.612 (0.496,0.755)	0.61 (0.526,0.706)	0.655 (0.553,0.774)
Quintile 5	0.705 (0.594,0.838)	0.652 (0.536,0.794)	0.725 (0.631,0.832)	0.754 (0.642,0.884)

[a] Adjusted for age, income, gender, ethnicity, smoking, years lived in the current neighbourhood, and regional accessibility.

4. Stratified analyses

4.1. Walkability

4.1.1. Income

Those in the lowest income group living in the most walkable neighbourhoods (quintile 5) were less likely to report a diagnosis of hypertension than income comparable participants living in car-dependent neighbourhoods (quintile 1). Living in a walkable neighbourhood was associated with 0.34 lower odds of self-reported hypertension in MHMC (OR = 0.66; 95% CI: 0.45, 0.98) and 0.39 lower odds in BC Gen (OR = 0.61; 95% CI: 0.42, 0.89).

4.1.2. Gender

Females living in walkable neighbourhoods (quintile 5) were less likely to report a diagnosis of hypertension than those living in car-dependent neighbourhoods (quintile 1) in both MHMC (OR = 0.70; 95% CI: 0.52, 0.92) and BC Gen (OR = 0.70; 95% CI: 0.55, 0.88) cohorts.

4.2. Park availability

4.2.1. Income

In subjects of the lowest income group, people with higher park availability (quintile 5) had 0.31 (OR = 0.69; 95% CI: 0.49, 0.98) and 0.31 (OR = 0.69; 95% CI: 0.50, 0.96) lower odds of reporting past diagnosis of high blood pressure than those living in areas with low park availability (quintile 1) in MHMC and BC Gen, respectively.

4.2.2. Gender

Both males and females living in areas with higher park availability (quintile 4 and 5) were significantly less likely to report a diagnosis of hypertension than those living in areas with low park availability (quintile 1). However, the confidence interval was not significant for males in the BC Gen cohort (with a lower proportion of males compared to MHMC).

Results that were significant in one of the data sets only are not reported in-text but are available for review as supplementary material (Tables S5–S8).

4.3. Path analysis

4.3.1. Walkability index

The path analysis showed a significant relation between walkability, transport walking (β = 0.022, 95% CI: 0.012, 0.032), obesity (β = −0.037, 95% CI: −0.049, −0.027), and hypertension (β = −0.014, 95% CI: −0.028, −0.001).

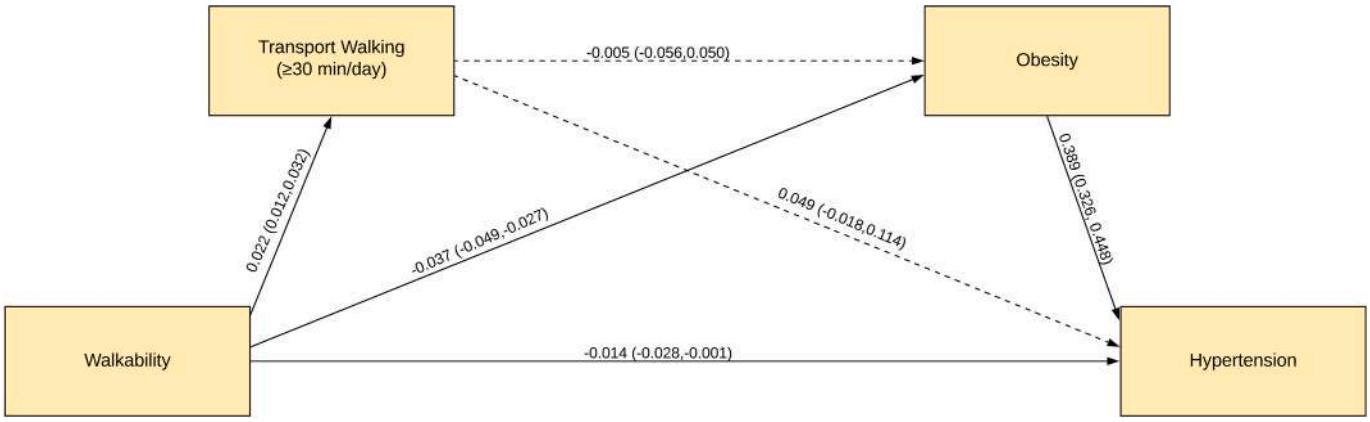

Fig. 2. Path coefficients for the associations between walkability and hypertension and their respective mediators, adjusted for income, age, gender, ethnicity, smoking, years lived in the neighbourhood, and regional accessibility (we accounted for sampling weights in the Model).

Table 5
Break down of the mediation effects and 95% bias-corrected confidence intervals explaining the relationship between neighbourhood walkability and hypertension in My Health My Community.

	Point estimate and 95% CI	P-value
Total effect	−0.028 (−0.041, −0.015)	<0.001
Direct effect	−0.015 (−0.028, −0.001)	0.035
Indirect effect	−0.013 (−0.019, −0.009)	<0.001
Specific Indirect Effects (Walkability to Hypertension)		
Walkability → Transport Walking → Hypertension	0.001 (0.000, 0.003)	0.175
Walkability → Obesity → Hypertension	−0.014 (−0.020, −0.010)	<0.001
Walkability → Transport Walking → Obesity → Hypertension	0.000 (−0.001, 0.000)	0.857

All numbers rounded to the closest 3 digits decimal place. Confidence interval (CI) obtained via bootstrapping; SE: standard error. All models controlled for income, age, gender, education, smoking status, ethnicity, regional accessibility, and time in the neighbourhood. Responses were weighted using the 2011 National Household Survey data by age, gender, education, and neighbourhood.

The direct relationships between the mediating variables—transport walking and obesity—were not statistically significant. However, obesity had a significant direct relationship with hypertension (Fig. 2). The analyzed pathways mediated 53.6% of the total effect of walkability on hypertension (Table 5). Missing data for the analyzed variables ranged from 4.6% (hypertension diagnosis) to 21.7% (leisure walking).

4.4. Park availability

The path analysis (Fig. 3) showed a significant relationship of park count with leisure walking (β = 0.013, 95% CI: 0.002, 0.024) and obesity (β = −0.048, 95% CI: −0.062, −0.036), but not hypertension (β = −0.015, 95% CI: −0.032, 0.001). Leisure walking and obesity were also significantly related (β = −0.048, 95% CI: −0.062, −0.036).

The analyzed pathways mediated 55.9% of the total effect of park availability on hypertension (Table 6).

Table 6
Break down of the mediation effects and 95% bias-corrected confidence intervals explaining the relationship between neighbourhood park count and hypertension in My Health My Community.

	Point estimate and 95% CI	P-value
Total effect	−0.034 (−0.049, −0.019)	<0.001
Direct effect	−0.015 (−0.030, 0.001)	0.060
Indirect effect	−0.019 (−0.025, −0.014)	<0.001
Specific Indirect Effects (Park Availability to Hypertension)		
Park Availability → Leisure Walking → Hypertension	0.000 (−0.001, 0.000)	0.520
Park Availability → Obesity → Hypertension	−0.018 (−0.025, −0.013)	<0.001
Park Availability → Leisure Walking → Obesity → Hypertension	0.000 (−0.001, 0.000)	0.073

All numbers rounded to the closest 3 digits decimal place. Confidence interval (CI) obtained via bootstrapping; SE: standard error. All models controlled for income, age, gender, education, smoking status, ethnicity, regional accessibility and time in the neighbourhood. Responses were weighted using the 2011 National Household Survey data by age, gender, education, and neighbourhood.

4.5. Sensitivity analyses

Both mediation analyses showed similar results once you controlled for the complementary exposure (Tables S13 and S14). However, adjusting for park count reduced the accuracy of the mediation estimate from walkability to hypertension. The proportion mediated for walkability and park count to hypertension changed to 41.1% and 57.7%, respectively.

5. Discussion

5.1. Summary of findings

Our analysis of two independent population-based Canadian cohorts found that higher levels of walkability and park accessibility were both

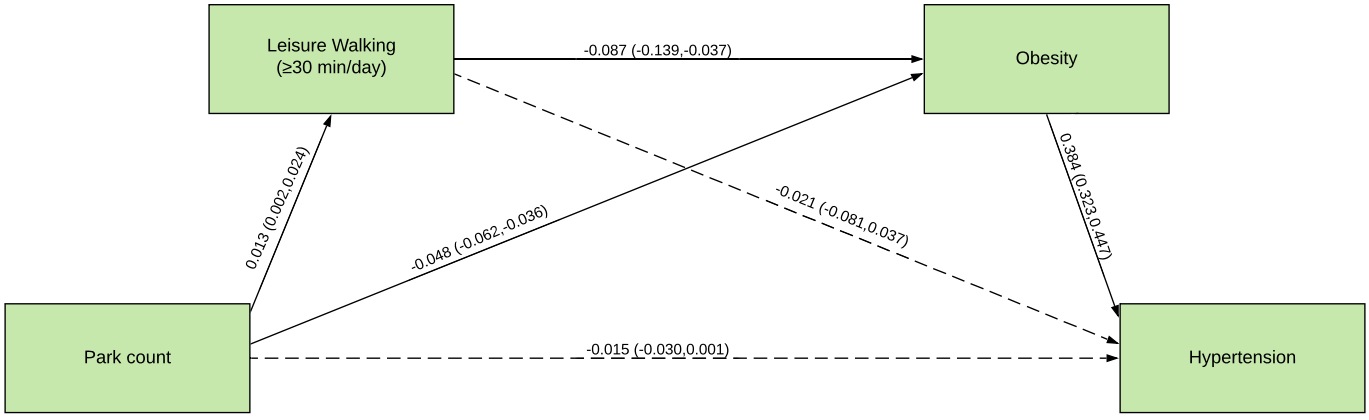

Fig. 3. Path coefficients for the associations between park count and hypertension and their respective mediators, adjusted for income, age, gender, ethnicity, smoking, years lived in the neighbourhood, and regional accessibility (we accounted for sampling weights in the Model).

associated with significantly lower odds of self-reported hypertension, particularly among females and people of low-income. Mediation analysis showed that obesity alone accounted for 50% and 52.9% of the total effect of walkability and park accessibility on hypertension, respectively. However, neither transportation walking nor leisure walking significantly mediated these effects. Our sensitivity analyses suggest that park count partially explains the effects of walkability, but not the other way around, given the number of parks derives from the urban structure (e.g., density of blocks and intersections).

5.2. Relationship with previous studies

Our main analysis for the relationship between walkability and hypertension concur with previous studies (Lindsay M Braun et al., 2016a, b; Chandrabose et al., 2019; Chiu et al., 2016; Howell et al., 2019b, 2019a; Loo et al., 2017; Sarkar et al., 2018). But unlike previous studies, we failed to find a significant role for transportation walking (Van Dyck et al., 2010). Obesity mediates most of the effect, which would suggest walkability is associated with obesity and hypertension by alternative pathways including reduced sedentary time in cars (Frumkin et al., 2004). In the Canadian CANHEART cohort study, environmental pollutants did offset the protective effects of walkability on hypertension. However, this effect seems to start at 40 ppb of NO_2 (Howell et al., 2019a), levels that are uncommon in the area of our study (Gan et al., 2014; Pinault et al., 2016).

Previous studies analysing the relation between park accessibility or green space exposure (or similar measures) and hypertension vary considerably across methods, data sources, and metrics used for greenness. Most studies have used either the Normalized Difference Vegetation Index (NDVI) or land use or land cover maps (Jarvis et al., 2020). The majority of existing studies support our findings, suggesting an association between greenness and a reduced risk of hypertension (Brown et al., 2016; Dzhambov et al., 2018; Jia et al., 2018; Lane et al., 2017; Paquet et al., 2013), although contradictory results have been found for urban versus rural greenspaces (2018) (Picavet et al., 2016). A Lithuanian study could not confirm a significant relationship between distance to greenspace and prevalence of hypertension (Tamosiunas et al., 2014), but the authors did not adjust for income or ethnicity, which are likely confounders in this relationship. There is a need for standardized procedures to research park availability and its effect on CVD and risk factors, independently of other open public spaces.

Similarly, there is a lack of research examining causal or mediating pathways from park accessibility to hypertension. Like ours, most studies are cross-sectional and have focused on the role of physical activity (Brown et al., 2016; Jia et al., 2018; Lane et al., 2017; Paquet et al., 2013; Picavet et al., 2016). Most used the NDVI index to measure neighbourhood greenness and use continuous measures of physical activity and BMI. Additionally, they also use air pollution as a mediator in their analyses (in addition to other socio-demographic covariates). The evidence from these studies, however, is not consistent. The paper by Huang et al. (2021) did not find a significant mediation by BMI and physical activity on neighbourhood greenness and hypertension relationship. Whereas Jia et al. (2018) found evidence of the mediation effect of total physical activity. Yang et al. (2020) found BMI partly mediating the greenness and hypertension relationship. Leng et al. (2020) identified seasonality as a mediator (2020). Other papers (Liu et al., 2021; Yang et al., 2020) did not find significant mediation by physical activity. Due to the difference in the measurement approach used by these studies compared to ours, we cannot make a direct comparison of the effect size. Furthermore, the focus of our paper is on the role of walking (leisure walking and transport walking) instead of total physical activity as measured by these studies. More recently, researchers have incorporated variables related to mental health (Brown et al., 2016; Dzhambov et al., 2018). Finally, longitudinal studies have revealed that exposure to air pollution during early infancy led to a higher prevalence of hypertension later in life, an effect that could potentially be alleviated by access or exposure to green spaces (Bijnens et al., 2017; Shen and Lung, 2016).

5.3. Strengths

The current study has several strengths. Foremost, the analyses employ two independent large samples in a dynamic, rapidly urbanizing highly varied region and a major destination for immigrants with various geographic, social, cultural, and economic backgrounds. The walkability data used for this study is built from parcel level land use data enabling the measurement of floor space and three-dimensional nature of the built environment. Land area data only captures what is at ground level and introduces considerable measurement error because we live in a three-dimensional (3D) world where buildings can have several floors. Parcel data is difficult to obtain and process and therefore, many studies to date have relied on two dimensional (2D) land area data to measure walkability (Manaugh and El-Geneidy, 2011). Our walkability index includes a retail-to-floor area ratio or the amount of floor space built on each retail parcel—a 3D measure built directly from floor space data. We used a highly varied urban form (densities included in the walkability index ranged from 0 to 210 dwelling units/acre), supporting the ability to test how variation in built form relates with variation in hypertension. Finally, the current study mapped out what could be "causal" pathways linking built and natural environment with hypertension through physical activity and obesity. These results are policy relevant given that these two lifestyle factors are where many population level policy discussions to reduce heart disease have been focused over the past two decades. Results are highly relevant to the policy discussions around the current pandemic which has taught us the critical importance that chronic diseases play in predicting severity of illness and mortality from COVID-19. Lower mortality rates from COVID-19 in more walkable areas (Hamidi et al., 2020) are a function of the mediating effect of obesity (marker for hypertension and diabetes) on the relationship between walkability and mortality (Wali et al., 2021). This result helps to explain extremely high COVID-19 mortality rates in lower income communities where obesity and other forms of chronic disease are highest where the need for active transportation investments and access to greenspace are the greatest.

5.4. Limitations

Our analysis faced a number of limitations. First, this is a cross-sectional study which limits the ability to establish causal inferences, even though structural equation methods employed supported the testing of hypothetical pathways. On a related note, the odds ratio for a common outcome like hypertension overestimates the true relative risk. Self-reported diagnosis of hypertension prevented us from estimating exact changes in systolic and diastolic blood pressure levels associated with walkability and park access and the mediating effects of walking and obesity. Self-reported data collected on walking for transport and leisure was not done through a travel diary and may likely suffer from considerable memory recall inaccuracies. Furthermore, we could not account for activities that increased overall physical activity while decreasing leisure or transportation walking. It is possible and even likely that more accurate forms of travel and physical activity data collection will yield different results. In addition, some bias could result from the clustering of study participants, which may bias the standard errors and lead to type 1 errors. We could not conduct subgroup analysis based on treatment allocation, although the region provides nearly universal health coverage. For our subgroup analysis, we should also note that most participants were female (particularly for BC Gen) and low-income (~53% for both cohorts) and that the lack of effect in males and higher income groups might derive from type 2 error. The high proportion of missing data for household income in one of the cohorts warrants further caution. While, on average, people moved to the study area before being diagnosed with hypertension, we could not establish

temporality because a high proportion (over 80%) of our sample did not report the date of diagnosis. We were not able to adjust for diet in our models, and there was a high proportion of missing data for smoking (over 40%). We added an extra category for missing data, which could have introduced bias in the analysis. Furthermore, based on the reported e-values (1.1 for walkability and 1.3 for park availability), relatively weak unobserved confounding could explain away the associations.

Researchers have mentioned the risk of self-selection bias in studies researching walkability (Nesbitt et al., 2019a), although others have suggested that walkable neighbourhoods are not necessarily associated with affluence (Booth et al., 2019; Dendup et al., 2019). On the other hand, park availability and green space exposure are typically associated with wealthy neighbourhoods (Nesbitt et al., 2019b). Ideally, residential self-selection should be accounted for in the study design and reported in the statistical analysis (Heinen et al., 2018), although we were unable to account for this in the current study. Nevertheless, we addressed socioeconomic variables by stratifying our sample by both income and age. Stratification by gender, while informative, should be interpreted with caution for BC Gen, as most of the sample was female. Walkability and park accessibility were associated with lower odds of hypertension; particularly among those with the least income. Compared to more commonly used green space measures, such as NDVI, the number of parks within a 1 km walkshed does not provide any information about the full greenness in the area. However, access to parks is a concept easily understood by the public and actionable by policy makers and captures spaces to recreate and to build social capital. Finally, the walkshed buffers used in this study are based on the road network and capture the effects of barriers and have been shown to better predict behavior in previous studies (Schipperijn et al., 2017).

5.5. Future studies

Our findings must be interpreted with caution since we used two cross-sectional samples from population-based cohorts. We suggest longitudinal research designs that incorporate variables that confound, modify, or mediate the effects of walkability and park accessibility on hypertension. For example, air pollution exposure is a key predictor of hypertension (Howell et al., 2019a) and should be captured when possible in future studies. Dynamic data captured at multiple time points will help to establish causal links between built and natural environment exposures and health outcomes. Retrospective residential history information will provide the ability to capture exposure to different types of environments over time. Usage of travel diaries to better capture travel and activity patterns or objective GPS data collection will greatly enhance the study of travel patterns in relation to built and natural environment exposures. Temperature, annoyance from traffic-related exposure, smoking, drinking, and diet are also related to both walkability and park access and hypertension and should be addressed in future studies either as confounders or mediators (Dzhambov et al., 2018; Frank et al, 2006, 2019; Howell et al., 2019a, 2019b). We suggest an integrated population health approach that considers many forms of co-morbidity as a result of exposure to car-dependent areas and the lack of green spaces. More importantly, greenspace and walkability act through similar pathways and may logically even be synergistic in their effect on health-related outcomes. City planners have known this for centuries – that is why so many old cities and towns had a "village green" with shops and eateries wrapped around a central public greenspace.

6. Conclusion

Findings demonstrate that living in a walkable neighbourhood and having higher park accessibility is associated with lower odds of hypertension, especially for lower income individuals. Interestingly, this observed association between built and natural environments and hypertension seemed significantly mediated by obesity. Neighbourhood walkability and park accessibility may help mitigate adverse health consequences of hypertension by reducing the number of individuals with body mass index above recommended guidelines. This study employed a set of walkability measures enforced through municipal zoning and subdivision regulations legally underpinned by health, safety, and welfare and our results further support these legally sanctioned measures and also suggest park availability as an asset for improved urban health.

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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.2021.113820.

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Detection of carbapenemase-producing, hypervirulent *Klebsiella* spp. in wastewater and their potential transmission to river water and WWTP employees

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ABSTRACT

Wastewater treatment plants (WWTPs) release drug-resistant microorganisms to water bodies (with effluents), and WWTP employees are exposed to bioaerosol emissions from the processed wastewater. Bacteria of the genus *Klebsiella*, in particular carbapenemase-producing (CP), hyper-virulent (Hvr) strains of *Klebsiella pneumoniae*, play a special role in this process. *Klebsiella* spp. strains isolated from wastewater, river water and the upper respiratory tract of WWTP employees were analyzed in this study. The isolated strains were identified as K. *pneumoniae* (K. pn) or K. non-*pneumoniae* (K. npn). The prevalence of nine types of genes encoding resistance to beta-lactams, nine genes encoding virulence factors and K1/K2 capsular serotypes, three genes encoding multi drug effluent pump systems, and the class 1 integron-integrase gene was determined by PCR. A total of 284 *Klebsiella* spp. isolates were obtained in the study: 270 environmental strains and 14 strains from the upper respiratory tract. Among environmental isolates 90.7% (245/270) harbored beta-lactam resistance genes, 17.4% (47/270) were classified as CP strains, 11.1% (30/270) were classified as Hvr strains, and 1.9% (5/270) were classified as CP-Hvr strains. CP-Hvr strains were also isolated from WWTP employees. Genes encoding β-lactamases (including carbapenemases), complete efflux pump systems and the K1 serotype were identified more frequently in K. pn strains. In turn, K. npn strains were characterized by a higher prevalence of bla_{SHV} and *intI*1 genes and K2 serotype gene. The strains isolated from wastewater and river water also differed in the abundance of drug resistance and virulence genes. The results of the study indicate that CP-Hvr K. pn strains are possibly transmitted from wastewater via bioareosol to the upper respiratory tract of WWTP employees. bla_{GES}-type carbapenemases significantly contributed to the spread of drug resistance in the environment.

1. Introduction

Biological treatment processes of wastewater in wastewater treatment plants (WWTPs) create a supportive environment for the horizontal transfer of antibiotic resistance genes (ARGs), and are not designed to eliminate microbial contaminants such as multidrug-resistant bacteria (MDRB) (Rizzo et al., 2013). WWTPs usually adopt different types of biological treatment processes (e.g. activated sludge, membrane bioreactor or anaerobic techniques) with various removal efficiency of antibiotic-resistant bacteria (ARB) and ARGs (Yuan et al., 2016). Treated wastewater is evacuated to natural water bodies, which significantly contributes to the spread of antibiotic resistance in the environment (Tesfaye et al., 2019; Alexander et al., 2020). In WWTPs, pathogenic microorganisms (including MDRB) are not confined to wastewater and are also highly abundant in bioaerosols that are generated at different stages of wastewater treatment. Sand traps, activated sludge bioreactors and sludge lagoons are the main sources of bioaerosol emissions (Lu et al., 2020; Breza-Boruta, 2010; Karra and Katsivela, 2007; Korzeniewska, 2011). K. *pneumoniae* may constitute up to 5% of the microbiological composition of bioaerosol in the proximity of mechanical and biological wastewater treatment sites (Korzeniewska and Harnisz, 2012). Bioaerosols pose a considerable health threat for WWTP employees who work long hours in hazardous conditions and are at high risk of infection. Microbial cells suspended in bioaerosols are

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inhaled with air and accumulate in the throat and the upper respiratory tract (Lou et al., 2021; Zieliński et al., 2020).

Gram-negative bacteria of the family *Enterobacteriaceae* deserve special attention in the group of MDRB present in wastewater and bioaerosols (Korzeniewska and Harnisz, 2012; Korzeniewska 2011). These bacteria are often resistant to all antibiotics or are sensitive only to older and more toxic antimicrobials (Magiorakos et al., 2012). The spread of antibiotic resistance in the family *Enterobacteriaceae,* in particular *Klebsiella pneumoniae*, gives serious cause for concern because these bacteria are responsible for a high percentage of hospital infections, including urinary tract infections, pneumonia, bacteremia and intra-abdominal infections (Sakkas et al., 2019). In 2017, the World Health Organization published a list of antibiotic-resistant priority pathogens for which innovative treatments are urgently needed. *Enterobacteriaceae* resistant to carbapenems and third-generation cephalosporins were listed as critical priority pathogens (WHO, 2017).

The production of β-lactamases encoded by, among others, bla_{TEM}, bla_{SHV}, bla_{CTX} and bla_{OXA} genes, is one of the key resistance mechanisms in *Klebsiella* spp. These genes may also be responsible for encoding the ESBL (extended-spectrum beta-lactamases) mechanism. ESBL-producing bacteria are resistant to most β-lactams, and the expression of ESBL mechanisms is particularly dangerous in highly virulent organisms (Gniadkowski 2008). To date, more than 350 different ESBL variants have been identified, which have been grouped into nine distinct families. The main types of ESBL include TEM, SHV, CTX-M and OXA (Bubpamala et al., 2018). Since ESBLs are not effective in hydrolyzing cephamycins or carbapenems, these antibiotic groups - particularly carbapenems, are recommended for the treatment of infections due to ESBL producers (Silago et al., 2021). However, a new group of β-lactamases – carbapenemases – has been identified in *Enterobacteriaceae* in the last decade (CDC 2013). Carbapenemases are enzymes that hydrolyze most β-lactams, including carbapenems, and have a broad substrate spectrum. Carbapenemase types such as *Klebsiella pneumoniae* carbapenemase (KPC), OXA β-lactamase (OXA-48), metallo-β-lactamases (MBL), including imipenemase (IMP), Verona integron-encoded metallo-β-lactamase (VIM) and New Delhi metallo-β-lactamase (NDM), have been increasingly detected in recent years (ECDC, 2019). Guiana extended-spectrum β-lactamases (GES) are also increasingly identified in Gram-negative bacteria, including *K. pneumoniae* (Gomi et al., 2018). Twenty-three GES types have been identified to date (Naas et al., 2016). Most of them are able to hydrolyze extended-spectrum cephalosporins, and nearly half show carbapenemase activity (Naas et al., 2016; Kim, 2016). Despite the above, bla_{GES} carbapenemases are not clinically monitored because of their presumably weak carbapenems hydrolyze and they are far less prevalent than other carbapenemases (Cave, 2019). A 2017 report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) demonstrated considerable variability in the national percentages of carbapenem resistance in *K. pneumoniae* isolates from invasive infections, ranging from 0% to 64.7%. In 2014–2017, the prevalence of carbapenem-resistant *K. pneumoniae* isolates increased in Slovakia, Poland and Portugal (ECDC, 2018). *Klebsiella pneumoniae* (as a group of bacteria), along with selected genetic determinants (including bla_{CTX-M}, bla_{TEM}, bla_{NDM-1}, bla_{VIM}, bla_{KPC}, and *int*I1) have been suggested as possible indicators to assess antibiotic resistance status in environmental settings (Berendonk et al., 2015). The global distribution of carbapenem resistance mechanisms is extensive. The spread of KPC-producing *K. pneumoniae* is considered to be endemic in the USA, China, Italy, Poland, Greece, Israel, Brazil, Argentina, Columbia and Taiwan. Endemic occurrence of NDM-producing K. *pneumoniae* is thought to occur in the Indian subcontinent, Pakistan and Bangladesh. For OXA-48-like-producing K. *pneumoniae,* endemic spread has also been reported in Turkey, Morocco, Tunisia, Libya, Egypt and India (Lee et al., 2016; Nordmann and Poirel, 2014). The other classes of carbapenemases also seem to be particularly frequent among members of the *Enterobacteriaceae* family, including GES-type enzymes (widely reported in Brazil, Turkey and South Korea), VIM-type (endemic in Greece, Spain, Italy, South Korea and Taiwan) and IMP-type (mainly in Japan, Taiwan and Eastern China) (Nordmann and Poirel, 2014).

In addition to antibiotic resistance genes, *K. pneumoniae* also produce virulence factors that are required for colonization, adhesion, invasion and infection progression. Virulence factors include lipopolysaccharides (LPS) in the cell wall, capsules, adhesins and siderophores. Hypervirulent strains of *K. pneumoniae* (HvK. pn.) which are responsible for serious cases of bacteremia and pyogenic liver abscesses have been increasingly described in the literature (Ali and Al-kakei Sana'a, 2019). Bacterial capsules are regarded as the main virulence factor of *Klebsiella* spp. because they protect the pathogen against phagocytosis and the bactericidal activity of the blood serum. Numerous capsular serotypes have been reported for *Klebsiella* spp. (including K1, K2, K5, K54 and K57), where serotypes K1 and K2 have the highest clinical significance (Jasim et al., 2020). The *rmp*A gene (regulator of the mucoid phenotype) is often linked with hypervirulent strains that cause liver abscesses (Hsu et al., 2011). Research has demonstrated that hypervirulent strains harbor a combination of iron acquisition systems, including enterobactin (Ent) – the prototype catechol siderophore; Kfu – iron (III) uptake system; aerobactin – a hydroxamate siderophore whose receptor is encoded by the *iut*A gene; and yersiniabactin (YbtS) – the phenolate siderophore that differs structurally from Ent and Kfu, participates in iron (III) uptake and is frequently identified in hypervirulent strains (Ali and Al-kakei Sana'a, 2019; Compain et al., 2014). The *allS* gene (encoding allantoin metabolism) is strongly correlated with *K. pneumoniae* isolates responsible for pyogenic liver abscesses. The remaining virulence factor genes in *K. pneumoniae* strains encode fimbrial and non-fimbrial adhesins. These include *mrk*D, the type 3 fimbrial adhesin gene which mediates the adhesion of bacterial cells to the extracellular matrix (Compain et al., 2014). A precise definition of hypervirulent K. *pneumoniae* does not exist in the international literature, but many researchers have argued that such a definition is urgently needed. In the present study, isolates harboring the aerobactin gene (*iut*A) and at least one hypermucoid phenotype gene (*magA*, K2, *rmp*A) were classified as hypervirulent based on the definition of Hvr K. pn strains proposed by Liu et al., (2019).

Efflux pumps that actively remove drugs from bacterial cells are an additional mechanism of antibiotic resistance in *Klebsiella* spp. In *K. pneumoniae,* efflux pump systems include AcrAB of the Resistance Nodulation Division (RND) family and MdtK of the Multi-Antimicrobial Extrusion (MATE) family. The AcrAB-TolC pump is composed of an outer membrane channel (TolC), a secondary transporter in the inner membrane (AcrB) and a periplasmic component (AcrA) (Wasfi et al., 2016). The advancement of molecular methods in the recent decades has led to the observation that the overexpression of efflux pumps is linked with clinically significant levels of multidrug resistance (Sun et al., 2014). The importance of efflux pumps in the context of β-lactam resistance has been investigated for several decades in *Enterobacteriaceae* (Maurya et al., 2019).

Hazardous working conditions in WWTPs and the risk of microbial transmission to the respiratory tract of WWTP employees have been extensively researched (Lu et al., 2020; Yang et al., 2018; Cyprowski et al., 2015; Han et al., 2019). However, the transmission of dangerous and clinically significant pathogens such as carbapenemase-producing, hypervirulent (CP-Hvr) *Klebsiella pneumoniae* including the genetic mechanisms of drug resistance and virulence has never been studied in detail. According to many researchers, the virulence potential of environmental strains of *K. pneumoniae* remains insufficiently investigated and requires further study (Barati et al., 2016; Podschun et al., 2001).

In view of the above, the present study set out to analyze the environmental strains of *Klebsiella* spp. isolated from wastewater, river water and the upper respiratory tract of WWTP employees. The main aim of the study was to identify environmental variants of carbapenemase-producing, hypervirulent (CP-Hvr) *Klebsiella pneumoniae*. The study focused on a WWTP as a potential source of CP-Hvr K. spp. emissions to

the upper respiratory tract of the plant's employees (bioaerosols in the activated sludge bioreactor) and natural water bodies (receptacles of treated wastewater). Quantitative and qualitative differences in the prevalence of antibiotic resistance genes and virulence were determined between the studied microbial groups (*Klebsiella pneumoniae* and *Klebsiella* non-*pneumoniae*) and environments (wastewater and river water). Untreated wastewater (reaching the WWTP) was also analyzed to determine whether it is a significant source of drug-resistant and hypervirulent strains of *Klebsiella* spp. The seasonality of antimicrobial resistance rates was investigated by analyzing strains isolated from samples collected in three seasons.

2. Materials and methods

2.1. Study site and sampling

The study area (WWTP and river) has been previously described (Zieliński et al., 2020; Gotkowska-Płachta et al., 2016). The WWTP operates a mechanical-biological treatment system without a disinfection process. The plant processes municipal sewage from the city of Olsztyn (including 20% of industrial and 2% of hospital wastewater). The treated effluent is evacuated to the Łyna River.

Samples of river water collected upstream from the wastewater discharge point (URW), untreated wastewater (UWW), wastewater from the activated sludge bioreactor (AS), treated wastewater (TWW), and river water collected downstream from the wastewater discharge point (DRW) were acquired in winter (February), summer (June) and autumn (September) of 2019. Samples of URW and DRW were collected approximately 600 m and 2000 m from the discharge point, respectively. A total of 15 samples (3 URW, 3 UWW, 3 AS, 3 TWW, and 3 DRW) were collected during the study. Samples of 1000 mL each were collected into sterile glass bottles. Five grab samples of river water of around 200 mL each were collected individually and combined into a composite sample. Wastewater for analysis was sampled at hourly intervals over a period of 24 h. Hourly samples (~45 mL) were pooled to provide a composite wastewater sample (1000 mL). The samples were transported to the laboratory at a temperature of 4 °C and were processed on the day of collection.

Nasal and throat swabs were obtained from the employees of the WWTP in Olsztyn (45 persons) on the day of wastewater sampling. The test group (employees of the wastewater treatment plant) routinely performed work activities. They were responsible for systematic observations at all stages of wastewater treatment (including within the activated sludge bioreactor). Samples were taken during their standard working days. The swabs were collected after the workers' morning inspections. The swab sampling was conducted by the authors of the study, who had been trained in the procedures of nasopharyngeal swab sampling. Both types of swabs were acquired from each employee. Swabs were collected from 15 individuals in each season, and they were pooled to produce composite samples for each sampling site. Swabs were also collected from a randomly selected healthy control group of individuals who were not WWTP employees (a total of 15 people). During the entire study, nasal and throat swabs were collected three times from WWTP employees and the control group.

2.2. Isolation of Klebsiella spp. and genomic DNA extraction

River water and WW samples were homogenized and diluted to isolate *Klebsiella* spp. strains. Sample specimens of 0.1 mL each and 0.1 mL of each serial dilution (10^{-1}, 10^{-2} and 10^{-3}) were inoculated on culture media. Klebsiella ChromoSelect Selective Agar (Sigma-Aldrich, Merck) supplemented with Klebsiella Selective Supplement (Sigma-Aldrich, Merck) was used for selective isolation and detection of *Klebsiella* species (Pérez-Díaz et al., 2018). After inoculation, the plates were incubated at 37 °C for 24 h. A chromogenic mixture incorporated in the media is cleaved specifically by *Klebsiella* species to produce purple-magenta (mucoid) colonies. Typical single colonies were transferred to Tryptone Soya Agar (Sigma-Aldrich, Merck) and incubated for 24 h at 37 °C. The obtained bacterial cultures were suspended in phosphate-buffered saline (PBS) (1.5 mL 1xPBS). Bacterial suspensions were centrifuged for 5 min at 8000 rpm to obtain pellets for DNA isolation.

Nasal and throat swab sticks were placed in 50 mL sterile falcon tubes to isolate *Klebsiella* spp. strains. 10 mL of 1xPBS was added to the falcon tubes. The tubes were placed in the Grant-bio PTR-60 360° Vertical Multi-Function Rotator (Grant Instruments) and shaken by vertical rotation (200 rpm for 5 h) (Zieliński et al., 2020). 0.1 mL of the extract was inoculated and successive steps were identical to those described in the procedure of isolating bacteria from water and wastewater samples. A total of 332 *Klebsiella* spp. isolates were obtained: 50 from URW, 64 from UWW, 67 from AS, 59 from TWW, 42 from DRW, 39 from throat swabs, and 11 from nasal swabs. Bacterial genomic DNA was extracted using the heat treatment method (Dashti et al., 2009) with minor modifications (Osińska et al., 2017). 0.5 mL of double-distilled water (ddH_2O) was added to the bacterial pellet and vortexed to obtain a suspension. The bacterial suspension was incubated at 95 °C for 10 min in the Grant QBD4 Block Heater (Grant Instruments) and centrifuged for 5 min at 4000 rpm. The supernatant (DNA) was transferred to a sterile Eppendorf tube. The quality and quantity of the obtained genetic material were evaluated with the Multiskan SkyMicroplate Spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA was stored at −20 °C for further analysis.

2.3. ERIC-PCR fingerprinting

The enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) method was used to generate DNA fingerprints of *Klebsiella* spp. isolates. The ERIC-PCR reaction was performed according to the procedure described by Versalovic et al. (1991) with minor modifications (Osińska et al., 2017) using ERIC 1 and ERIC 2 primers (Table S1). PCR products were separated on 1.5% agarose gel (Sigma-Aldrich, Merck) stained with ethidium bromide (0.5 mg/mL). Digital image data from electrophoresis gels were obtained using the Gel Doc EZ System and Image Lab™ Software (Bio-Rad Laboratories, CA, USA). Selected *Klebsiella* spp. isolates had identical profiles in ERIC-PCR fingerprinting. Identical isolates from the same sampling sites and sampling series were excluded from further analyses. Finally, a total of 284 unique isolates (40 from URW, 64 from UWW, 65 from AS, 59 from TWW, 42 from DRW, 5 from throat swabs, and 9 from nasal swabs) were selected for analysis – 270 environmental isolates and 14 strains collected from the upper respiratory tract.

2.4. Identification of Klebsiella pneumoniae

Klebsiella pneumoniae strains were identified by PCR based on the 16S–23S internal transcribed spacer (ITS) region according to the method described by Liu et al. (2008) (Table S1). PCR products were separated on 1.5% agarose gel (Sigma-Aldrich, Merck), stained with ethidium bromide (0.5 mg/mL).

2.5. Detection of resistance and virulence genes

The presence of nine beta-lactam resistance genes (bla_{CTX}, bla_{SHV}, bla_{TEM}, bla_{OXA}, bla_{KPC}, bla_{GES}, bla_{NDM}, bla_{IMP}) and one subtype ($bla_{OXA\text{-}48}$), nine genes encoding virulence factors and K1/K2 capsular serotypes (*ybtS, mrk*D, *ent*B, *rmp*A, *K2, kfu, all*S, *iut*A, *mag*A), three genes encoding multidrug efflux pump systems (*tolC, acrAB, mdtk*), and one class 1 integron-integrase gene (*int*I1) were determined by standard PCR in genomic DNA of 284 *Klebsiella* spp. isolates (Compain et al., 2014; Wasfi et al., 2016; Kim et al., 2009; Goudarzi et al., 2019; Monteiro et al., 2012; Goldstein et al., 2001). PCR was performed in a reaction mix with a volume of 15 μL, containing a pair of specific primers (Table S1), 1 μL

of genomic DNA of each sample, and the NZYTaq II 2xGreen Master Mix. PCR products were separated electrophoretically by transferring 5 μL of each amplified DNA fragment to 1.5% agarose gel stained with ethidium bromide (0.5 μg/mL) (Sigma, St. Louis, MO, USA). Electrophoresis was conducted for 1 h at 100 V in 0.5 × TBE buffer. A positive control for individual genes was used for each of the PCR reactions. Up to 10 randomly selected reaction products were sequenced for each gene to verify the accuracy of the reaction results.

2.6. Data analysis

A bubble plot showing the relative abundance (%) of the analyzed genes was developed in RStudio using the ggplot2 package. To measure the similarity between data sets (binary variables), the Jaccard coefficient of Gower & Legendre was calculated in RStudio using the dist.binary (ade4) package, and hierarchical cluster analysis was performed. The plotted tree was saved in Newick (parenthetic) format. The dendrograms were displayed, annotated and managed using the Interactive Tree Of Life (iTOL) online tool (Letunic and Bork, 2019). Data were processed statistically in the STATISTICA 13.1 software package (StatSoft Inc.). The Chi-square test was used to detect statistically significant differences in the occurrence of the studied genes between the research seasons ($p \leq 0.05$). The Principal Component Analysis (PCA) was based on the similarity of bacterial isolates in each sampling site. Spearman's rank correlation coefficient was calculated to determine the correlations between the prevalence of the analyzed genes ($p < 0.05$). The results of the correlation analysis were visualized in Gephi 0.9.2 software using Force Atlas 2 and Expansion layouts. The Edge Weight Filter was set at 0.2 to cut off negligible correlation values. The mean cumulative number of virulence factors per isolate was determined by summing up the total number of virulence genes in selected groups of bacteria/sampling sites and dividing the result by the number of the analyzed strains (Podschun et al., 2001).

3. Results

3.1. Environmental isolates

3.1.1. The prevalence of Klebsiella pneumoniae in environmental samples (K. pneumoniae 16S–23S ITS gene)

A total of 270 environmental strains of the genus *Klebsiella* (K. spp.) were isolated, including 102 identified as *K. pneumoniae* (*K. pn*) based on the presence of the ITS gene, and 168 strains without the ITS gene, identified as *Klebsiella* non-*pneumoniae* (*K.* n*pn*). *Klebsiella pneumoniae* was isolated from the samples collected in all sites during all seasons (Fig. 1). The highest prevalence of *K. pn* in the environmental strains of K. spp. was observed in AS (51.5%) and in summer samples (42.2%). *Klebsiella pneumoniae* was least abundant in DRW and TWW isolates at 39.9% and 25.4%, respectively.

3.1.2. The prevalence of genes encoding carbapenemases (types bla_{KPC}, bla_{GES}, bla_{NDM}, bla_{IMP} and subtype bla_{OXA-48}) and beta-lactamases (types bla_{CTX}, bla_{TEM}, bla_{OXA}, bla_{SHV}) in Klebsiella spp. isolates

The bla_{GES} gene was the most prevalent carbapenemase gene which was identified in 8.2% of all isolates. The bla_{GES} gene was the only carbapenemase gene in strains isolated from all sampling sites and in all seasons (Fig. 1). The prevalence of the bla_{GES} gene differed between isolates acquired in various seasons, and it was determined at 13.3% in winter samples, 6.9% in summer samples and 2.5% in autumn samples ($p < 0.05$ for W and A). This gene was also abundant in the strains isolated from TWW (15.3%). Interestingly, the bla_{GES} gene was more frequently detected in *K. pn* isolates (14.4%) than in *K.* n*pn* isolates (3.9%). The bla_{VIM} gene was identified in 6.0% of all isolates, and it was most frequently noted in UWW (12.3%) and AS isolates (9.1%). This gene was not detected in isolates from TWW. Similarly to bla_{GES}, the bla_{VIM} gene was more prevalent in *K. pn* (12.5%) than in *K.* n*pn* strains (1.7%). The prevalence of bla_{KPC}, bla_{IMP} and bla_{OXA-48} genes was similar (2.2%, 1.9% and 2.2% of all isolates, respectively). It should be noted

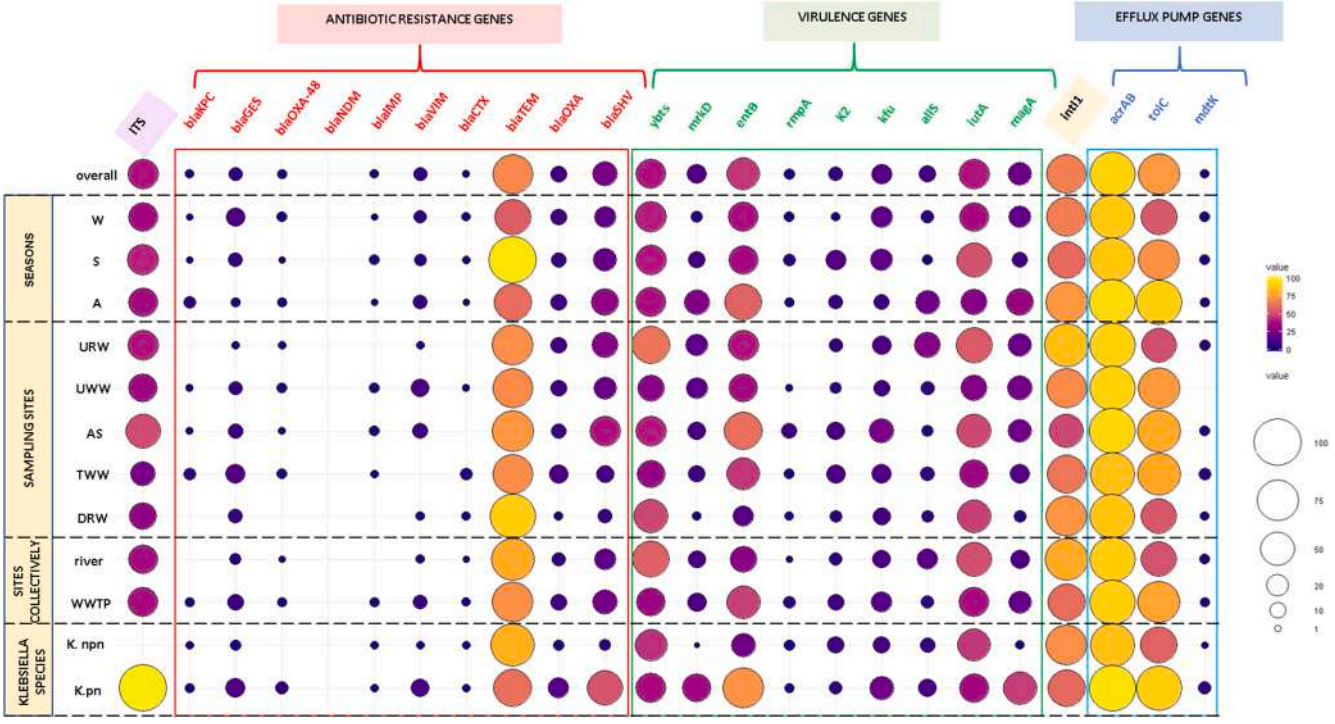

Fig. 1. A bubble plot showing the relative abundance (%) of the analyzed genes in *Klebsiella* spp. strains isolated in different seasons (W – winter, S – summer, A – autumn) from different sampling sites (URW – upstream river water, UWW – untreated wastewater, AS - activated sludge bioreactor, TWW – treated wastewater, DRW – downstream river water) and from pooled samples (river – URW, DRW; WWTP – UWW, AS, TWW), with a division into *Klebsiella pneumoniae* (K. pn) and *Klebsiella* non-*pneumoniae* (K. npn).

that bla_{KPC} and bla_{IMP} were not detected in any of the isolates from river water. The bla_{OXA-48} gene was present only in *K. pn* isolates. Regardless of season and sampling site, none of the isolates harbored the bla_{NDM} gene.

In *Klebsiella* spp., bla_{TEM} was the most prevalent β-lactamase gene (69.8%) which was more frequently detected in summer (99.0%) than winter (56.7%) or autumn (61.7%). The bla_{TEM} gene was the only ARG that was more abundant in isolates from river water (80.4%) than from wastewater (72.1%) isolates, and in *K.* n*pn* (82.0%) than in *K. pn* (62.5%) strains. In turn, bla_{SHV} (25.4%) and bla_{OXA} (9.9%) were more prevalent in *K. pn* (53.8% and 17.3%, respectively) than in *K.* n*pn* (4.5% and 3.7%) strains. The bla_{CTX} gene was the least abundant β-lactamase gene (1.3%), and it was not identified in URW and AS isolates.

3.1.3. The prevalence of virulence genes (ybtS, mrkD, entB, rmpA, K2, kfu, allS, iutA, magA) in Klebsiella spp. isolates

*ent*B, *iut*A and *ybt*S were the most prevalent virulence genes that were identified in 43.3%, 40.0% and 40.0% of all examined environmental isolates of *Klebsiella* spp., respectively. The abundance of the *ent*B gene was 50% higher in *K. pn* isolates and 18% higher in wastewater isolates than in *K.* n*pn* and river isolates, respectively. In turn, *iut*A and *ybt*S were more prevalent in river water isolates and *K.* n*pn* strains (by 15% on average). The frequency of *mag*A and *mrk*D was high in *K. pn* strains (48.0% and 35.3%, respectively), but very low in the remaining K. spp. isolates. (2.4% and 0.6%, respectively). The *kfu* gene (16.0% in all isolates) was also more frequently identified in *K. pn* (24.0%) than in *K.* n*pn* (10.7%). The *allS* (10.0% in all isolates) was most prevalent in isolates from autumn samples and its frequency was highest in URW isolates. The prevalence of the *K2* gene was highest in wastewater (maximum abundance of 13.7% in TWW), but it was more frequently detected in *K.* n*pn* than in *K. pn* isolates. The *rmp*A gene (3.7%) was the least abundant virulence gene, and it was not identified in URW isolates.

3.1.4. The prevalence of genes encoding class 1 integrase (intI1) and efflux pumps (acrAB, tolC, mdtK) in Klebsiella spp. isolates

On average, 67.2% of *Klebsiella* spp. isolates harbored the class 1 integrase gene. The prevalence of the *int*I1 gene was similar in all seasons (from 60.8% in summer to 74.0% in autumn). The *int*I1 gene was most frequently detected in river water isolates (86.0% in URW and 73.8% in DRW), and it was least frequently noted in AS isolates (50.0%). *Klebsiella* non-*pneumoniae* strains harbored the *int*I1 gene more frequently (71.9%) than *K. pn* strains (60.6%). The prevalence of genes encoding the AcrAB-TolC efflux pump system was high in the analyzed *Klebsiella* spp. strains. The *acr*AB gene was identified in 92.2% of all isolates, and its prevalence was as high as 98.1% in *K. pn* strains. However, fewer isolates harbored the *tol*C gene (74.6% on average). The *tol*C gene was least frequently detected in river water (53.1%) and in *K.* n*pn.* isolates (58.4%). Both efflux pump genes (*acr*AB and *tol*C) were identified in 68.5% of all isolates. These genes were present in 90.2% (92/102) of *K. pn* isolates and in 55.4% (93/168) of *K.* n*pn* isolates. The *mdt*K efflux pump gene was present in only 8/270 of the tested strains (2.9%), and it was not detected in UWW isolates. Six strains, including five *K. pn* strains and one *K.* n*pn* strain, harbored a total of three efflux pump genes.

The principal component analysis (PCA) revealed that the examined variables were grouped based on differences between sampling sites and *K. pn* vs. *K.* n*pn* strains (Fig. 2).

The analyzed genes formed two distinct clusters corresponding to the main sampling sites: river water – URW and DRW (marked in blue) and wastewater – UWW, AS and TWW (marked in yellow). River water isolates were characterized by a prevalence of *ybtS, iut*A and *allS* virulence genes, the *int*I1 gene and the bla_{TEM} drug resistance gene. Virulence genes were more frequently identified in URW isolates, whereas the bla_{TEM} gene was more abundant in DRW isolates. In the cluster of the genes detected in river water isolates, *K.* n*pn* strains from DRW isolates were located closely to the bla_{TEM} gene. The cluster of genes characteristic of wastewater isolates contained all carbapenemase genes, bla_{SHV}, $bla_{OXA,}$ efflux pump genes, *mrk*D, *mag*A, *ent*B, *kfu, rmp*A and *K2* virulence genes, and the ITS gene (used in the identification of *Klebsiella pneumoniae*). The bla_{CTX} gene was not grouped in either cluster.

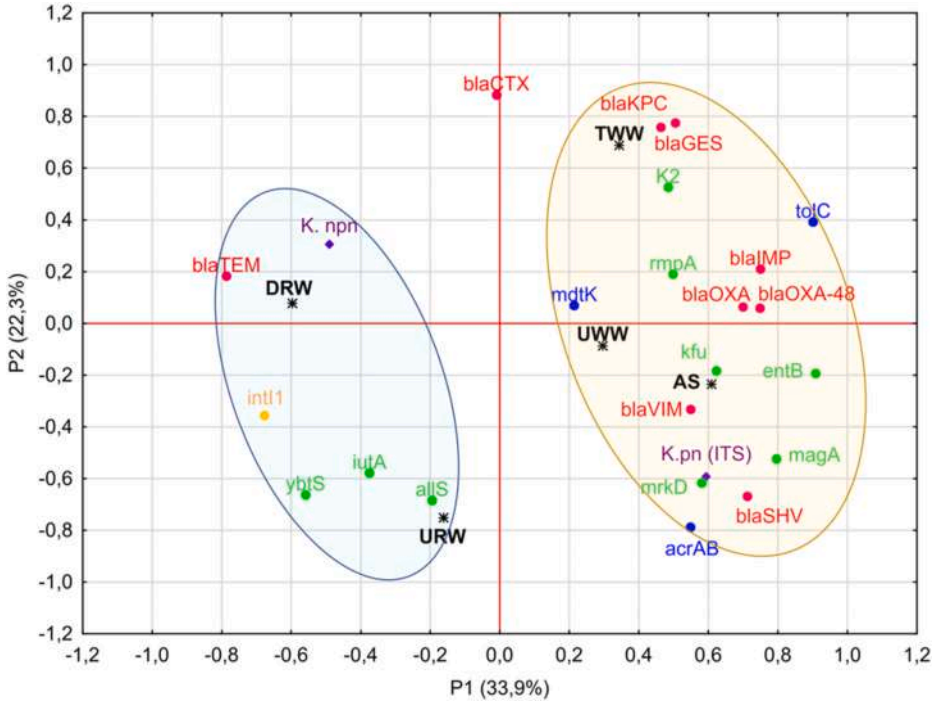

Fig. 2. Principal component analysis (PCA) based on the distribution of the analyzed genes and K.pn and K. npn groups in sampling sites. The genes isolated from WWTP samples and samples of river water formed two distinct groups that are marked in yellow and blue, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.1.5. Hypervirulent (Hvr) and carbapenemase-producing (CP) strains of Klebsiella spp.

90.7% (245/270) of the analyzed isolates harbored at least one antibiotic resistance gene, and 86.3% (233/270) harbored at least one virulence gene. In the group of 270 *Klebsiella* spp. strains, 17.4% (47/270) harbored carbapenemase genes, of which 17% (8/47) harbored more than one carbapenemase gene. Among CP-K. spp. strains, 70.2% (33/47) were identified as *K. pn*. The number of CP strains was similar in the samples acquired in different seasons: W – 18 (19.8%); S – 15 (15.3%); A – 14 (17.3%), but it differed across sampling sites: URW – 4 (10.0%), UWW – 15 (23.4%), AS – 13 (20.0%), TWW – 12 (20.3%), and DRW – 3 (7.1%). Among *Klebsiella* spp. strains 11.1% (30/270) were classified as hypervirulent. Within the 30 Hvr strains, 46.7% (14/30) were identified as *K. pn*. The highest number of Hvr strains was isolated in summer: 16 (16.3%), followed by autumn: 8 (9.9%) and winter: 6 (6.6%). The frequency of Hvr *Klebsiella* spp. strains varied across sampling sites: URW – 4 (10.0%), UWW – 4 (6.3%), AS – 11 (17.0%), TWW – 9 (15.6%), and DRW – 2 (4.8%). Five Hvr *Klebsiella* spp. strains were classified as CP-Hvr *Klebsiella* spp., including three *K. pn* (CP-Hvr K. pn.) strains and two *K. npn* (CP-Hvr *K. npn*) strains. Clusters of similarity among *Klebsiella* spp. isolates were identified in each sampling season (Fig. 3). The majority of *K. pn* isolates were grouped in 3 clusters: IIA, IIB and IIC. These clusters were characterized by the highest abundance as well as the highest diversity of the examined genes. Many isolates contained both bla_{SHV} and *ent*B genes, and they frequently harbored genes encoding the AcrAB-TolC efflux pump system. *iut*A and *mag*A were the predominant co-occurring hypervirulence determinant genes (8/10). These clusters were also characterized by the highest abundance of CP strains containing all carbapenemase genes (excluding NDM). The discussed clusters featured all *K. pn* strains harboring all three efflux pump genes. Clusters IA, VIB and VC were highly abundant in *int*I1 (100%), bla_{TEM} (97.0%), *ybt*S (91.2%) and *uit*A (75.0%) genes characteristic of river water isolates (Fig. 2). In these clusters, hypervirulence was determined by the combination of *iut*A and *K2* genes (11/12). It should be noted that clusters IB, IVB and IVC contained the only strains whose mechanism of hypervirulence relies on the *rmp*A gene (all strains were isolated from AS).

The cumulative number of virulence genes per strain was calculated to determine whether *K. pn* and *K. npn* strains differed in virulence

Table 1
Distribution of virulence factors in *Klebsiella* spp. isolated from wastewater and river water.

Virulence factor	Number (%) of positive isolates			
	Wastewater *K. pn* (n = 69)	River water *K. pn* (n = 33)	Wastewater *K. npn* (n = 119)	River water *K. npn* (n = 49)
ybtS	24 (35)	15 (46)	41 (34)	28 (57)
***mrk*D**	25 (36)	11 (32)	1 (1)	0 (0)
***ent*B**	54 (78)	21 (63)	36 (30)	6 (12)
***rmp*A**	3 (4)	0 (0)	6 (5)	1 (2)
K2	5 (7)	1 (3)	14 (12)	3 (6)
kfu	20 (29)	5 (15)	12 (10)	6 (12)
allS	4 (6)	9 (27)	7 (6)	7 (14)
***iut*A**	24 (35)	12 (36)	46 (39)	26 (53)
***mag*A**	36 (52)	13 (39)	4 (3)	0 (0)
Mean cumulative no. of virulence factors per isolate	**2.8**	**2.6**	**1.4**	**1.5**

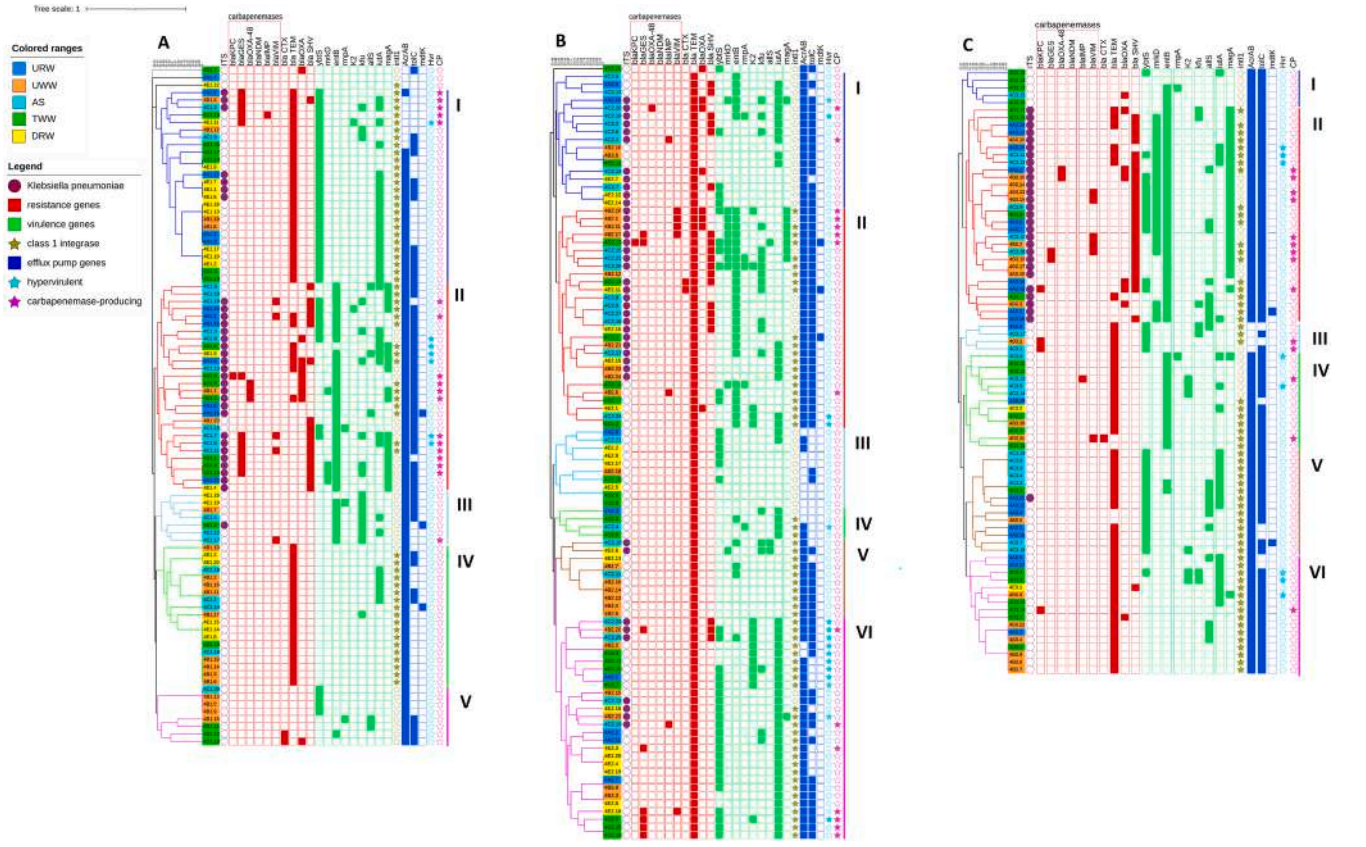

Fig. 3. Phylogenetic trees of *Klebsiella* spp. isolates collected in winter (A), summer (B) and autumn (C). Each column represents the genes listed at the top, and gene types indicated in the legend are marked with different colors. A filled box indicates a given gene was detected. Hypervirulent (Hvr) and carbapenemase-producing (CP) strains are additionally marked with stars at the end of the gene list. The resulting clusters are numbered on the right side of each graph. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

factors and whether differences were observed across sampling sites (Table 1). No significant differences in the abundance of virulence genes were noted between sampling sites. However, significant differences were observed between the examined microbial groups (*K. pn* > *K.* n*pn*).

3.2. *Klebsiella spp. strains isolated from the upper respiratory tract of WWTP employees*

Klebsiella spp. strains were isolated from throat and nasal swabs only in winter; therefore, they were analyzed separately from the environmental strains isolated in all seasons. The strains isolated from swabs were compared only with the strains isolated from the AS bioreactor, which is considered as the sampling site with the highest potential risk of bioaerosol transmission (Fig. 4). A total of 14 unique *Klebsiella* spp. isolates were obtained, including 7 (50.0%) from WWTP employees and 7 (50.0%) from the control group. In the group of seven strains isolated from the employees, six were isolated from nasal swabs (NW) and one was isolated from throat swabs. In the control group, 3/7 strains were isolated from nasal swabs (NC) and 4/7 strains were isolated from throat swabs (TC). Seven of the nine nasal isolates (77.7%) were classified as *K. pn*, and all throat isolates (5/5) were classified as *K.* n*pn.* Thirteen out of 14 swab isolates (92.9%) harbored the *ybt*S virulence gene. All nasal swab isolates (9/9) harbored the *ent*B virulence gene. The isolates from employee swabs were characterized by a low prevalence of efflux pump genes (only the *acr*AB gene was detected in 2/7 isolates). None of the strains isolated from various sampling sites had an identical genetic profile.

The strains isolated from employee swabs were characterized by a higher prevalence of drug resistance and virulence genes than the control group isolates. Six of the seven strains isolated from employee swabs harbored carbapenemases genes (100% NW strains). All of these strains harbored the bla_{GES} gene. Two of the seven strains isolated from employee swabs were hypervirulent, and they differed in virulence determinants (*iut*A + *mag*A and *iut*A + *rmp*A). CP and hypervirulent strains were not detected in the control group.

Swab isolates formed four main clusters containing strains that were isolated from AS samples in winter (Fig. 4). All Hvr and CP strains were grouped in three clusters: II, III and IV. Cluster II was characterized by the highest frequency of drug resistance genes. All strains in cluster II harbored carbapenemase genes. Cluster I contained all TC strains and one strain from AS samples, and it was characterized by the lowest frequency of resistance genes.

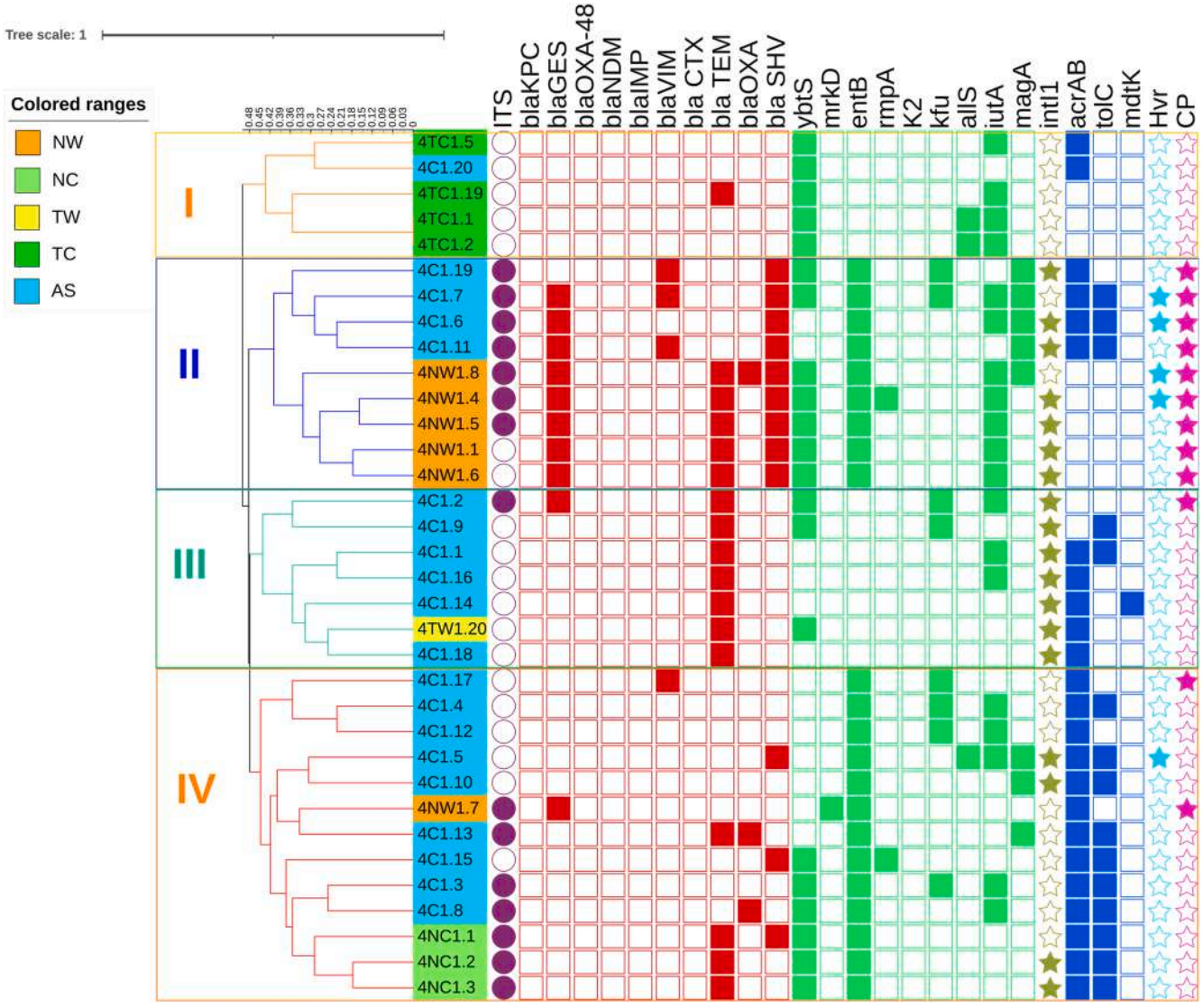

Fig. 4. A phylogenetic tree of *Klebsiella* spp. isolates from AS and swabs (NW - nasal swabs from employees; TW - throat swabs from employees; NC - nasal swabs from the control group; TC - throat swabs from the control group). Refer to Fig. 3 for the legend. The resulting clusters are numbered on the left side of the graph.

3.3. Correlations between the genes present in environmental K. spp. and Klebsiella isolated from the upper respiratory tract

Two groups of genes bound by positive correlations inside each group and negative correlations with the other group were identified (Fig. 5). Group I contained *int*I1, bla_{TEM}, *iut*A and *ybt*S, and group II contained *ITS*, *tol*C, *acr*AB, *ent*B, *mrk*D, *mag*A, bla_{VIM}, bla_{SHV}, bla_{OXA} and bla_{0XA-48}. The bla_{GES} gene was present in both groups, and it was bound by both positive and negative correlations with group II genes.

A strong correlation was observed only between *mag*A and *mrk*D (0.63). The *ITS* gene (*K. pn*) was bound by a moderate positive correlation with bla_{SHV}, *mag*A, *ent*B and *mrk*D, and a weak positive correlation with bla_{GES}, bla_{VIM}, bla_{OXA} and *tol*C. Positive correlations were noted between two subunits of the AcrAB-TolC efflux pump system. The *acrAB* gene was bound by a weak negative correlation with bla_{GES}, whereas *tol*C was positively correlated with *ITS*, *ent*B, *mrk*D, bla_{SHV} and *mag*A. The *int*I1 gene was bound by a weak positive correlation with bla_{TEM}. Weak negative correlations were observed between the bla_{TEM} gene and bla_{SHV}, *mrk*D, *ent*B and *mag*A, between bla_{GES} and *acrAB*, between *ybt*S and *ent*B, and between the *iut*A gene and *mrk*D and *ent*B. The correlation matrix is presented in the Supplementary Materials (Table S2).

4. Discussion

Beta-lactamase-producing *Enterobacteriaceae* strains are ubiquitous in the environment (Debabza et al., 2018). The prevalence of environmental *Klebsiella* spp. isolates harboring β-lactam resistance genes was very high (90.7%) in the present study. It may be due to the presence of hospital wastewater in the wastewater inflowing to the WWTP (Cahill et al., 2019; Zhang et al., 2020). *Klebsiella* spp. isolated from river water and wastewater did not differ in the cumulative number of the main β-lactamase-encoding genes (bla_{CTX}, bla_{SHV}, bla_{TEM}, bla_{OXA}). Approximately 90.0% of the strains isolated from both environments harbored at least one β-lactamase gene, but bla_{CTX}, bla_{SHV} and bla_{OXA} were more frequently identified in wastewater isolates. In a study by Falodun et al. (2018), *Klebsiella* spp. was the most prevalent Gram-negative and β-lactam-resistant bacterium in samples of river water collected in Nigeria. The results of the present study are similar to the findings reported by Adesoji and Ogunjobi (2016). In the cited study, bla_{TEM} and bla_{SHV} genes were most prevalent in *Klebsiella* spp. isolated from the water distribution system, whereas the bla_{CTX} was noted only sporadically. In contrast, Al-Kareem et al. (2015) detected the bla_{CTX} gene in 87.5% of *K. pn* river water isolates. Similar observations were made by Makowska et al. (2020) who found that bla_{CTX} was the most abundant β-lactamase gene in bacteria isolated from wastewater. These differences suggest that the prevalence of the bla_{CTX} gene could be geographically conditioned (Gundran et al., 2019; Rossolini et al., 2008). Considerable variations in the prevalence of the main β-lactamase-encoding genes were observed between *K. pn* and *K.* n*pn* isolates. bla_{SHV} and bla_{OXA} were significantly more prevalent in *K. pn*, and they were positively correlated with the gene used in species identification. The bla_{TEM} gene was more prevalent in *K.* n*pn*, and it was negatively correlated with bla_{SHV}.

In Poland, the prevalence of carbapenemase-producing *Enterobacteriales* has been increasing steadily for more than ten years. Strains producing NDM, KPC, VIM and OXA-48-type carbapenemases have been most frequently reported (KORLD, 2019). The fact that the bla_{GES} and bla_{IMP} are not included in the reports of the National Reference Center for Susceptibility Testing (KORLD) gives serious cause for concern, and it suggests that the abundance of carbapenemase-producing strains could be much higher than reported. The prevalence of the above strains as well as strains producing GES and IMP carbapenemases was investigated in the present study. Despite the fact that NDM carbapenemases are most abundant in Poland (KORLD, 2019), the bla_{NDM} gene was not identified in any of the analyzed isolates. The most prevalent carbapenemase-encoding genes were bla_{GES} (8.2%) and bla_{VIM} (6.0%). Similar results were reported by Makowska et al. (2020) who found that bla_{GES} (30.3%) and bla_{VIM} (7.8%) were the most frequently occurring CP

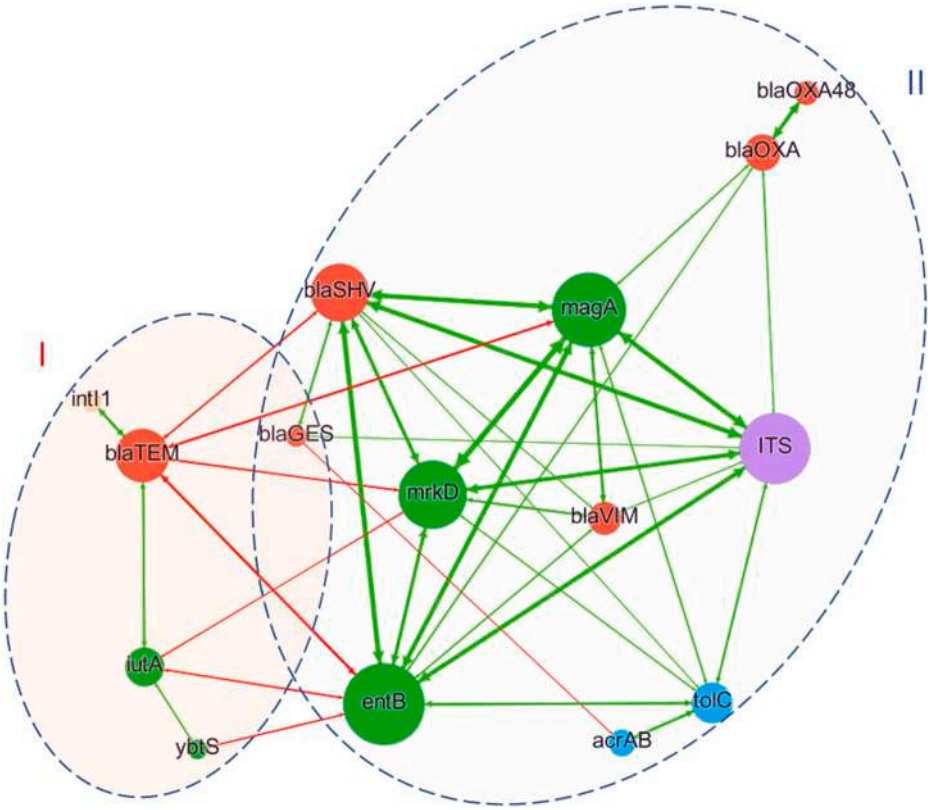

Fig. 5. Network analysis of the correlations between the analyzed genes. Network nodes represent the analyzed genes. Groups of genes that are identical to those presented in Figs. 2 and 3 are denoted by differently colored nodes. Red edges represent negative correlations, and green edges represent positive correlations. The size of the node corresponds to the weight of the correlated attributes. Edge thickness denotes the strength of the correlation between nodes. The network is based on Spearman's rank correlation coefficient and includes values that are significant at $p < 0.05$. The identified correlations were described as weak (0.2–0.4), moderate (0.4–0.6), strong (0.6–0.8) or very strong (0.8–1.0). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

genes in carbapenem-resistant *Enterobacteriaceae* (CPE) isolated from wastewater. Jendrzejewska (2018) detected the bla$_{GES}$ gene in 20.0% of the isolates from an aeration tank. Piotrowska et al. (2019) reported numerous variants of the *bla*$_{GES}$ gene (11 types) in wastewater samples from an aeration tank. In the present study, all CP genes were more prevalent in *K. pn* strains (70.0%) than in *K. npn* strains (30.0%), and in wastewater isolates (85.0%) than in river water isolates (15.0%). A positive correlation was also noted between *K. pn* and CP genes (*bla*$_{GES}$ and *bla*$_{VIM}$). *bla*$_{IMP}$ and *bla*$_{KPC}$ were not identified in river water isolates, which could suggest that these genes are not common in environmental *Klebsiella* spp. in Poland. However, the strains isolated from DRW samples were characterized by a higher prevalence of *bla*$_{GES}$ and *bla*$_{VIM}$ genes than URW isolates. In other studies, CPE were identified in river water in Switzerland (Bleichenbacher et al., 2020), Sweden (Khan et al., 2018), Spain (Piedra-Carrasco et al., 2017), Portugal (Kieffer et al., 2016), Austria (Zarfel et al., 2017), Ireland (Mahon et al., 2017), USA (Aubron et al., 2005), Brazil (Oliveira et al., 2014) and the Danube River (Kittinger et al., 2016). The *bla*$_{GES}$ gene seems to play the key role in the spread of CP-Hvr *Klebsiella* spp. from WWTPs. The above gene was detected in CP-Hvr *K. pn* strains isolated from WWTP employees and in the only CP-Hvr strain isolated from DRW samples. The prevalence of the *bla*$_{GES}$ gene increased significantly during wastewater treatment (UWW - 6.2%; TWW - 15.3%). In a study by Makowska et al. (2020), *bla*$_{GES}$ was the only CP gene whose abundance increased during wastewater processing. Similar observations were made by Jendrzejewska (2018). Such a widespread presence of *bla*$_{GES}$ in Polish WWTPs may indicate its specific geographical conditions or relation with treatment methods based on activated sludge. The reason for such a frequent occurrence and increasing abundance along the wastewater treatment stages may be due to the frequent *bla*$_{GES}$ position on class 1 integrons (Gatica et al., 2016). In this study, however, no correlations between the *int*I1 and *bla*$_{GES}$ genes in *Klebsiella* spp. were observed. Integrons carrying *bla*$_{GES}$ were reported in activated sludge from German (Girlich et al., 2011) and Portuguese WWTPs (Manageiro et al., 2014). Other authors suggest that *bla*$_{GES}$ may be more abundant in southeastern Mediterranean countries (Gatica et al., 2016). Activated sludge abiotic factors seem to play a role in determining resistomes. Nutrients and oxygen are heavily consumed by activated sludge biomass and, consequently, may act as drivers for the microbial community and resistome composition (Ju et al., 2019). Moreover, the method based on active sludge itself seems to be the least effective in reducing *bla*$_{GES}$ compared to other carbapenemase genes (Makowska et al., 2020). Cave (2019) also observed a surprisingly high prevalence of *bla*$_{GES}$ at all stages of wastewater treatment and in a river acting as a receptacle of treated wastewater in the USA. According to the cited author, the fact that the prevalence of the *bla*$_{GES}$ gene is not monitored by the US Centers for Disease Control and Prevention (CDC) is highly worrying. The number of known GES variants increases rapidly, and the number of point mutations of *bla*$_{GES}$ and their geographic distribution indicate that this family of enzymes continues to evolve and spread around the world. In some cases, variants that do not display carbapenemase activity have expanded their substrate spectrum to include carbapenems, aztreonam and cephamycin. This observation gives cause for serious concern because these enzymes could become potent carbapenemases as a result of single point mutations (Naas et al., 2016; Bogaerts et al., 2010). These alarming reports call for further research into *bla*$_{GES}$ gene variants and their phenotypic determinants of resistance to carbapenems.

Bacteria of the genus *Klebsiella* occur naturally in river estuaries, including in areas that are located remotely from human settlements, and river estuaries can be regarded as a natural reservoir of these species (Barati er al. 2016). In the present study, 35.9% of river water isolates were identified as *K. pn*. Similar values were reported in analyses of transitional water (48.8%) (Barati et al., 2016), fresh water (31.6%), brackish water (25.0%) and sea water (25.4%) (Podschun et al., 2001). Despite the fact that *Klebsiella pneumoniae* is ubiquitous in nature, very little is known about its virulence determinants in aquatic environments (Barati et al., 2016). An analysis of the mean number of virulence genes revealed that strains isolated from river water were equally virulent to the strains isolated from wastewater (Table 1). However, the mean number of virulence genes were higher in *K. pn* strains (by 1.3 on average) relative to the remaining isolates. Identical values of the above parameter were reported in *K. pn* strains by Podschun et al. (2001), and they did not deviate significantly from the values noted in typical clinical isolates (2.9) (Podschun et al., 2000). According to Struve and Krogfelt (2004), environmental and clinical *K. pn* strains are characterized by similar virulence. The mean number of virulence genes is a purely quantitative parameter. In the present study, virulence genes were also analyzed qualitatively by linking them with specific sampling sites. Statistical analyses (Fig. 2) demonstrated that three virulence genes (*allS, ybtS, iut*A) were highly associated with the strains isolated from river water and that hypervirulent strains were more abundant in wastewater.

Capsular serotypes K1 and K2 are associated with the dominant virulent *K. pn* strains. In the current study, serotypes K1 and K2 accounted for 19.6% and 8.5% of all *Klebsiella* spp. isolates. Similar results were reported by Wasfi et al. (2016) who identified 28.5% of K1 serotypes and 7.1% of K2 serotypes in clinical isolates. In a study by Feizabadi et al. (2013), K1 and K2 serotypes accounted for 11.2% and 14.6% of all clinical *K. pn* isolates, respectively. Virulence factors such as fimbriae, capsules, enterobactin and biofilm have been identified in nearly all isolates, and they are involved in the pathogenesis of bacterial infections (Hennequin and Robin, 2015). Hypervirulent *K. pn* strains cause more severe and disseminated infections than classical *K. pn* strains. Hypervirulent strains are also more resistant to the bactericidal activity of the blood serum and the phagocytic activity of neutrophils and macrophages (Harada and Doi, 2018). According to some authors, Hypervirulence was also defined based on the presence of a mucoidal phenotype or the *rmp*A gene (Shankar et al., 2018). The cited authors identified genes responsible for the Hvr phenotype, in particular *mag*A (mucoid phenotype A gene associated with the K1 capsular serotype), *rmp*A (regulator of the mucoid phenotype A gene) and *K2* (capsular serotype K2 and hypermucoid phenotype) (Harada and Doi, 2018; Liu and Guo, 2019; Fang et al., 2004). In recent years, Hvr *K. pn* was identified based on the *iut*A gene encoding aerobactin (Zhang et al., 2016). According to many authors, the *iuc*A/*iut*A gene also plays a particularly important role in hypervirulent strains (Russo et al., 2014, 2015). A total of 30 environmental strains (river water and WWTP) and two strains isolated from swabs were classified as hypervirulent based on the above criteria. The most common combination of Hvr determinants was *iut*A + *K2* (17/32), followed by *iut*A + *mag*A (11/32) and *iut*A + *rmp*A (3/32). One isolate (1/32) harbored three genes characteristic of hypervirulent strains (*iut*A + *rmp*A + *mag*A). The vast majority of Hvr strains were associated with the WWTP environment. Interestingly, the aerobactin gene (*iut*A) was grouped in an opposite direction to hypermucoid phenotype genes (Fig. 2). These genes were also located in different clusters, but they were not bound by direct negative correlations (Fig. 4). It has been found that carbapenemase-encoding plasmids can be transferred to Hvr *K. pn* strains, which poses a serious public health threat (Siu et al., 2014). In the present study, five environmental Hvr K. spp. isolates harbored a CP-encoding gene. One CP-Hvr strain was isolated from river water, which gives particular cause for concern. This strain was isolated from DRW samples, whereas the remaining four CP-Hvr strains were isolated from the WWTP environment, which could indicate that WWTPs play a critical role in the spread of CP-Hvr *K. pn* strains. A positive correlation was also noted between a CP-encoding gene (*bla*$_{VIM}$) and a hypervirulence factor (*mag*A). In this study, a worrying trend was observed related to the more frequent detection of hypervirulent strains during the biological treatment (AS) and in treated (TWW) wastewater compared to untreated wastewater (UWW). Gotkowska-Płachta (2021) noticed a similar dependence while studying virulence determinants in *Enterococci* occurring in sewage. This may suggest that during the treatment process, *Klebsiella* spp. acquired

certain hypervirulence determinants or that the hypervirulent strains demonstrated enhanced survivability throughout the biological purification process. It seems that the determinants of hypervirulence not only allow Hvr *Klebsiella* to survive inside the host but also in the natural environment. The most important siderophore, i.e. aerobactin, seems to be of particular importance. It is responsible for iron acquisition from the environment, contributes to biofilm formation and increases the expression of active enzymes against toxic reactive oxygen species (ROS) (Zhu et al., 2021).

Employees of WWTPs are at higher risk of exposure to harmful biological factors which can exert a negative impact on the respiratory tract (Cyprowski et al., 2015). Aeration tanks (AS) are considered to be the main source of bioaerosols in WWTPs, and the respirable fraction contains up to 70% of bacteria in the area of an aeration tank (Han et al., 2019). These findings imply that most bacterial aerosols in the work environment can be potentially accumulated in the upper respiratory tract. The accumulation of pathogens such as *K. pneumoniae* can lead to acute infections in susceptible hosts. *Klebsiella pneumoniae* and *K. oxytoca* appear to be ubiquitous in bioaerosols emitted by the aeration tank and mechanical treatment systems (Lu et al., 2020). Seasonal variations in the composition and concentration of biological factors in bioaerosols emitted by WWTPs have been widely documented (Lou et al., 2021; Michalkiewicz, 2018; Han et al., 2020). In the current study, *Klebsiella* spp. was identified only in nasal and throat isolates collected in winter. According to research, exposure to a cold environment increases the risk and severity of respiratory infections. Body cooling can lead to involuntary vasoconstriction in the nose and the upper respiratory tract, mucosal dryness, dysfunction of respiratory cilia, and higher susceptibility to infections (D'Amato et al., 2018; Mäkinen et al., 2009; Mourtzoukou and Falagas, 2007). According to general practitioners, infections of the upper respiratory tract are most prevalent in winter, and *K. pneumoniae* is one of the most common causes of bacterial respiratory infections outside the hospital setting (Pietrzykowska, 2017). In the present study, seven *Klebsiella* spp. strains were isolated from WWTP employees, including six strains from nose swabs and one strain from throat swabs. The strain isolated from throat swabs was least virulent, and it harbored only the *ybtS* gene and one β-lactamase-encoding gene (bla_{SHV}). All strains isolated from nasal swabs (6/6) harbored the bla_{GES} gene which encodes resistance to carbapenem. Most of these strains (5/6) also harbored β-lactamase-encoding genes bla_{TEM} and bla_{SHV}. The presence of the bla_{OXA} gene was additionally confirmed in one nasal swab isolate. The strains isolated from nasal swabs harbored *ent*B (6/6), *ybt*S (5/6) and *iut*A (5/6) virulence genes. The gene encoding type 3 fimbriae (*mrk*D) was identified in one nasal swab isolate, which gives serious cause for concern. The *mrk*D gene encodes adhesin that enables bacterial pathogens to adhere to collagen molecules in mammalian cells (Ranjbar et al., 2019). Two of the six strains isolated from nasal swabs harbored *mag*A (1/6) and *rmp*A (1/6) genes encoding the hypermucoid phenotype, and they were classified as CP-Hvr *K. pn*. In the group of hypervirulent environmental strains, the presence of *rmp*A was detected only in *Klebsiella* strains isolated from AS samples, which could indicate that the strains isolated from employees were also strongly correlated with AS isolates. However, unlike AS isolates, the strains isolated from nasal and throat samples were characterized by a low prevalence of genes encoding efflux pump systems. The presence of carbapenemase-producing strains and hypervirulent strains, in particular CP-Hvr *K. pn* strains from nasal swabs, is highly worrying. Carbapenemase-producing, hypervirulent and CP-Hvr strains were not identified in the control group. However, strains isolated from the control group harbored beta-lactam resistance genes (bla_{TEM} and bla_{SHV}). These genes were also found in URW isolates. This proves that the above-mentioned drug resistance mechanisms are widespread in the natural environment, without the impact of WW.

Effluent pumps export antibiotics and other substances, including dyes and detergents (Hennequin and Robin, 2015). Strains devoid of efflux pumps were found to be non-pathogenic in animal models (Buckley et al., 2006; Bunikis et al., 2008; Nishino et al., 2006). Similar observations were made in the present study, where the *tol*C gene was positively correlated with virulence genes (*ent*B, *mrk*D and *mag*A). A complete efflux pump system (presence of both *acr*AB and *tol*C genes) was identified in 68.5% of all isolates, including in 92.2% (92/102) *K. pn isolates* and 55.4% (93/168) *K.* n*pn* isolates. Subunits of the AcrAB-TolC system were not detected in any of the swab isolates. Positive correlations were noted between the genes encoding efflux system subunits. The *tol*C gene was also correlated with the bla_{SHV} resistance gene and *K. pneumoniae* (ITS gene). The gene encoding subunit AcrAB was negatively correlated with bla_{GES}. The AcrAB-TolC pump (68.5%) was identified far more frequently than *mdt*K (2.9%), which is consistent with the findings of Wasfi et al. (2016) and Mirzaie and Ranjbar (2020). Despite the above, the role of the AcrAB-TolC pump in the pathogenesis of respiratory infections caused by *K. pneumoniae* has not been fully elucidated to date (Padilla et al., 2010).

This study is subject to limitations. Only one WWTP was studied, and only seven Cp-Hvr strains were detected in total. Moreover, it was possible to successfully obtain strains from swabs from only one of the three research seasons. This makes the claim of the transmission of *Klebsiella* spp. to WWTP employees less reliable compared to the transmission to river water. Further studies of the bioaerosol emitted in the WWTP area are planned to explore the transmission aspect in more detail.

5. Conclusions

The results of this study indicate that WWTPs are a significant source of carbapenemase-producing, hypervirulent *Klebsiella* spp. A qualitative analysis revealed that strains isolated from wastewater and river water harbored different virulence and antimicrobial resistance genes. Differences in the prevalence of antimicrobial resistance and virulence genes were also observed between *K. pn* and *K.* n*pn* strains. *Klebsiella pneumoniae* strains and the strains isolated from wastewater were the major carriers of carbapenemase genes and hypervirulence determinants. The study demonstrated that CP-Hvr *K. pn* strains are possibly transmitted to the upper respiratory tract of WWTP employees. These observations indicate that WWTP employees may be at high risk of exposure to harmful biological factors. The study also revealed that bla_{GES} carbapenemases, which are generally neglected in antimicrobial resistance reports, play a very important role in the spread of drug resistance in the environment and require further research. The prevalence of virulence genes and genes encoding resistance to beta-lactams did not fluctuate across seasons.

Author contributions

Conceptualization, M.H. and E.K.; methodology, M.H. E.K. and D.R; software, D.R.; validation, D.R., M.B., J.H. and W.Z.; formal analysis, D. R.; investigation, D.R., M.B., J.H. and W.Z.; resources, M.H. and E.K.; writing—original draft preparation, D.R.; writing—review and editing, M.H. and E.K.; visualization, D.R.; supervision, M.H and E.K.; funding acquisition, M.H and E.K. All authors have read and agreed to the published version of the manuscript.

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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.2021.113831.

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Factors associated with safe child feces disposal in Ethiopia, India, and Zambia

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ABSTRACT

Safe child feces disposal (CFD) is defined as a child or caregiver placing or rinsing child feces into an improved sanitation facility. In low- and middle-income countries (LMICs), 48% of households with children under five report that child feces were safely disposed. Despite its widespread prevalence and harmful health effects, little is known about the determinants of safe CFD. We analyzed determinants of CFD across three countries that differently address safe CFD in their policies. We used data from a cross-sectional survey of 3737 households in rural areas of Ethiopia, India, and Zambia. Multivariable logistic regression models were used to identify factors associated with safe child feces disposal (CFD) in these countries. Safe CFD was positively associated with whether a female head of household attended primary school in Zambia and India, whether someone spoke to households about cleanliness in Ethiopia, and whether a community had a WaSH committee that met in the past year in Ethiopia. In all three countries, households with a member who practiced open defecation were significantly less likely to practice safe CFD. Increasing the education level of female head of households, reducing open defecation, speaking to a household, and having an active WaSH committee are important programmatic considerations for actors who seek to address CFD in low resource settings. Unsafe CFD is a substantial challenge to transformative WaSH, and more studies should be conducted to evaluate the causes, determinants, and behaviors of CFD.

1. Introduction

Child feces disposal (CFD) is considered unsafe when the feces of a child five years or younger is not disposed in an improved sanitation facility. Unsafe CFD practices include feces left out in the open, throwing feces into the garbage, burying or leaving feces on the ground, and placing or rinsing feces into drains or ditches (Bain and Luyendlijk, 2015; Rand et al., 2015). Unsafe CFD poses a substantial public health risk yet it is commonly practiced by households in low- to middle-income countries (LMICs) with more than 50% of households disposing child feces unsafely (Rand et al., 2015). In India, Ethiopia, and Zambia, safe CFD is practiced by 16%, 31%, and 67% of households respectively (Rand et al., 2015).

While child feces may be perceived to be less harmful than adult feces, evidence shows that children's feces pose a higher health risk than that of adults due to a higher prevalence of certain pathogens such as hepatitis A, rotavirus, and other pathogens (Almedom, 1996; Bawankule et al., 2017; Lanata et al., 1998). When feces are left out in the open, there is a higher risk that people, especially children, may come into direct contact with fecal pathogens (Majorin et al., 2017). Children are at higher risk of exposure to fecal-oral pathogens because they play on the ground and put their hands near their face and into their mouths

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(Bawankule et al., 2017; George et al., 2016; Majorin et al., 2017). Fecal-oral pathogens can cause diarrheal illnesses which can lead to stunting, a condition that affects 162 million children worldwide. The link between unsafe CFD and stunting illustrates the severe and lasting effects of this behavior, as stunting leads to diminished cognitive and physical development, reduced productive capacity, and other adverse health outcomes (de Onis and Branca, 2016; Worley, 2014). However, Majorin et al. (2019) found that there is little evidence on safe CFD and soil-transmitted helminths and there is a need for rigorous evidence to assess whether interventions to improve safe CFD are effective in preventing soil-transmitted helminth infections. Islam et al. (2020) found that children with unsafe CFD had higher rates of diarrhea, but this association was not statistically significant. Because unsafe CFD leads to increased exposure of fecal-oral pathogens, this practice will negatively affect human health, especially children.

Most known determinants of safe CFD are demographic factors. Wealthier households were significantly more likely to practice safe CFD (Azage and Haile, 2015; Bawankule et al., 2017; Sahiledengle, 2019). Mothers' education and/or literacy was positively associated with safe child feces disposal (Ayele et al., 2018; Azage and Haile, 2015; Bawankule et al., 2017; George et al., 2016; Preeti et al., 2016). Both water and sanitation infrastructure may affect whether one is more likely to practice safe CFD. Households with an improved latrine were more likely to dispose of child feces safely (Bawankule et al., 2017; Azage and Haile, 2015; Sahiledengle, 2020). This association may occur because those who invest in sanitation infrastructure understand and generally care more about sanitation and sanitary behaviors (Curtis et al., 1995). However, George et al. (2016) did not find that those who have an improved sanitation facility were more likely to practice safe CFD. In a study where subsidies for improved latrines were provided, the intervention increased safe disposal of child feces to 10% in intervention households, compared to 3% in the control households. Providing households with improved sanitation facilities is not the sole barrier to safe CFD (Freeman, 2016). Preeti et al. (2016) found that households with an improved water source were more likely to practice safe CFD. Likewise, Sahilendengle (2020) found that households having improved drinking water were less likely to dispose of feces unsafely. In India, most households that safely disposed of child feces had water on the compound (Majorin et al., 2014), while a previous study in Ethiopia found no such association (Azage and Haile, 2015). Evidence from Ethiopia has shown that urban residents with improved drinking water facilities were less likely to practice unsafe CFD (Sahiledengle, 2020).

Despite the widespread prevalence and health impacts of unsafe CFD, it is not universally addressed in sanitation policies. In Zambia, the Ministry of Local Government and Housing (MLGH) developed the National Rural Water Supply and Sanitation Programme (NRWSSP) in 2015, which focuses on decentralizing organizational structures, creating an enabling environment, and strengthening organizational and individual capacity (Government of the Republic of Zambia, 2015). The sanitation strategy works across ministries, local authorities, civil society organizations, non-governmental organizations, and private sector partners. There is no mention of CFD specifically across any programs or guidelines.

Ethiopia's One WASH Nation Program (OWNP) was established in 2013 as a multi-sectoral program that brings together four ministries (Health, Finance, Water Resources, and Education) along with private and nongovernmental stakeholders. Ethiopia's National Hygiene and Sanitation strategy has three pillars. The first is enabling a favorable legislative and political environment to expand Water, Sanitation, and Hygiene (WaSH) improvements, particularly regarding sanitation and health extension workers (Federal Democratic Republic of Ethiopia Ministry of Health (FMOH), 2017). The second pillar is promoting sanitary and hygienic behavior through communication campaigns, social marketing, and incentives. The final pillar is improving access and affordability of necessary products and services. Ethiopia's "Baby and Mother WASH Implementation Guideline" (BabyWaSH) is meant to be integrated into the pillars of this overall strategy. Safe CFD is a central target of the BabyWaSH approach, and BabyWaSH is meant to be integrated into each administrative level (Federal Democratic Republic of Ethiopia Ministry of Health (FMOH), 2017).

India's national sanitation campaign, Swachh Bharat Mission (SBM), began in 2014 and includes significant sanitation infrastructure investments at household level and community-centered, collective behavioral change approaches. SBM is split into urban and rural missions named SBM (Urban) and SBM (Gramin), respectively. SBM (Gramin) has two methods to improve sanitation in India: providing and facilitating access to physical and monetary resources and encouraging behavior change through information, education, and communication (IEC) programs. Child feces disposal is not mentioned in the SBM (Gramin) guidelines, and there are no programming, information campaigns, or guidelines specifically focusing on reducing unsafe CFD (Ministry of Drinking Water and Sanitation, 2017; Standing Committee on Rural Development, 2017).

Despite its widespread practice, there is little evidence describing the determinants of safe CFD and comparison of this practice across countries. We analyzed determinants of safe CFD such as whether a household member practiced open defecation, whether a female head of household had attended primary school, whether someone had spoken to a household about cleanliness, whether a respondent washed their hands after cleaning a baby, and whether a community had an active WaSH committee. This study examined these determinants across Ethiopia, India, and Zambia which differently address safe CFD in policies.

2. Methods

2.1. Survey data summary

This study used data from a survey conducted by World Vision (WV) and the Water Institute (WI) at the University of North Carolina at Chapel Hill (UNC). The survey used a stratified, cluster-randomized study design to examine WV program areas and comparison areas in 14 LMICs. The WI created the evaluation design, methods, sampling plan, ethical protocol, data collection plan, data collection tools, and data analysis plan. This study examined household data from Ethiopia, India, and Zambia. Binary and multivariable logistic regression models were used to identify factors associated with safe CFD.

2.2. Survey population

The survey was conducted in 14 countries which were selected by WV based on the level of in-country programming. WV generally operates in rural areas and 56 WV program clusters and 56 non-WV clusters were randomly selected from rural areas in each country. This study restricted analysis to the 3737 households that answered the survey questions on CFD.

2.3. Sample selection and design

2.3.1. Selected countries

Ethiopia, India, and Zambia were selected because of their high rates of unsafe CFD, high rates of open defecation, and relatively expansive community health worker programs. India was selected because of its extensive national sanitation program that has little emphasis on safe CFD. Ethiopia was chosen because it has a national sanitation program that has guidelines on safe CFD. Zambia was selected because of the high rates of unsafe CFD, high rates of open defecation, its community healthcare worker program, and its proximity to Ethiopia.

2.3.2. Sampling design

Data were collected using a cluster-randomized, population-based sampling strategy. For Zambia and Ethiopia, enumeration areas (EAs)

were identified from lists of administrative units compiled by national statistics offices from each country. EAs were divided into two strata – areas with WV programs and comparison areas without WV programs. Primary sampling units (PSUs) were randomly selected from each stratum using a probability-proportional-to-size method (PPS) (See Fig. 1). PSUs were overlaid with a map of WV program areas and then sampling areas were selected. Comparison areas were selected using a similar procedure from districts outside the WV program area boundaries.

India differed in sampling strategy in that districts containing WV programs were identified and WV administrative blocks and non-WV administrative blocks within these districts were selected and used as clusters. A list of blocks, both comparison areas and WV areas, was created and then sampled using PPS to select the PSUs. Fifty-six comparison area clusters and 56 WV program area clusters were randomly selected in each country.

PSUs were randomly selected and further divided if there were more than 200 households in the unit. This division resulted in secondary sampling units (SSUs) which were randomly sampled. Final clusters for data collection contained 100 to 200 households (See Fig. 1).

2.3.3. Survey instruments and personnel

The survey instrument contained interview questions and direct observations on water, sanitation, and hygiene infrastructure and practices as well as demographic characteristics of respondents and households. The WI led workshops to train research supervisors who used this material to train enumerators with previous experience in mobile surveys and survey data collection. The surveys were translated into the local languages and validated by either research personnel or WV staff in each country. Survey answers were logged using mWater, a mobile survey tool. Female heads of households were interviewed unless unavailable. If unavailable, an available adult was interviewed. If no adult was available, another household from the same cluster was selected.

2.4. Data entry, processing, and analysis

Descriptive statistics were calculated and indicators were created using the survey questions (Table 1). Indicators were calculated for "at the time of survey" responses.

2.4.1. Data analysis

Binary and multivariable logistic regression analyses were used to examine factors associated with unsafe disposal of child feces. Independent variables were tested for possible multicollinearity. The logit

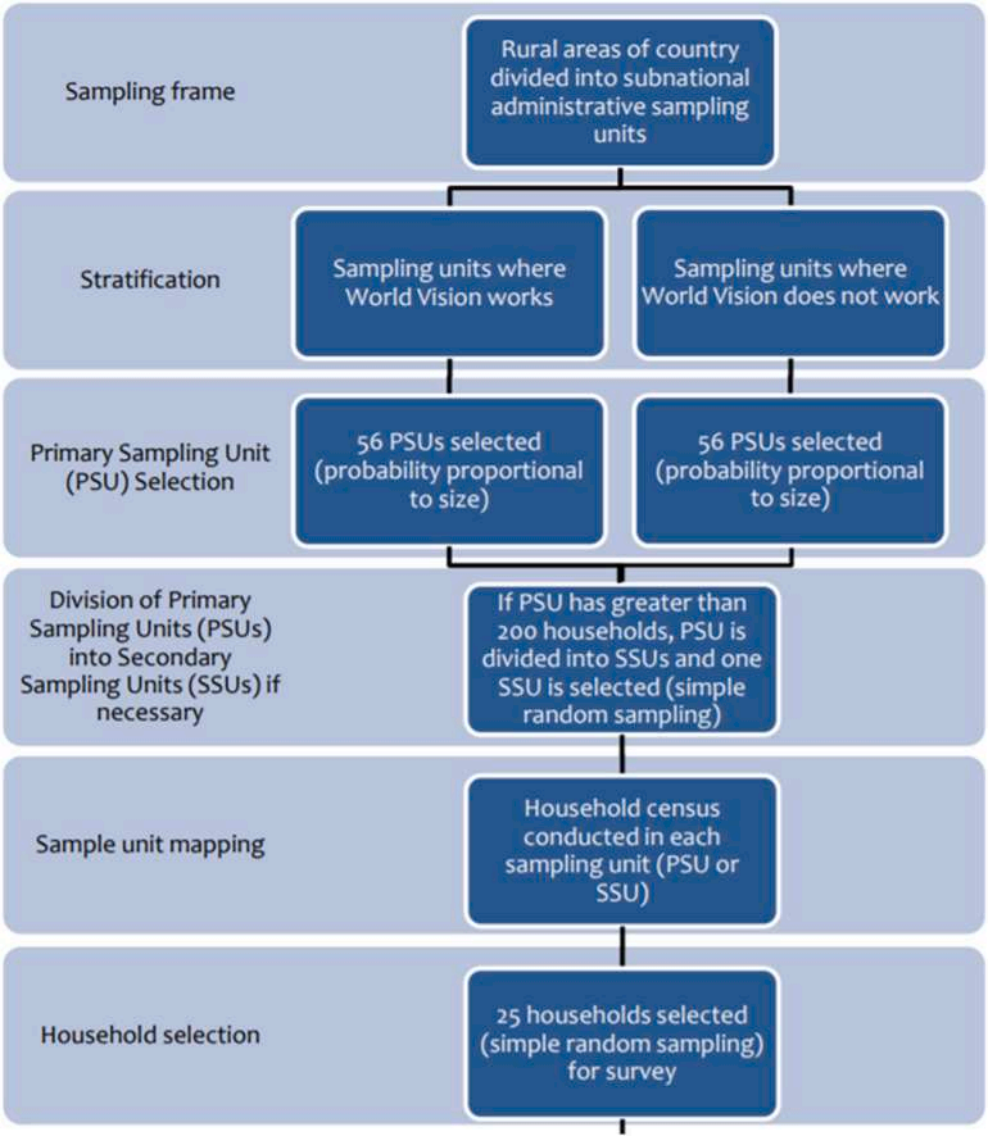

Fig. 1. Schematic diagram for survey population.

Table 1
Variables and corresponding survey questions and responses.

Variable topic	Variable definition or survey question	Categorization of survey responses
Child feces disposal	How do you dispose of your child's feces? Put/rinsed into toilet or latrine was coded as safe. Put/rinsed into drain or ditch, thrown into garbage bin or pile, buried, left in the open, and "other" were coded as unsafe.	• Unsafe disposal • Safe disposal
Wealth quintile	Wealth indexes were created separately for each country using variables for household services and possessions, such as electricity, vehicles, and electronics, as well as housing construction materials, such as flooring, roof, and walls. The principal components analysis method outlined by the Demographic and Health Surveys Program was employed for wealth index construction (Rutstein, 2015).	• Poorest • Poor • Middle • Wealthy • Wealthiest
Female head of household education level	Has the female head of household finished primary school?	• Yes • No
Open defecation	Does at least one member of your household defecate in the open?	• Yes • No
Talked about Cleanliness	Can you remember the last time someone talked to you about cleanliness?	• Yes • No
WaSH committee - activity	Has the water/WaSH committee held a community meeting in the past year?	• Yes • No
Sanitation facility – shared	Is this facility shared with other families who are not relatives?	• Yes • No
Handwash – defecation	Does the respondent wash their hands after defecating?	• Yes • No
Handwash-cleaning baby	Does the respondent wash their hands after cleaning their baby?	• Yes • No
Main water source improved	What is the main type of water point that your household uses to get drinking? Responses then coded into improved/unimproved drinking water source	• Unimproved • Improved

regression was used to calculate odds ratios and to identify associations between safe CFD and demographic factors, sanitation infrastructure, health behaviors, contact with community health workers, and household participation in the community. Control variables were used in all multivariable analyses were wealth quintile, female head of household's education, if any household member open defecates, and improved versus unimproved sanitation facility. All analysis was conducted using Stata 15.1.

2.5. Variables

There were some modifications to questions about WaSH committees in the survey conducted in India. Indian households who had on-premises piped water as their main water source were excluded from questions about committees which decreased the number of respondents. Community water points were available in each of the PSUs and community members were available to look after the water points and report in cases of malfunctioning to appropriate authorities. The households in India were not organized into any water committees. As a result, respondents in most of the PSUs were not able to properly answer the questions related to water committees.

3. Results

The prevalence of safe CFD was 17%, 40%, and 54% for India, Ethiopia, and Zambia, respectively (Table 2). The prevalence of unsafe CFD ranged from 75% among poorest wealth quintile households to 41% among the richest wealth quintile households. In the three studied countries, 59% of female heads of households had finished primary school. Twenty-seven percent of households had an improved sanitation facility. Rates of improved sanitation facility coverage were similar across all countries ranging from 26% to 28% (Table 2). Open defecation was highest in India (63%). Households in Ethiopia, India, and Zambia that had an improved sanitation facility practiced safe CFD at rates of 28%, 27%, and 27% respectively (Table 2). Fourteen percent of Indian households recalled talks about cleanliness versus 42% of Ethiopian households and 37% of Zambian households. There were few active WaSH committees in India with 2% holding meetings within the year, compared to Ethiopia and Zambia which had 31% and 21%, respectively (Table 2).

All wealth quintiles for India were positively associated with safe CFD relative to the poorest households. In the three studied countries, there was a negative association between a household member open defecating and safe child feces disposal (Ethiopia: OR = 0.43, p = 0.0001; India: OR = 0.33, p = 0.0001; Zambia: OR = 0.35, p = 0.0001) (Table 3). In India and Zambia, if a female head of household attended primary school, they were more likely to practice safe CFD (India: OR = 1.69 p = 0.007; Zambia: OR = 1.64, p = 0.013) (Table 3). In Ethiopia, if a respondent could recall speaking to someone about cleanliness, they were significantly more likely to dispose of child feces safely (OR = 2.35,

Table 2
Safe disposal of child feces by socioeconomic and demographic characteristics by country.

Characteristics	Ethiopia		India		Zambia	
	Safe	n	Safe	n	Safe	n
Prevalence of safe CFD	40.0	541	17.3	187	54.0	704
Wealth quintile						
Poorest	29.7	63	2.1	5	43.5	101
Poor	32.0	93	15.3	37	46.7	121
Middle	35.7	97	14.4	31	48.3	130
Wealth	41.3	123	24.2	44	55.9	152
Wealthiest	59.4	165	36.2	68	73.3	200
Female head of household finished primary school						
No	33.2	213	11.8	80	46.0	103
Yes	46.3	328	26.4	107	55.6	601
At least one household member open defecates						
No	71.3	361	46.7	147	80.2	595
Yes	21.3	180	5.2	40	19.3	108
Improved sanitation facility						
None	40.7	550	30.3	394	62.8	679
Unimproved	31.8	429	42.9	559	10.0	108
Improved	27.5	372	26.8	349	27.2	294
Respondent can recall when someone last spoke to them about cleanliness						
No	31.4	238	15.8	144	49.2	389
Yes	52.8	288	23.0	35	61.5	283
WaSH committee has held a meeting in past year						
No	37.2	294			52.7	328
Yes	51.2	178			56.4	194
Closest sanitation facility is shared with non-family members						
No	72.8	397	43.0	119	80.2	446
Yes	77.5	55	25.8	8	72.7	85
Washes hands after defecation						
No	30.3	168	11.5	9	40.4	76
Yes	46.9	373	17.8	178	56.2	628
Washes hands after cleaning baby						
No	35.6	273	18.1	115	52.2	530
Yes	45.8	268	16.18	72	60.2	174
Improved year-round main water point						
Unimproved	26.0	58	12.2	12	58.6	190
Improved	43.2	435	1838.0	172	52.3	487

Table 3
Multivariable logistic regression of safe child feces disposal.

Variables	Ethiopia		India		Zambia	
	OR (CI)	p-value	OR (CI)	p-value	OR (CI)	p-value
Wealth Quintile (vs. Poorest)						
Poor	0.96 (0.56–1.69)	0.912	4.43 (1.62–12.12)	0.004	1.20 (0.74–1.94)	0.452
Middle	0.56 (0.33–0.96)	0.036	3.81 (1.37–10.56)	0.010	1.51 (0.93–2.45)	0.098
Wealthy	0.39 (0.23–0.66)	0.0001	5.28 (1.92–14.51)	0.001	1.96 (1.20–3.22)	0.007
Wealthiest	1.05 (0.62–1.78)	0.866	6.59 (2.43–17.89)	0.0001	2.06 (1.27–3.53)	0.003
Female attended Primary school	1.28 (0.96–1.72)	0.092	1.69 (1.15–2.49)	0.007	1.64 (1.11–2.42)	0.013
Any member of the household open defecates	0.43 (0.32–0.60)	0.0001	0.33 (0.19–0.58)	0.0001	0.35 (0.24–0.50)	0.0001
Sanitation Facility (vs. None)						
Unimproved	28.43 (16.86–47.96)	0.0001	5.32 (2.48–11.42)	0.0001	44.12 (22.05–88.30)	0.0001
Improved	40.27 (23.34–69.49)	0.0001	6.55 (3.26–13.17)	0.0001	58.38 (28.79–118.39)	0.0001
Can recall last time someone talked to them about cleanliness	2.35 (1.74–3.18)	0.0001	1.22 (0.74–2.02)	0.429	1.24 (0.89–1.73)	0.200
WaSH committee has met in past year	1.30 (0.92–1.83)	0.133			1.02 (0.71–1.48)	0.879
Closest sanitation facility shared with non-family members	1.45 (0.78–2.70)	0.236	0.52 (0.21–1.29)	0.157	0.73 (0.45–1.18)	0.205
Washes hands after defecation	1.23 (0.91–1.66)	0.186	1.34 (0.58–3.12)	0.491	1.07 (0.67–1.72)	0.762
Washes hands after cleaning baby	1.66 (1.23–2.24)	0.001	0.96 (0.65–1.42)	0.828	1.39 (0.96–2.03)	0.083
Main water source is improved	1.50 (0.99–2.29)	0.058	1.50 (0.72–3.12)	0.274	0.87 (0.61–1.24)	0.449

$p = 0.0001$). If a respondent in Ethiopia reported washing their hands after cleaning a baby, they were significantly more likely to practice safe CFD (OR = 1.66, $p = 0.001$). In Ethiopia, a community that had a WaSH committee that met in the past year was significantly associated with the practice of safe CFD (OR = 1.47, $p = 0.017$) (Table 3).

4. Discussion

In the three studied countries, sixty-two percent of all respondents did not dispose of child feces safely, and India had the highest rates of unsafe CFD at 83% (Table 2). The high rates of unsafe CFD observed in India are comparable to the 81% and 79% reported by Freeman et al. (2016) and Majorin et al. (2014) respectively. Coverage of improved sanitation facilities was similar across countries, so this difference in safe CFD rates is not attributed to lack of available toilets since latrine access alone is insufficient to encourage safe disposal of child feces (Bauza et al., 2019; Freeman et al., 2016). A study in Ethiopia found that households with access to an improved sanitation facility failed to use them for disposal of child stool (Sahiledengle, 2019). An intervention in Odisha, India that gave government subsidies to build improved latrines saw an substantial increase of safe CFD to 10% in intervention households, compared to 3% in control households (Freeman et al., 2016). There is an urgent need to strengthen efforts on behavioral changes regarding safe CFD to minimize adverse health outcomes (Preeti et al., 2016).

Adult members of Indian households were much more likely to openly defecate, despite comparable rates of coverage of sanitation facilities (Table 3). In these three countries, if the household had a family member that openly defecated, they were significantly less likely to practice safe CFD (Table 3). Similar results were found by Miller-Petrie et al. (2016) but with a smaller odds ratio of 0.09.

Rates of handwashing after CFD in India were relatively small compared to Ethiopia and Zambia with 11% of Indian caregivers washing hands after CFD. Majorin et al. also found similarly low rates of handwashing after child feces disposal (2017). In Ethiopia, those who washed their hands after cleaning their baby were more likely to dispose of child feces safely (Table 3). Handwashing may have indicated a recognition of the danger associated with child feces. While the effect of handwashing on safe CFD is not well understood nor the reasons why caregivers dispose of feces either safely or unsafely, this association may suggest that if caregivers are made to understand the harms of child feces, they may be more likely to dispose of them safely.

Education about cleanliness may have had an influence on whether someone disposes of child feces safely (Table 3). Respondents in Ethiopia were more likely to practice safe CFD if they had talked to someone about cleanliness in the past six months. Several studies have found that talking to community health workers about cleanliness increases awareness of risky health behaviors and can encourage behavior change (Biemba et al., 2016). Azage and Haile (2015) found that being visited by Health Extension Workers (HEWs) in the past was not significantly associated with safe CFD (Federal Democratic Republic of Ethiopia Ministry of Health (FMOH), 2017). Another study by Gebru et al. (2014) found that model families, which are families selected and taught by HEWs to serve as role models for the community, were more likely to dispose of child feces safely compared to non-model families (93% versus 58%). Although it could not be determined if the training material contained information about CFD, this study suggests that the training and information may encourage safe CFD.

There were no positive associations between WaSH committees meeting in the past year and safe CFD (Table 3). This may have occurred because WaSH committees are more focused on providing working infrastructure rather than behavior change.

The association between wealth and safe CFD was consistent with the results of other studies (Azage and Haile, 2015; Bawankule et al., 2017; Preeti et al., 2016; Rand et al., 2015; Majorin et al., 2019). Those within higher wealth quintiles were more likely to have improved sanitation facilities, making safe CFD possible, and were more likely to have a better standard of living, motivating them to dispose of child feces safely (Azage and Haile, 2015; Sahiledengle, 2020).

Studies show that females who attended primary school were more likely to safely dispose of child feces in Ethiopia, India, and Bangladesh (Ayele et al., 2018; Azage and Haile, 2015; Bawankule et al., 2017; George et al., 2016; Preeti et al., 2016). Preeti et al. (2016) found that 61% of mothers with 12 or more years of education disposed of child feces safely compared to the 9% of mothers with no education in India. Maternal and paternal education was associated with lower odds of unsafe child feces disposal (Azage and Haile, 2015). This may be because educated mothers were more likely to understand the causes of childhood illness, and therefore were more likely to address those causes through practicing more hygienic behavior (Mwambete and Joseph, 2010).

4.1. Limitations

While the study population covered much of the rural areas within these three countries, generalizations cannot be made for all rural areas of all countries. All household survey results were unweighted because the data needed for household weighting was not collected in the three countries, and thus demographic characteristics should not be considered representative of all rural areas within these countries. There were several potential sources of bias in the survey questionnaire. However, the method of asking these questions was consistent with that used by

others (Elliot and Valliant, 2017). Households may not have had the technical knowledge to answer some questions. Additional questions about safe CFD could have addressed the reasons people choose a specific method of CFD or a determination of whether the caregiver understood that child feces are dangerous to improve the understanding of motivations behind the practice of unsafe CFD.

4.2. Implications

In Ethiopia, caregivers who washed their hands after cleaning a baby were more likely to practice safe CFD (Table 3). It is possible that those that washed their hands after cleaning a baby did so because they understand that feces pathogens pose a risk to their health. If people are aware of the dangers of child feces, they may be more likely to dispose of child feces safely. Further research should be conducted to determine the nature of this relationship. This research may be able to reveal whether this association occurs because people who wash their hands after cleaning a baby understand the risk of child feces or if people who wash their hands are more likely to dispose of child feces safely due to other factors.

Ethiopia was the only country where a respondent who talked to someone about cleanliness was more likely to practice safe CFD (Table 3). This may be partially attributed to the fact that Ethiopia was the only country to have a policy specifically promoting safe CFD. This suggests that policies that specifically address safe child feces may be useful in encouraging the practice of safe CFD.

Little is known about the influence of beliefs about child feces on the choice to dispose of children's feces safely or unsafely. No research has been conducted on whether increased knowledge of the dangers of child feces affect the likelihood of safe CFD. More research should be conducted to determine these beliefs to better cater information on child feces to encourage behavior change.

5. Conclusions

This study draws attention to the scale of unsafe CFD in India, Ethiopia and Zambia and adds to a growing body of evidence raising questions about the effectiveness of sanitation programs to minimize exposure to fecal-oral pathogens without an adequate and specific focus on safe disposal of child feces. The study identified several factors associated with safe child feces disposal that should be considered when developing WaSH programs to promote safe CFD. Behavior change messages promoting safe CFD should be included in sanitation programs and disseminated by community health workers alongside efforts to reduce open defecation more generally. Further studies investigating the link between knowledge, attitudes, and beliefs related to child feces and the practice of safely disposing child feces would be beneficial to inform the development of more effective solutions.

Declaration of competing interest

There is no conflict of interest with regards to this manuscript – the authors have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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Hair mercury levels, intake of omega-3 fatty acids and ovarian reserve among women attending a fertility center

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ABSTRACT

Objective: To investigate the association of hair mercury (Hg) levels with antral follicle count (AFC), as a marker of ovarian reserve, and evaluate whether this relationship differed among women with high vs. low total intake of long chain omega-3 polyunsaturated fatty acids (n3PUFA) from foods and supplements.
Design: We included 353 women attending an academic fertility center (2007–2019) who had data on hair Hg levels, total n3PUFA intake, and AFC.
Methods: Hair Hg levels were assessed using a Direct Mercury Analyser, total n3PUFA intake was estimated using an extensively validated food frequency questionnaire, and AFC was assessed by transvaginal ultrasonography. Poisson regression models adjusted for potential confounders were used to evaluate the association of hair Hg levels (divided into tertiles, and as above vs below EPA reference (1 ppm)) with AFC. Associations were also evaluated after stratification by median n3PUFA intake (≤0.124% vs. >0.125% calories/week).
Results: Women's median hair Hg level was 0.60 ppm (range = 0.001–8.60 ppm), with more than 30% > 1 ppm (EPA reference level). Hair Hg was positively related to AFC after adjusting for age, BMI, smoking status, infertility diagnosis, and alcohol intake. However, associations became attenuated after adjustment for intake of total n3PUFA. The positive associations of hair Hg and AFC were observed only among women above the median total n3PUFA intake. Specifically, women who consumed >0.125% calories/week of total n3PUFA had mean AFCs of 11.9, 13.2 and 14.1, respectively, across increasing tertiles of hair Hg (p,trend = 0.004). Similar results were found when hair Hg was divided above vs below EPA reference (mean AFC = 12.7 vs. 14.1, p = 0.008).
Conclusions: In these women, positive associations of hair Hg with AFC may be reflective of beneficial effects of n3PUFA on ovarian reserve rather than a beneficial effect of Hg per se. Our findings highlight the importance of considering diet when exploring Hg effects on women's reproductive health in urban settings.

1. Introduction

Mercury (Hg) is a ubiquitous and persistent toxicant (EPA 2020) and is currently ranked as one of the three pollutants of highest concern to human health by the U.S. Agency for Toxic Substances and Disease Registry (ATSDR 2019). Methylmercury (MeHg) is the primary form of non-occupational exposure to Hg in the general population and occurs primarily through the consumption of contaminated fish and shellfish. Nearly all US residents have detectable levels of MeHg in their bodies, reflecting the widespread presence of MeHg in the environment. Although no level of MeHg exposure has been designated as safe given the lack of any physiological benefit, the US Environmental Protection

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Agency (EPA) has set 1 ppm of MeHg in hair as the level in which the most concerning adverse effects can appear. Exposure to Hg has been associated with adverse reproductive health effects, including infertility and increased incidence of menstrual and hormonal disorders (EPA 2020; Mergler et al., 2007). However, fish intake is also the primary source of long-chain n-3 polyunsaturated fatty acids (n3PUFA), which have well-known benefits on reproductive health, including fertility and pregnancy outcomes (Gaskins et al., 2018; Gil and Gil 2015; Moran et al., 2016; Mozaffarian and Rimm 2006; Mumford et al., 2016; Nesheim, 2007; Wise et al., 2018). Therefore, it is important to consider the potential beneficial effects of n3PUFA intake when investigating health effects of Hg exposure at environmental levels in urban settings.

A systematic review on Hg and human reproductive health concluded that, overall, exposure to Hg was associated with infertility as well as increased incidence of menstrual and hormonal disorders in women (Henriques et al., 2019). However, most of the detrimental effects of Hg exposure on women's reproductive function were observed in occupational settings, which differs from exposure to MeHg at environmental levels through fish consumption in urban settings. It is also understudied whether exposure to Hg is associated with ovarian aging. Thus, we investigated the association of Hg levels (measured in hair samples) with ovarian reserve (measured as antral follicle count (AFC)) among women attending a fertility center. We also evaluated whether this relationship differed among women above vs. below the median of intake of total (foods and supplements) n3PUFA. Ovarian reserve reflects women's reproductive potential and declines irreversibly during a woman's life (Tal and Seifer 2017). AFC, defined as the number of visible antral follicles >2 mm present in the ovaries when assessed by transvaginal ultrasonography in the early follicular phase, has been proposed as a well-stablished and reliable marker to measure a woman's ovarian reserve (Broekmans et al., 2010).

2. Methods

2.1. Study population

We evaluated women enrolled in the Environment and Reproductive Health (EARTH) Study, a prospective cohort established to assess environmental and dietary determinants of fertility at the Massachusetts General Hospital (MGH) Fertility Center (Minguez-Alarcon et al., 2016). Women between 18 and 45 years old were eligible to participate and approximately 60% of those contacted by the research staff enrolled. Between 2007 and 2019, 353 female participants provided a hair sample for the assessment of mercury, had a transvaginal ultrasonography performed at the Massachusetts General Hospital (MGH) Fertility Center yielding a measurement of AFC, and also completed a food frequency questionnaire (FFQ) before the ultrasonography was performed. We included women with hair mercury samples that were collected less than 5 months before and no more than 1 year after the ultrasonography because hair mercury is an integrated measure of exposure during the 5 months prior to sample collection. Women included in this analysis had similar demographic and reproductive characteristics to women who enrolled but were not able to be included in the current analysis due to lack of hair Hg, total n3PUFA, or AFC measures (Supplemental Table 1).

The participant's date of birth was collected at entry, and weight and height were measured by trained study staff. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared. At enrollment, research staff administered sociodemographic, lifestyle, and medical history questionnaires to participants. Study participants also completed a comprehensive questionnaire on family, medical, reproductive and occupational history, consumer products use, smoking history, and physical activity. Infertility was diagnosed using the Society of Assisted Reproductive Technology definitions (SART, 2015). The study was approved by the Human Subject Committees of the Harvard T.H. Chan School of Public Health and MGH. Participants signed an informed consent after the study procedures were explained by trained research study staff and all the study questions were answered.

2.2. Hair collection and mercury assessment

We chose hair as the preferred biomarker for Hg levels in our participants because it reflects exposure over several months (Grandjean and Weihe 2002b). The hair sample was collected after recruitment into the study and mailed by participants to study staff loose in an envelope. We cleaned the hair sample before analysis to remove extraneous contaminants by sonication for 15 min in a 1% Triton X-100 solution. After sonication, samples were rinsed with distilled deionized water and dried 5 times at 60 °C for 48 h. Total Hg in parts per million (ppm) was measured using 0.02 g of the proximal 2 cm of hair (where 1 cm of length represents approximately 1 month of exposure) using a Direct Mercury Analyzer 80 (Milestone Inc, Monroe, CT) with a matrix matched calibration curve. We used certified reference material GBW 07601 (human hair; Institute of Geophysical and Geochemical Exploration, China) containing 360 ppm mercury as the quality control standard. The limit of detection (LOD) for Hg was 0.01 ppm with the percentage recovery for quality control standards ranging from 90 to 110 percent. The percentage difference in duplicate samples (collected at the same time point) analysis was <10%.

2.3. Diet assessment

We assessed diet using an extensively validated 131-item food frequency questionnaire [FFQ; (Rimm et al., 1992; Salvini et al., 1989; Yuan et al., 2017; C. Yuan et al., 2018)]. We asked participants how often, on average during the previous year, they consumed specific foods and beverages. The FFQ also included information on dose and frequency of supplement intake, including fish oil or n3PUFA. Nutrient contents for each item were obtained from a database maintained by the Research Dietetics Group at the Department of Nutrition, Harvard T.H Chan School of Public Health. This database is based on the US Department of Agriculture nutrient database (United States Department of Agriculture & Agricultural Research Service, 2008) and supplemented with data obtained from food and dietary supplement manufacturers, and direct measurements of foods as necessary to complete gaps from these other sources. We calculated nutrient intakes, including total n3PUFA (sum of foods and supplements), by adding across all foods and supplements included in the FFQ, the product between the frequency of intake for each food item and the nutrient composition for its specified portion size (foods) or dose (supplements). Fish intake was defined as the sum of canned tuna fish, dark meat fish (e.g. fresh tuna, salmon, blue fish), white meat fish (e.g. cod, haddock), breaded fish cakes or fish sticks, and shellfish (e.g. shrimp, scallops).

Intake of energy-bearing nutrients was adjusted for total energy intake using the multivariate nutrient density method while non-energy-bearing nutrients were adjusted using the nutrient residual method (Willet 2013). Among women in other studies, assessment of n3PUFA intake using this FFQ were validated against 2 fasting blood samples collected over a 15-month period, and prospectively collected 7-day diet records (Changzheng Yuan et al., 2018), as well as the assessment of fish intake using this FFQ, which showed high correlation ($r = 0.66$) with 1 year average of prospectively collected dietary records (Salvini et al., 1989). We calculated the total intake of caffeine and alcohol by summing the caffeine and alcohol content for the specific items multiplied by weights proportional to the frequency of use of each item.

To summarize overall food choices, we used two data-derived dietary pattern scores, the Prudent and Western patterns (Gaskins et al., 2012), with higher scores reflecting greater adherence to the corresponding dietary pattern. Specifically, we used factor analysis to derive these food patterns based on 40 pre-defined food groups. Orthogonal transformations were used to achieve uncorrelated factors (dietary patterns) with simpler structures and greater interpretability. In determining the

number of factors to retain, we considered eigenvalues (>1) (the amount of variance explained by the factor), the Scree plot (a plot of all the eigenvalues for the derived factors in descending order) and the interpretability of the factors. The substantive meanings of the rotated factors were considered in conjunction with the above empirical criteria and the derived factors were labeled on the basis of our interpretation. For every woman we calculated factor scores on each of the two retained factors by summing the frequency of consumption multiplied by factor loadings across all food items. Thus, each participant was given a score for the 'Prudent' and 'Western' patterns according to their consumption of items from each. The Prudent pattern is generally characterized by healthy foods (e.g. high intakes of fish, chicken, fruit, cruciferous vegetables, tomatoes, leafy green vegetables, legumes and whole grains) while the Western pattern is characterized by non-healthy foods (e.g. high intakes of red and processed meat, butter, high fat dairy, refined grains, pizza, snacks, high energy drinks, mayonnaise and sweets).

2.4. AFC assessment

All women participating in the study underwent an evaluation of ovarian AFC through transvaginal ultrasonography by one of the MGH reproductive endocrinology and infertility physicians in early follicular phase of an unstimulated cycle. No fertility medications were used in the cycle preceding the ultrasonographic determination of the AFC. This analysis included one AFC per woman. Of the 353 women, 14 (4%) women had AFC>30. Because women in the current study had a median (interquartile range, IQR) AFC of 13 (9, 18), in order to reduce the influence of these high values, AFC>30 were set to 30.

2.5. Statistical analysis

We presented demographic, dietary and baseline reproductive characteristics of the women using median ± interquartile ranges (IQRs) or percentages. Hair Hg levels was categorized into tertiles and also in two groups according to the US Environmental Protection Agency (EPA) reference level of 1.0 ppm (EPA 2020), with the lowest group considered as the reference group. We evaluated associations of hair Hg levels, in tertiles, with demographics, dietary and baseline reproductive characteristics using Kruskal–Wallis tests for continuous variables and chi-squared tests for categorical variables (or Fisher's exact test where appropriate). We used Poisson regression models to estimate the association of hair Hg levels, as a categorical variable and also as a continuous exposure variable, with AFC. We conducted tests for linear trends when hair Hg was categorized in tertiles using the hair Hg levels as an ordinal level indicator variable of each quartile, simulating a continuous variable. To allow for better interpretation of the results, we presented population marginal means (Searle et al., 1980) (except when we used hair Hg levels as a continuous variable that we presented β (95% CI)), adjusting for all the covariates in the model (at the mean level for continuous variables and for categorical variables at a value weighted according to their frequencies).

Confounding was assessed using both prior knowledge regarding biological relevance and descriptive statistics from our study population. The variables considered as potential confounders included factors previously related to ovarian reserve, and factors associated with Hg exposure and ovarian reserve in this study (Rooney and Domar 2014; Sharma et al., 2013). We initially adjusted models for age (years), BMI (kg/m^2), smoking status (ever and never smoked), infertility diagnosis at enrollment (female, male, unexplained), and alcohol intake (gr/day). We fitted an additional multivariable model further adjusted for total n3PUFA intake (% calories/week) and total calorie intake (kcal/day), to minimize the possibility of residual confounding due to intake of other foods, and to evaluate their independent effects. Furthermore, associations were also evaluated after stratification by total n3PUFA intake (≤0.124 vs. >0.125% calories/week) and fish intake (divided by the median for our study population, ≤1.40 servings/week vs. >1.40 servings/week). Statistical analyses were performed with SAS (version 9.4; SAS Institute Inc., Cary, NC, USA).

3. Results

Women had a median (interquartile range [IQR]) age of 35 (32, 38) years and were predominantly Caucasian (84%) (Supplemental Table 1). Their median (IQR) BMI was 23.1 (21.4, 26.1) kg/m^2 and 74% had never smoked. Around half of the women (45%) had undergone a previous fertility evaluation and 38% had been previously pregnant. The median hair Hg level of the women was 0.60 ppm and ranged from 0.001 to 8.60 ppm (Fig. 1). More than 30% of the women had hair Hg levels >1 ppm (US-EPA 2001). Women included in our study had considerable higher hair Hg levels compared to women in Sweden (median = 0.34, range = 0.01–1.53 ppm) (Kippler et al., 2021), but lower than those reported among Korean (median = 0.97, range = 0.92–1.03 ppm) (Seo et al., 2020) and Surinamese women (median = 0.83, range = 0.001–31.9 ppm) (Gokoel et al., 2020). Hair Hg levels were positively correlated with women's intakes of n3PUFA and total fish intake over the past year (r = 0.43 and r = 0.55, respectively). Compared to women in the lowest tertile (range = 0.001, 0.39 ppm), women in the highest tertile of hair Hg (range = 1.00, 8.60 ppm) were older (median = 36.0 vs. 34.0 years), leaner (median = 22.5 vs. 23.7 km/m^2), and had higher intakes of alcohol (median = 8.8 vs. 3.0 g/day), total fish (median = 2.2 vs. 0.8 servings/day) and total n3PUFA (median = 0.17 vs. 0.07% calories/day) (Table 1). No other demographic, dietary, or reproductive characteristics differed substantially across tertiles of hair Hg levels (Table 1).

In models adjusted for age, BMI, smoking status, abstinence time and alcohol intake, hair Hg levels were positively related to AFC (Table 2). Specifically, adjusted means (95% CI) were 13.0 (12.3, 13.7), 14.0 (13.4, 14.7) and 14.1 (13.4, 14.8), respectively, across increasing tertiles of hair Hg (p, trend = 0.03). Further adjustment for total n3PUFA and total calorie intake slightly attenuated these associations. We observed similar positive associations and attenuations after considering intake of total n3PUFA when modeling hair Hg levels as a binary variable (above vs below the EPA reference level) as well as a continuous exposure (Table 2).

To further separate the potentially deleterious effects of Hg from the potentially beneficial effects of total n3PUFA intake on AFC, we evaluated associations after stratification by total n3PUFA intake (≤0.124 vs. >0.125% calories/week) (Fig. 2). The positive associations of hair Hg and AFC were observed only among women with above the median of total n3PUFA intake. Specifically, women who consumed >0.125% calories/week of n3PUFA had an adjusted mean (95% CI) AFC of 11.9 (10.9, 13.1), 13.2 (12.3, 14.3) and 14.1 (13.3, 14.9), respectively, across increasing tertiles of hair Hg (p = 0.004). In contrast, among women below the median of total n3PUFA intake, hair Hg was unrelated to AFC. Stratified analyses yielded similar results when modeling hair Hg levels

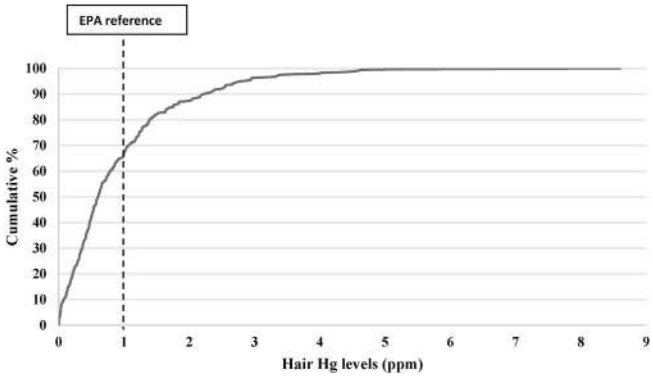

Fig. 1. Distribution of hair Hg levels among 353 women in the EARTH Study.

Table 1
Demographic, dietary, and reproductive characteristics by tertiles of hair Hg levels among 353 women in the EARTH Study.

	T1 (n = 117)	T2 (n = 118)	T3 (n = 118)	p-value[a]
		Median (IQR) or N (%)		
Hair Hg levels, ppm	0.18 (0.05, 0.29)	0.60 (0.50, 0.76)	1.64 (1.23, 2.39)	
Demographic characteristics				
Age, years	34.0 (31.0, 37.0)	34.5 (32.0, 39.0)	36.0 (33.0, 38.0)	0.04
White (race), N (%)	102 (88)	99 (84)	96 (82)	0.48
Body Mass Index, kg/m^2	23.7 (21.5, 26.7)	23.5 (21.5, 26.0)	22.5 (21.2, 25.4)	0.18
Ever smoked, N (%)	31 (27)	24 (20)	35 (30)	0.24
Total physical activity, hours/wk	5.50 (2.68, 12.4)	5.75 (2.50, 9.20)	5.00 (2.50, 9.49)	0.72
Diet				
Total energy intake, kcal/day	1692 (1473, 2071)	1638 (1286, 2028)	1704 (1317, 2075)	0.62
Caffeine intake, mg/day	102 (36.2, 158)	104 (33.4, 215)	108 (55.8, 231	0.22
Alcohol intake, g/day	3.02 (0.90, 8.67)	4.38 (1.39, 11.4)	8.77 (3.58, 15.5)	<0.0001
Total fish intake, servings/wk[b]	0.84 (0.14, 1.68)	1.40 (0.98, 2.24)	2.17 (1.68, 3.08)	<0.0001
Total omega-3, % of calories	0.07 (0.03, 0.15)	0.11 (0.07, 0.21)	0.17 (0.12, 0.28)	<0.0001
Prudent pattern score	−0.13 (−0.74, 0.52)	−0.05 (−0.67, 0.45)	−0.21 (−0.57, 0.47)	0.90
Western pattern score	−0.03 (−0.51, 0.76)	−0.02 (−0.80, 0.57)	−0.23 (−0.65, 0.30)	0.12
Reproductive history				
Day 3 FSH Levels, IU/L	6.80 (5.90, 8.65)	7.05 (6.20, 8.50)	6.70 (5.90, 7.80)	0.27
Initial infertility diagnosis, n (%)				0.03
Male factor	17 (15)	34 (29)	32 (27)	
Female factor	37 (32)	27 (24)	23 (19)	
Unexplained	63 (53)	57 (47)	63 (53)	
History of prior pregnancy, n (%)	45 (39)	47 (40)	42 (36)	0.76
History of infertility treatment, n (%)	42 (41)	51 (52)	43 (42)	0.21

[a] From Kruskal-Wallis test for continuous variables and chi-squared tests for categorical variables.
[b] Total fish defined as the sum of dark meat fish (including canned tuna fish and other dark meat fish such as salmon and bluefish), white meat fish (including breaded fish cakes and other white meat fish such as cod, haddock, and halibut), and shellfish (including shrimp, lobster, scallops, and clams as a main dish).

as a binary variable (above vs below the EPA reference level) (Fig. 2) as well as a continuous exposure (p-interaction = 0.13; β (95%CI) = 0.03 (0.006–0.06) for total n3PUFA below the median; β (95%CI) = 0.04 (0.004–0.07) for total n3PUFA above the median). Additionally, we found similar positive trends, although no significant associations, when we adjusted for total fish intake (instead total n3PUFA) in the main models. When we stratified by total fish intake, we observed overall higher antral follicle count among women above the median of fish consumption, compared to those with below the median, but no associations between hair Hg and AFC in either stratum (data not shown).

4. Discussion

We investigated the association of hair Hg levels with ovarian reserve, as reflected by AFC, among women attending a fertility center in Boston, MA. We found that hair Hg levels were positively related to AFC. This association became attenuated after adjusting for intake of n3PUFA and it was only observed among women above the median of total n3PUFA intake. Thus, in this group of women, positive associations of hair Hg with AFC may be reflective of beneficial effects of n3PUFA on ovarian reserve rather than a beneficial effect of Hg per se. These results highlight the need to account for diet when investigating Hg exposure at environmental levels on reproductive health outcomes in non-occupational settings, where fish is the main source of both Hg and n3PUFA.

Table 2
Adjusted mean antral follicle count[a] by hair Hg levels among 353 women in the EARTH Study.

	Estimated Mean Antral Follicle Count, n	
Hair mercury levels, ppm (range)	Adjusted for age, BMI, smoking, infertility diagnosis and alcohol intake.	Adjusted for age, BMI, smoking, infertility diagnosis, and intakes of alcohol, total omega-3 and calories.
Tertiles		
T1 (0.001–0.39)	13.0 (12.3, 13.7)	13.1 (12.4, 13.8)
T2 (0.40–0.99)	14.0 (13.4, 14.7)*	14.0 (13.3, 14.7)‡
T3 (1.00–8.60)	14.1 (13.4, 14.8)*	13.9 (13.3, 14.7)‡
p-trend	0.03	0.09
EPA Ref		
G1 (0.001–1.00)	13.4 (13.0, 13.9)	13.5 (13.0, 14.0)
G2 (1.01–8.60)	14.1 (13.5, 14.9)	14.0 (13.3, 14.7)
p-value	0.10	0.25
β (95% CI)	0.03 (0.01, 0.05)	0.02 (0.008, 0.05)
p-value	0.001	0.01

‡p-value <0.10 when compared that tertile with the lowest tertile of exposure.
*p-value <005 when compared that tertile with the lowest tertile of exposure.
[a] Data are presented as predicted marginal means (95% CI) except when using hair Hg levels as a continuous exposure variable that data are presented as β (95% CI).

Results from this study are in agreement with those found among men in the EARTH Study. Briefly, we previously observed that hair Hg levels were positively related to some semen quality parameters, but only among those men who had high consumption of fish (Minguez-Alarcon et al., 2018). The overall interpretation that hair Hg levels are unrelated to fertility or makers thereof and that their apparent relation with better fertility is a function of their relation with fish intake is also in agreement with previous work from our group and others. In previous reports from this study, we found no associations of hair Hg levels with intermediate and clinical pregnancy outcomes among women from the same study cohort (Wright et al., 2015), even though we also found a positive association between pre-conception fish intake and the probability of achieving a live birth in the same women (Nassan et al., 2018). Similarly, in the LIFE Study, which followed couples without a history of infertility as they tried to become pregnant, blood Hg concentrations were unrelated to time to pregnancy (Buck Louis et al., 2012) even though seafood consumption was associated with a shorter time to conception and a lower risk of infertility (Gaskins et al., 2018). On the other hand, and in contrast with our results, a small study including 30 subfertile women undergoing IVF showed an inverse association between hair Hg levels and AFC (Dickerson et al., 2011). Compared to women in our study, women in this small study had similar mean age (33 vs. 35 years), BMI (25 vs. 23 kg/m^2), hair Hg levels (0.89 vs. 0.93 ppm) and AFC (13 vs. 14). However, our study included over 10 times more female participants and also accounted for a broad spectrum of potential confounders including important demographic, reproductive and dietary characteristics. While studies conducted among female workers have shown a detrimental reproductive health effect of Hg (measured also in urine and blood), it is not as clear that the same negative effect exists outside of occupational settings where fish intake represents the main source of exposure (Henriques et al., 2019). Fish intake is also the primary source of n3PUFA in the general population, which have well-known benefits on relevant health outcomes, such as reproductive

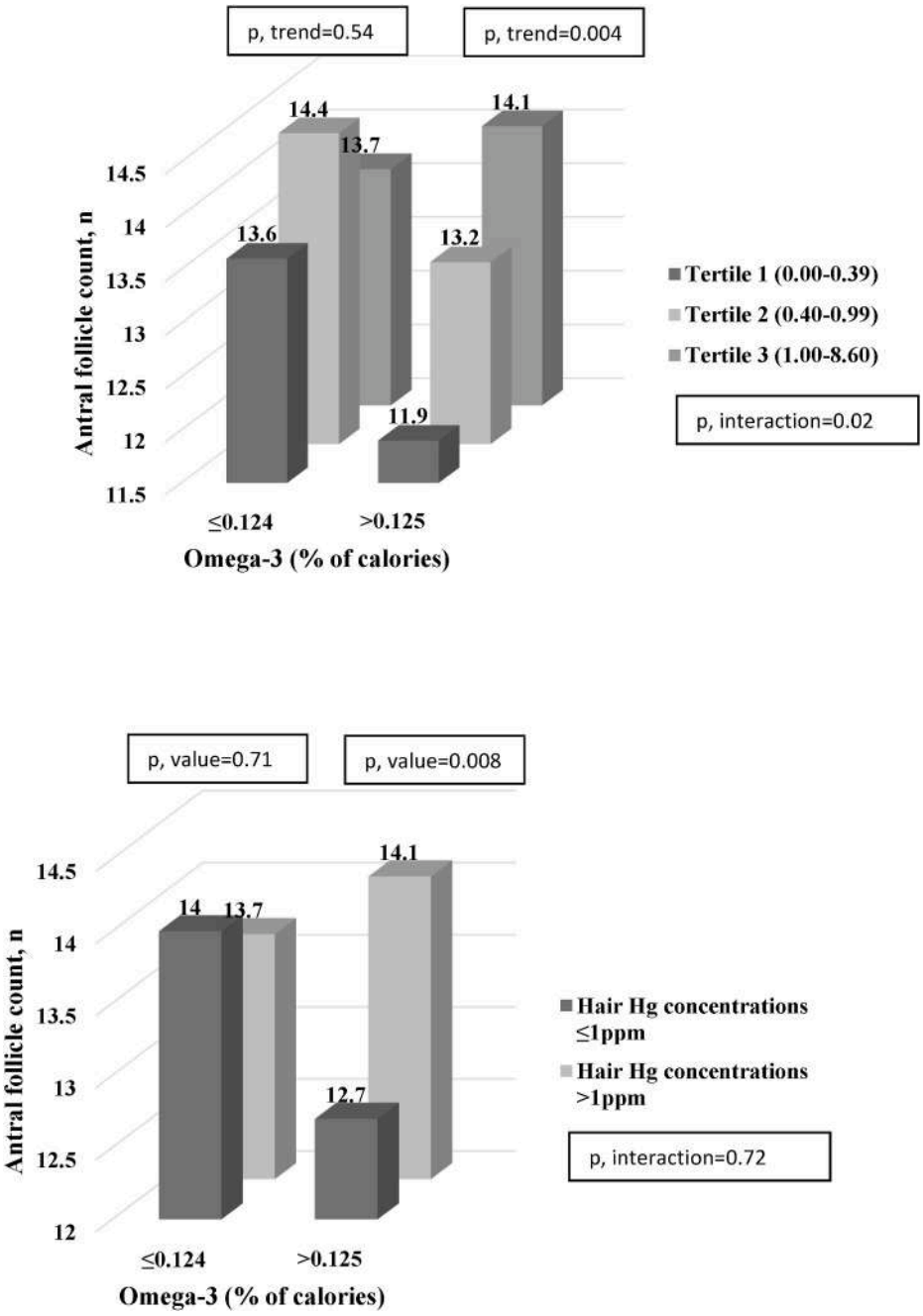

Fig. 2. Effect modification by total omega-3 intake on the relationship between hair Hg levels and estimated mean antra follicle count (marginal means, 95% CI) among 353 women in the EARTH Study. Models are adjusted for age (continuous), BMI (continuous), smoking status (ever and never smoked), infertility diagnosis (male, female and unexplained), alcohol (g/day), and total calorie (kcal/day) intakes.

health including fertility and pregnancy complications (Gaskins et al., 2018; Gil and Gil 2015; Moran et al., 2016; Mozaffarian and Rimm 2006; Mumford et al., 2016; Nesheim, 2007; Wise et al., 2018). We hypothesize that the positive associations of hair Hg with AFC may be reflective of beneficial effects of n3PUFA on ovarian reserve rather than a beneficial effect of Hg per se since the positive associations between hair Hg and AFC are attenuated and in some models no longer significant after adjustment for total n3PUFA. Also, the positive associations between hair Hg and AFC were only found among women above the median of total n3PUFA intake, ant not among women below the median of total n3PUFA intake. Future research on Hg exposure and ovarian reserve in urban settings should also evaluate the balance of risk and benefits given shared exposure profiles associated with fish consumption. Similarly, Hg is well-known for its neurotoxic effects on the fetus, newborns and children, but also the beneficial effects of fish consumption on fetal outcomes, especially on fetal brain development, has been reported (Oken et al., 2005). Thus, the beneficial/detrimental balance of maternal fish consumption have been evaluated over time to protect children's health (FDA 2017; Oken et al., 2003; Taylor et al., 2018).

In experimental studies including several animal models, the ovary has been shown to be particularly sensitive to Hg exposures, specifically affecting oogenesis and folliculogenesis (Massányi et al., 2020). For example, in an animal study using rats, those that were Hg-exposed showed lower ovarian weight and their ovaries displayed irregular ovarian follicular development, leading to reduced number of ovarian antral follicle count and increased number of atretic ovarian follicles,

compared to controls (Merlo et al., 2019). However, it is always difficult to compare results from animal studies to those found in epidemiologic studies of humans because of the physiological differences among species, exposure periods and doses, which all lead to variation in the effect. It has been shown that the Hg kinetics (absorption, metabolism, distribution, and excretion) and toxicokinetics in humans are not comparable with those in animals (Carrier et al., 2001). In addition, some of the differences in reproductive health effects by Hg exposure may also exist in human studies assessing occupationally vs. non-occupationally Hg exposure. Consequently, it is important to differentiate Hg reproductive effects in animal vs. human studies as well as occupational vs. urban settings in human studies.

Limitations of this study include limited generalizability of these results to women in the general population since this study includes subfertile women attending a fertility center. Subfertile couples represent a vulnerable and important public health subpopulation given the decreasing birth rates in the U.S. general population (CDC 2019) and growing number of babies born using medically assisted reproduction in the US, estimated to be > 250,000 births per year (Dyer et al., 2016; Schieve et al., 2009; Zegers-Hochschild et al., 2014) and over 1 million over the next 10 years. Also, this study design is not prospective in terms of Hg and AFC which may limit causal inference. However, it has been demonstrated that measurement of Hg in hair samples reflects Hg exposure over several months (Grandjean and Weihe 2002a). In addition, as is the case of all studies based on diet questionnaires, measurement error and misclassification of n3PUFA intake are a concern. Nevertheless, intake measured with this FFQ has been validated against biological markers of intake, as already mentioned (Changzheng Yuan et al., 2018). Moreover, because intake of total n3PUFA and diet were assessed prior to the AFC, it is unlikely that the error was related to the outcomes. A concern specific to this particular study is that FFQs are designed to optimize the accrual of nutritional data. Hence, questions regarding fish intake were chosen to obtain information on sources of marine fatty acids, but not to distinguish fish in terms of their Hg content. For example, intakes of salmon (low Hg, high omega-3) and swordfish (high Hg, high omega-3) are combined in a single question. Hence, while simultaneous consideration of fish intake and a biomarker of Hg are a significant improvement over previous work addressing this question, residual confounding is still possible. Furthermore, we were not able to investigate women with a diagnosis of polycystic ovarian syndrome (PCOS) as noted in their medical records because these observations were given a code, not a count. Finally, we did not investigate Hg effects on other reliable markers of ovarian reserve, such as assess anti-mullerian hormone levels, because of the small number of women with available data. The main strengths of our study are the relatively large size and the comprehensive adjustment of possible confounding variables, dietary and non-dietary related, due to the standardized assessment of a wide range of participant characteristics.

In summary, we observed positive associations between hair Hg levels and ovarian reserve, measured as AFC, among women attending a fertility center. These associations became attenuated after adjusting for n3PUFA intake and were only observed among women who consumed high intakes of total n3PUFA (foods and supplements). Thus, in these group of women, positive associations of hair Hg with AFC may be reflective of beneficial effects of n3PUFA on ovarian reserve rather than a beneficial effect of Hg per se. These results highlight the need to account for diet when investigating Hg exposure at environmental levels on reproductive health outcomes in non-occupational settings, where fish is the main source of both Hg and n3PUFA.

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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.2021.113825.

Competing financial interests

None of the authors has any conflicts of interest to declare.

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HBM4EU combines and harmonises human biomonitoring data across the EU, building on existing capacity – The HBM4EU survey

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ABSTRACT

As part of the Human Biomonitoring for Europe (HBM4EU) initiative a human biomonitoring (HBM) survey is conducted in 21 countries. This survey builds on existing HBM capacity in Europe by aligning national or regional HBM studies. The survey targets 3 age groups (i) children aged 6–11 years, (ii) teenagers aged 12–19 years and (iii) young adults aged 20–39 years and includes a total of 9493 participants (3151 children, 2953 teenagers and 3389 young adults). Depending on the age group, internal exposure to phthalates and substitute Hexamoll® DINCH, brominated and organophosphorus flame retardants, per-/poly-fluorinated compounds, cadmium, bisphenols and/or polycyclic aromatic hydrocarbons are assessed. The main goal of the programme is to obtain quality controlled and comparable HBM data of exposure to chemicals, prioritized under HBM4EU, with European wide coverage to inform the development of environment and health policies. This paper describes the framework of the HBM4EU survey and the approach that has been applied to align European HBM initiatives across Europe.

1. Introduction

In June 2004 the European Commission recognized in its Environment and Health Action Plan the relevance of human biomonitoring (HBM) and the need for more harmonized approaches in Europe to allow for better comparability of results and more efficient use of resources (European Commission, 2004). In 2005 the Expert team to Support BIOmonitoring in Europe (ESBIO) project was launched followed by the first joint European HBM initiative COPHES (Consortium to Perform Human Biomonitoring on a European Scale) together with the feasibility study DEMOCOPHES (Demonstration of a Study to Coordinate and Perform Human biomonitoring on a European Scale) which were conducted between 2009 and 2012 (Schindler et al., 2014). These projects were very successful and laid the foundation for future projects. In 2017 a joint European HBM initiative (HBM4EU) was launched. The HBM4EU project runs from 2017 to 2021 and is co-financed under Horizon 2020. The project's main goal is to coordinate and advance human biomonitoring in Europe to provide science based evidence for chemical policy development and improve chemical management (Ganzleben et al., 2017). As part of the HBM4EU initiative a joint HBM survey is conducted laying the foundation of a

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sustainable European HBM platform.

Human biomonitoring measures trace levels of multiple environmental chemicals, their metabolites, or reaction products in human biological matrices, such as blood and urine. HBM data directly reflects the actual internal chemical exposure of a sampled population at a given time covering all routes of exposure and taking into account the kinetics of chemicals in the body including bioaccumulation of persistent chemicals (Angerer et al., 2007). HBM data can be used for a number of objectives such as: establishing reference ranges for selected chemicals in the general population or in specific targeted populations such as occupational populations or pregnant women, identifying highly exposed populations, monitoring time trends and spatial patterns of internal exposure and evaluating the effect of policy measures. Connecting HBM data to personal information regarding lifestyle, diet, behavior and health enables the mapping of internal doses with potential exposure sources and/or health outcomes. It is recognized that HBM data provides an important tool to support environment and health policy development through regulatory actions but also by awareness raising campaigns or remediation actions (Bahadori et al., 2007). Some EU countries have national programs that collect, for a wide variety of chemicals, HBM data which are representative of specific characteristics of their populations such as age, sex and socio-demographic factors. Examples are the German GerES studies (Schulz et al., 2007), the Czech Environmental Health Monitoring System Czech-HBM (Cerna et al., 2012), the French national biomonitoring programme (ELFE) (Dereumeaux et al., 2017), the Italian PROBE (Alimonti, 2011), the Spanish BIOAMBIENT. ES (Perez-Gomez et al., 2013) and the Flemish Human Biomonitoring Studies (FLEHS) representative for the Flemish region (Choi et al., 2015; Schoeters et al., 2012). Most countries lack such a programme and collect HBM data in the frame of specific research projects. Hence the studies are fragmented and heterogeneous. There is no overarching strategy within Europe and current studies are not harmonized or aligned to meet common goals. Therefore, to generate comparable HBM data with European wide coverage of exposure to HBM4EU prioritized chemicals (David et al., 2020), ongoing and planned HBM studies from different countries and regions all over Europe have been aligned and brought together under the joint HBM4EU survey. All HBM4EU priority substances are summarised under https://www.hbm4eu.eu/the-substances/. In the hereby reported survey the focus is on a subset of the first set of priority substances of HBM4EU, phthalates and substitute Hexamoll® DINCH, brominated and organophosphorus flame retardants, per-/poly-fluorinated compounds (PFAS), cadmium (cd), bisphenols and polycyclic aromatic hydrocarbons (PAHs). The data resulting from the HBM4EU survey will feed into the evaluation of following research objectives: the derivation of European exposure values, geographical comparisons of the four European regions, identification of determinants of internal exposure (personal characteristics, external sources), comparing exposure levels to health based guidance values, associating exposure biomarkers with personal health data and linking these associations through effect markers in a causal pathway analysis.

2. Method: aligning European HBM surveys

2.1. Sampling frame

The main focus of the joint HBM4EU survey is to establish reference ranges for HBM4EU priority substances that are representative for the exposure distribution of the European population and to facilitate the use and translation of the scientific results into policy actions. The survey builds on existing HBM capacity in Europe by aligning HBM studies/initiatives targeting the general population. A sampling frame was developed to facilitate the selection of participating studies that could be aligned to obtain comparable HBM data across the EU. In a first step the target populations were defined for whom HBM data representative of the current exposure variability across Europe should be collected. Current exposure was defined as samples collected between 2014 and 2019 (later extended to 2020). Due to logistic and financial constraints we could only include a 'sample' of the European population in our study. However, representativeness means that this sample reflects the composition of the European population for some prespecified criteria. A representative study population can only be achieved by the use of a probability sampling method which is most commonly used in HBM studies. The ideal concept to obtain a representative sample of the European population would be a multistage probability sampling method among the participating EU countries with proportional representation of sex, age groups, SES and geographical spread. In all study areas we aimed for an equal participation of both sexes in the surveys cfr. National Health and Nutrition Examination Survey (NHANES) and Canadian Health Measures Survey (CHMS) (Statistics Canada, 2018; CDC, 2017). Additional sampling domains for which representation is considered important in HBM4EU are socio-economic status (SES) and residential degree of urbanisation. In each primary sampling unit (country) each level of SES and degree of urbanisation should be at least 10% represented in the participating surveys to cover variation in SES and degree of urbanisation. The categorisation of study participants in low – medium – high level of education was based on Eurostat's online tables that refer to the International Standard Classification of Education (ISCED) developed by United Nations Educational, Scientific and Cultural Organization (UNESCO) (UNESCO Institute for Statistics, 2012). Lower educational level denotes individuals with no to lower secondary education (ISCED 0–2), medium level of education includes individuals with upper secondary to post-secondary non-tertiary education (ISCED 3–4), and high level of education represents individuals with tertiary education and higher (ISCED $\geq$5). A subject's living environment is classified according to the degree of urbanisation (DEGURBA) classification of Eurostat distinguishing three levels of urbanisation. i.e. densely populated area (cities), intermediate density area (towns and suburbs) and thinly populated area (rural area) (Lewis Dijkstra, 2014).

2.2. Age categories

For the joint HBM4EU survey several age groups were considered for sampling including newborns (0-2y), toddlers (3-5y), children (6-11y), teenagers (12-19y), young adults (20-39y), adults (40-59y), elderly (60-79y) and 80 + y. Ideally, exposure data from all age groups should be collected. However, for this programme of work it was only feasible to sample certain age groups. Newborns and young children (age 0-5y) are considered as a vulnerable and important subgroup. However, newborns were not included since a lot of information is already available on early life stressors, including environmental chemicals, that has been collected in European birth cohorts (Maitre et al., 2018; Vrijheid et al., 2012). Toddlers were not included in this first step as the related field work for recruitment and sampling is more complex compared to older age groups. Therefore, the joint HBM4EU survey focuses on collecting recent data from three age groups: (i) children aged 6–11 years, (ii) teenagers aged 12–19 years and (iii) young adults aged 20–39 years. Restricting the adult age group to 39 years provides a more homogeneous group with a more similar health status (reproductive age group). Additionally, this classification mirrors the age stratification used in NHANES and CHMS (CDC, 2017; Statistics Canada, 2018).

2.3. EU wide coverage

To obtain complete European coverage, a maximal scenario for Europe would be to sample each of the 27 EU countries of the HBM4EU consortium. To lower the resolution and reduce the maximal scenario, Europe was stratified into four geographical regions i.e. North, East, South and West, according to the United Nations geoscheme (United Nations, 1999). The number of PSU (countries) to include per geographical region should be proportional to the number of inhabitants of that region. Given that the North, West, South and East represent 21%,

40%, 28% and 11% of the EU inhabitants respectively, we proposed to include 2 countries to represent the North, 3–4 countries to represent the West, 3 countries to represent the South and at least 1 country to represent the East of Europe. In Supplementary Figure 1 the countries contributing to the joint HBM4EU survey for at least one of the age groups are coloured according to the EU geographical region they are attributed to.

2.4. Sample size

The main objective of the HBM4EU survey is to establish EU exposure values of internal exposure to priority chemicals with their confidence intervals. As calculated by Poulsen et al. to obtain percentiles with reasonably narrow confidence intervals, at least 120 measurement

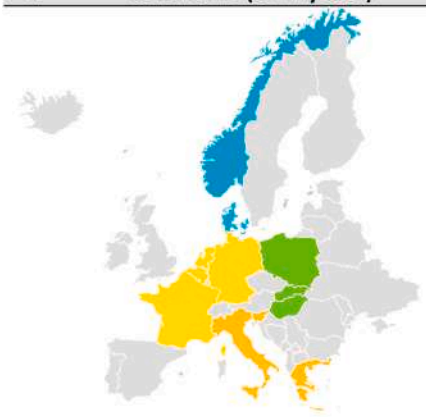
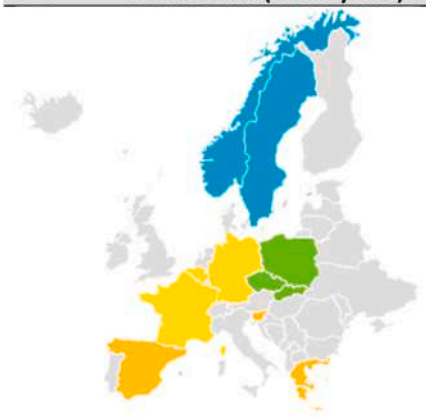
	Age group	EU region	Cohort	Time period covered	No. subjects
A	CHILDREN (6-11 years)				3151
		North	NEBII	2016-2017	300
			OCC	2018-2019	300
		East	InAirQ	2017-2018	262
			PCB cohort	2014-2017	300
			POLAES	2017	300
		South	SLO CRP	2018	149
			CROME	2020-2021	161
			NAC II	2014-2016	300
		West	ESTEBAN	2014-2016	544*
			GerES V-sub (unweighted)	2015-2017	300
			3xG	2019-2020	133
			SPECIMEn-NL	2020	102
B	TEENAGERS (12-19 years)				*2953*
		North	NEB II	2016-2017	181
			Riksmaten Adolescents 2016-17	2016-2017	300
		East	POLAES	2017	281
			CELSPAC: Teenagers	2019	300
			PCB cohort (follow-up)	2019-2020	297
		South	SLO CRP	2018	97
			CROME	2020-2021	150
			BEA	2017-2018	300
		West	ESTEBAN	2014-2016	447*
			FLEHS IV	2017-2018	300
			GerES V-sub (unweighted)	2014-2017	300
C	ADULTS (20-39 years)				*3389*
		North	CPHMINIPUB (parents) / DYMS	2018	292
			Diet_HBM	2019-2020	205
			FinHealth	2017	300
		East	POLAES	2017	228
			(C)ELSPAC: YA	2019	300
		South	HBM survey in adults in Croatia	2019-2020	300
			INSEF-ExpoQuim	2019-2020	296
		West	ESTEBAN	2014-2016	393*
			HBM4EU-study for Switzerland	2020	300
			ESB	2014-2019	565*
			Oriscav-Lux2	2016-2018	210

Fig. 1. Studies participating in the joint HBM survey targeting children (A), teenagers (B) and adults (C) representing all 4 geographical subregions in Europe North, East, South and West Europe based on United Nations geoscheme subregions of Europe. * For ESTEBAN and ESB the total number of subjects >300 because the group of 300 subjects differs per substance group.

Table 1
Sampling design: clustering of the Primary Sampling Units (PSU), selection of the PSUs, and sampling within the PSU

Age groups
Target: 3 age groups: • children aged 6-11 years • teenagers aged 12- 19 years • young adults aged 20-39 years
Primary sampling unit (PSU)
Country based, 27* EU HBM4EU participating countries
EU wide coverage (clustering of PSUs)
PSU are clustered per EU geographical region**: • Northern Europe (DK, FI, SE, IS, NO, LV, LT, IE, UK, EE) • Western Europe (AT, BE, NL, FR, DE, CH, LU) • Southern Europe (HR, CY, EL, IT, PT, Sl, ES, MK) • Eastern Europe (CZ, PL, SK, HU)
EU wide coverage (selection of PSU)
Inclusion of number of PSU (countries) per region proportional to number of inhabitants of the region. • 2 PSU for Northern Europe: 21% • 3-4 PSU for Western Europe: 40% • 3 PSU for Southern Europe: 28% • At least 1 PSU for Eastern Europe: 11%
National representativeness
• PSU representative on national level preferred, PSU representative on regional level also accepted.
Sampling design within the PSU
• Min 240 - max 300 subjects with a 1:1 male female ratio

*Current HBM4EU consortium consists of 30 countries including Israel (non-EU) and Estonia and Republic of North Macedonia who joined the HBM4EU consortium in a later stage, ** following the United Nations geoscheme subregion of Europe. DK = Denmark, FI = Finland, SE = Sweden, IS = Iceland, NO = Norway, LV = Latvia, LT = Lithuania, IE = Ireland, UK = United Kingdom, EE = Estonia, AT = Austria, BE = Belgium, NL = The Netherlands, FR = France, DE = Germany, CH = Switzerland, LU = Luxembourg, HR = Croatia, CY = Cyprus, EL = Greece, IT = Italy, PT = Portugal, Sl = Slovenia, ES = Spain, MK = North Macedonia, CZ = Czech Republic, PL = Poland, SK = Slovakia, HU = Hungary.

values are needed (Poulsen et al., 1997). Therefore, to establish exposure values for specific subpopulations of the EU sample i.e. male vs. female, low vs. medium vs. high SES, residents of rural vs. urban vs. semi-urban areas, and residents of the 4 geographical regions, a minimum of measurements from 120 subjects per stratum is required. Since we want to be able to also compare male vs. female subjects within a region a minimum of 240 subjects (120 male and 120 female) per region is required. For Eastern Europe a region with at least 1 PSU this implies a minimum sample size of 240 subjects from the PSU. Because of EU co-funding availability the maximum contribution of samples per PSU was limited to 300. For PSU belonging to those regions with more than 1 PSU (N, S, W) an exception was granted to contribute with a reduced sample size per PSU given that the minimum of 240 is reached for their respective region (Fig. 1). The resulting sampling strategy of the joint HBM4EU survey is a compromise, in order to build maximally on already existing studies and expertise (Table 1).

2.5. Final study selection

In practice the selection of HBM studies to be included in the HBM4EU survey started from an inventory of HBM studies in Europe composed at the start of the HBM4EU project (Institute of Environmental Health, 2019). Then inclusion and exclusion criteria presented in Table 2 were applied to evaluate the fitness of the studies to be aligned/included in the HBM4EU survey. A first selection was made to keep only studies that: (i) fit the proposed time period and age groups, (ii) targeted general population, no residents of hotspots (for the prioritized chemicals), no patient groups, no institutionalized civilians or specific occupational groups, (iii) included both male and female study subjects with a 50:50 ratio and (iv) are representative on a national (country) level. Based on these basic criteria a first overview of potential contributing studies was made. In a next step it was checked if per age group all 4 geographical regions were represented with a number of PSU (countries) per region that is proportional to the number of inhabitants of the region. 2 PSU for Northern Europe, 3–4 PSU for Western Europe, 3 PSU for Southern Europe, at least 1 PSU for Eastern Europe. At this point there were gaps for each age group. Ideally countries need to contribute with a representative sample within their country. Unfortunately, few nationally representative studies were identified that were ongoing or planned in Europe. Although national representativeness is the gold standard, it was not feasible to initiate a large number of nationally representative studies within the scope of HBM4EU. Therefore, to fill the gap we considered to also include regional studies, provided that they fulfilled all other aforementioned inclusion criteria. In an ideal scenario we would also use SES and residential degree of urbanisation as inclusion criteria i.e. each level (low, medium, high educational level and thinly, medium and highly populated areas) at least 10% represented. However, the candidate HBM studies for inclusion were limited and therefore it was decided to consider those as nice to have but not to keep them as strict criteria as it would result in too few studies that could be included.

Table 2
Inclusion and exclusion criteria for participation in the joint HBM4EU survey.

Inclusion criteria
• Sampling period: samples collected between 2014 and 2020. • More specifically this may include: (i) Completed studies with available biobanked samples (ii) Studies that were initiated before the start of the HBM4EU project but sampling fell within the stipulated timeframe (iii) New studies, adopting the HBM4EU protocols • Study targets general population and includes both male and female subjects within the selected age groups (children 6-11y, teenagers 12-19y, young adults 20-39y) • Availability of a basic set of variables (see Supplementary Table 1) • Analyse HBM4EU priority substance groups as proposed per age group • Analysis performed in a laboratory that successfully passed the HBM4EU quality assurance quality control (QA/QC) programme • Sampling conditions and sample storage conditions are consistent with specific technical requirements as specified by the quality assurance unit of HBM4EU • Demonstrated ethical approval and permission to transfer the individual data for statistical analysis at EU level • Compliance with the HBM4EU data management plan and data policy
Exclusion criteria
• Residents of hotspot areas for 1st set HBM4EU priority chemicals (e.g. industrially contaminated sites or known historical contamination sites) • Patient populations • Institutionalized citizens • Targeted occupational groups

2.6. Selection of exposure biomarkers

During the planning of HBM4EU, eight chemical groups were prioritized for studying environmental exposure in the European population. This was based on a systematic input from European and national policy makers and scientists. The specific policy needs for each of these chemical groups were identified in a consultation round (Ougier et al., 2021). Recent and comparable HBM data for Europe were requested for non-persistent organic pollutants (phthalates and Hexamoll® DINCH, bisphenols, PAHs, organophosphorus flame retardants (OPFRs)) and persistent pollutants (PFAS, brominated flame retardants (BFRs) and cadmium). To reduce costs it was decided to not analyse all exposure biomarkers in all age groups, but to analyse specific exposures

in selected age groups taking into account (i) the potential exposure risk of the age groups, (ii) filling knowledge gaps and responding to policy questions, and (iii) interest of the participating countries.

The chemical exposures assessed in each age group are presented in Table 3. An expert group within HBM4EU selected the most relevant biomarkers and matrices (Vorkamp et al., 2021). Estimated exposure of children to BFRs and OPFRs via house dust and diet raises concern (Rantakokko et al., 2019; Van den Eede et al., 2011) therefore these analysis were prioritized in children in blood and urine respectively. Teenagers were selected as target group for PFAS exposure in blood because of their endocrine properties causing concern during puberty development (Terry et al., 2019). In addition, both children and teenagers were prioritized for urinary analysis of phthalates and Hexamoll® DINCH as EU wide information in this age group was lacking while modelled intake and HBM data from US and Germany suggest relatively high uptake in children (Li et al., 2019; Schwedler et al., 2020; Wang et al., 2019). In the adult age group exposure to cadmium, PAHs and bisphenols were prioritized in urine samples. Cadmium accumulates with age hence, the adults were selected as target population for analysis of cadmium. Exposure biomarkers of PAHs were measured in adults to obtain a comprehensive overview of internal exposure through air and dietary pathways. Exposure to bisphenols was measured in adults to cover a diversity of exposure pathways and vulnerability at reproductive age. Each country/HBM study could choose the age group in which they would participate. A summary of the targeted substances per PSU is shown in Table 3.

2.7. Standardization and harmonization of procedures for sample transport and chemical analysis

Despite national studies entering the project in different phases, harmonization and quality assurance of some key aspects was undertaken for all participating studies. Standard protocols for recruitment, sampling, questionnaire development and sample transport were developed within HBM4EU and made available online at the HBM4EU website for each of the study phases (https://www.hbm4eu.eu/deliverables/, Deliverable 7.3 and Deliverable 7.6 (for standard operating procedures (SOPs) and guidelines for recruitment, sampling and questionnaires, Deliverable 7.2 (for sample transport)) (Fiddicke et al., 2021).

Some studies already collected their samples in the period between 2014 and 2019, those samples were biobanked (frozen at min. storage temp of −20 °C preferably −80 °C) and made available to the project. Other studies were ongoing and could not adjust their protocols as they were already approved by an ethics committee, while some studies still needed to be initiated and could develop their protocols according to the HBM4EU guideline protocol.

Transport of samples to the laboratories followed the appropriate SOP, which was developed within the framework of the HBM4EU initiative (Lermen et al., 2020). Additionally, the comparability and quality of the biomarker measurements performed within the joint HBM4EU survey is controlled at the EU level. Briefly, the biomarkers must be analysed in laboratories that successfully participated in the

Table 3
Overview of first set HBM4EU priority substance groups covered in joint HBM4EU survey.

Substance group	Proposed analytes – included in QA/QC programme of HBM4EU	Age group	Sampled matrix	Country included in joint HBM4EU survey
Organophosphorus flame retardants	DPHP, BDCIPP, BCEP, BCIPP	children	urine	NO, DK, SK, Sl, FR, BE, DE
Brominated flame retardants	BDE-209, TBBPA, DBDPE, 2,4,6-TBP, BDE-47, BDE-153, DP-syn, DP-anti, α-HBCD, γ-HBCD	children	serum, plasma	NO, Sl, EL, FR
Phthalates	MEP, MBzP, MiBP, MnBP, MCHP, MnPeP, MEHP, 5OH-MEHP, 5oxo-MEHP, 5cx-MEPP, MnOP, OH-MiNP, cx-MiNP, OH-MiDP, cx-MiDP	children	urine	NO, DK, HU, SK*,PL, Sl, EL, IT, FR, DE*, NL, BE
		teenagers		NO, SE, SK,PL, CZ, Sl, EL, ES, FR, DE*, BE
Hexamoll® DINCH	OH-MINCH, cx-MINCH	children	urine	NO, DK, HU, SK,PL, Sl, EL, IT, FR, DE*, NL, BE
		teenagers		NO, SE, SK,PL, Sl, EL, ES, FR, DE*, BE
Per-/polyfluorinated compounds	PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFBS, PFHxS, PFHpS, PFOS (sum of all isomers)	teenagers	serum, plasma	NO, SE*, SK, Sl, EL, ES, FR*, DE, BE
Cadmium	Cd	adults	urine	DK, IS, PL, CZ, HR, PT, FR*, DE, LU
Polyaromatic hydrocarbons	1-naphthol, 2-naphthol, 1,2 DHN, 2-FLUO, 3-FLUO, 9-FLUO, 1-PHEN, 2-PHEN, 3-PHEN, 4-PHEN, 9-PHEN, 1-PYR, 3-BaP	adults	urine	DK, IS, PL, CZ, HR, PT, FR*, CH, DE, LU
Bisphenols	BPA, BPS, BPF	adults	urine	DK, IS, FI, PL, CZ, HR, PT, FR*, CH, LU, DE

* studies with data generated outside HBM4EU project i.e. PCB cohort (phthalates), ESTEBAN (PFAS in teenagers and PAH, bisphenols and cadmium in adults), Riksmaten Adolescents 2016–17 (PFAS), GerES V (phthalates and DINCH in children and teenagers, PFAS in teenagers). QA/QC = Quality assurance/Quality control, DK = Denmark, FI = Finland, SE = Sweden, IS = Iceland, NO = Norway, BE = Belgium, NL = The Netherlands, FR = France, DE = Germany, CH = Switzerland, LU = Luxembourg, HR = Croatia, EL = Greece, IT = Italy, PT = Portugal, Sl = Slovenia, ES = Spain, CZ = Czech Republic, PL = Poland, SK = Slovakia, HU = Hungary; MEP = Mono-ethyl phthalate, MBzP = Mono-benzyl phthalate, MiBP = Mono-isobutyl phthalate, MnBP = Mono-n-butyl phthalate, MCHP = Mono-cyclo-hexyl phthalate, MnPeP = Mono-n-pentyl phthalate, MEHP = Mono(2-ethylhexyl) phthalate, 5OH-MEHP = Mono(2-ethyl-5-hydroxyhexyl) phthalate, 5oxo-MEHP = Mono(2-ethyl-5-oxo-hexyl) phthalate, 5cx-MEPP = Mono(2-ethyl-5-carboxypentyl) phthalate, MnOP = Mono-n-octyl phthalate, OH-MiNP = 7-OH-(Mono-methyl-octyl) phthalate, cx-MiNP = 7-Carboxy-(mono-methylheptyl) phthalate, OH-MiDP = 6-OH-Mono-propyl-heptyl phthalate, cx-MiDP = Mono(2,7-methyl-7- carboxy-heptyl) phthalate, OH-MINCH = cyclohexane-1,2- dicarboxylate-mono-(7- hydroxy-4-methyl)octyl ester, cx-MINCH = cyclohexane-1,2- dicarboxylate-mono-(7- carboxylate-4- methyl) heptyl ester, DPHP = Diphenyl phosphate, BDCIPP = Bis(1,3-dichloro-2-propyl) phosphate, BCEP = Bis(2-chloroethyl) phosphate, BCIPP = bis(1-chloro-2-propyl) phosphate, BDE-209 = Polybrominated diphenylether 209, TBBPA = Tetrabromobisphenol A, DBDPE = Decabromodiphenylethane, 2,4,6-TBP = 2,4,6-Tribromophenol, BDE-47 = Polybrominated diphenylether 47, BDE-153 = Polybrominated diphenylether 153, DP-syn = Syn-dechlorane plus, DP-anti = Anti-dechlorane plus, α-HBCD = Hexabromocyclododecane alpha, γ-HBCD = Hexabromocyclododecane gamma, PFPeA = Perfluoropentanoic acid, PFHxA = Perfluorohexanoix acid, PFHpA = Perfluoroheptanoic acid, PFOA = Perfluorooctanoic acid, PFNA = Perfluorononanoic acid, PFDA = Perfluorodecanoic acid, PFUnDA = Perfluoroundecanoic acid, PFDoDA = Perfluorododecanoic acid, PFBS = Perfluorobutane sulfonic acid, PFHxS = Perfluorohexane sulfonic acid, PFHpS = Perfluoroheptane sulfonic acid, PFOS = Perfluorooctane sulfonic acid (sum of all isomers), cd = cadmium, BPA = Bisphenol A, BPS = Bisphenol S, BPF = Bisphenol F, 1-naphthol = 1-hydroxynaphthalene, 2-naphthol = 2-hydroxynaphthalene, 1,2 DHN = 1-,2-dihydroxynaphthalene, 2-FLUO = 2-hydroxyfluorene, 3-FLUO = 3-hydroxyfluorene, 9-FLUO = 9-hydroxyfluorene, 1-PHEN = 1-hydroxyphenanthrene, 2-PHEN = 2-hydroxyphenanthrene, 3-PHEN = 3-hydroxyphenanthrene, 4-PHEN = 4-hydroxyphenanthrene, 9-PHEN = 9- hydroxyphenanthrene, 1-PYR = 1-hydroxypyrene, 3-BaP = Benzo[a]pyrene.

interlaboratory comparison investigation/external quality assurance scheme (ICI/EQUAS) organized as part of the HBM4EU initiative which is described by Esteban et al. (Esteban López, 2021). Participating analytical laboratories had to participate in at least two proficiency tests. This quality assurance scheme safeguards the reliability and comparability of analytical results. Laboratories from all European countries could participate in the ICI/EQUAS programme. HBM4EU provided an opportunity to check and improve the analytical methods and facilitated capacity building in Europe. Results of the cadmium and FR ICI/EQUAS are described by Nübler et al. and Dvorakova et al. respectively (Nubler et al., 2021; Dvorakova et al., 2021). All data coming from the joint HBM4EU survey have a data quality label assigned. Compounds for which the analysing laboratory obtained successful results are labelled as "Biomarker data quality assured by HBM4EU QA/QC programme". Specific compounds for which the analyzing laboratory did not obtain successful results are labelled as "Biomarker data not quality assured by HBM4EU QA/QC programme". Some of the contributing studies had already analysed some of the selected substance groups outside HBM4EU context. Those studies, indicated in Table 3, provided the already available data. As no real retrospective assessment of the proficiency and comparability of the data is possible, the Quality Assurance Unit (QAU) could not consider these results at the same level as those obtained within the QA/QC programme and therefore, the QAU provided a recommendation on how to deal with these available data. Different labels were attributed to those data generated before HBM4EU to distinguish them from the data analysed under HBM4EU. If the data was analysed in laboratories that later on obtained successful results under the QA/QC programme and used the same method with continuous internal quality assurance the data are labelled "Biomarker data generated before HBM4EU QA/QC programme but deemed comparable", data generated by laboratories that did not participate or did not obtain successful results under the QA/QC programme are labelled "Biomarker data generated before HBM4EU QA/QC programme but not deemed comparable". For the derivation of European exposure values only data quality approved under HBM4EU will be included.

2.8. Post-harmonization of questionnaire data

All participating studies were requested to collect information on socio-demographics and on substance specific environmental- and lifestyle-related exposure sources and exposure routes through questionnaires. The method applied to conduct the questionnaires differed between the individual studies (e.g. telephone assisted interview, paper questionnaires, …). New or ongoing studies that were still in the planning phase were requested to adapt their questionnaires to the standard HBM4EU questionnaire. Since the joint HBM4EU survey aligned both new/ongoing studies and recently conducted studies, a post-harmonization approach was applied to harmonize the collected questionnaire data. A specific expert working group provided advise on the harmonization rules for each variable based on prior experience in the process of post-harmonizing variables from the OBELIX (OBesogenic Endocrine disrupting chemicals: LInking prenatal eXposure to the development of obesity later in life) (Legler et al., 2011) and HELIX (Human Early-Life Exposome) project (Maitre et al., 2018). All the studies report their individual data in a similar way using a harmonized HBM4EU codebook which was developed centrally and is available online at the HBM4EU website (https://www.hbm4eu.eu/online-library/).

2.9. Exchange of personal HBM data on EU level conform the general data protection regulation

Since human biomonitoring data is considered as sensitive personal data, the collection and exchange of these type of data are subject to the general data protection regulation (GDPR, Regulation (EU) 2016/679) as of May 2018. Each of the participating studies of the HBM4EU survey collected personal characteristics, socio-demographic and lifestyle information of their study participants and the individual exposure biomarker levels. Subsequently, the data were transferred to a central database in encrypted format through an established HBM4EU web-portal to ensure GDPR compliant exchange of individual personal data. The data is subjected to an extensive quality control process, checking for consistency between the provided variables and ensuring the data is correctly harmonized according to the HBM4EU codebook. After the quality control the individual data are integrated into a central database, hosted at the Flemish Institute for Technological Research NV (VITO), to create a pooled EU wide dataset per age group. Consequently, the data are processed to create additional variables in a uniform way such as the calculation of creatinine or specific gravity corrected biomarker values, imputed values for measurements below the limit of detection or quantification or calculation of sum parameters for biomarkers belonging to the same parent compound.

The role and responsibilities of VITO as a Data Processor (for the hosting activity) are stated in a bilateral processing agreement. Access to the individual data within the HBM4EU consortium is regulated via a single collaboration agreement i.e. joint data controller agreement between all supplying data controllers and receiving data controllers (Supplementary Figure 2). Access to the single measurement data is controlled at the individual user level. Users are provided with encrypted extracts of the database via the HBM4EU webportal, that only contain those variables required for the research question defined by the user in order to adhere to the data minimization principle laid down by the GDPR (Art5) (European Commission, 2016). In addition, sample and sample associated data exchange were covered with data and material transfer agreements signed by the responsible institutions.

2.10. Joint statistical analysis at EU level

Statistical analysis plans (SAPs) were developed describing the statistical approach that will be applied on the pooled European datasets. Within HBM4EU the following research objectives were defined: derivation of European reference values of internal exposure, comparing internal exposure levels between geographical regions, examination of determinants of internal exposure (personal characteristics, exposure sources, exposure routes), associating exposure biomarkers with personal health data and linking these associations through effect markers by causal pathway analysis. The use of the data for the research purposes described in the SAPs were formulated as part of the collaboration agreement established between all parties involved in the data exchange (i.e. supplying and receiving data controllers). For testing additional research hypotheses, a request should be submitted to the supplying data controllers, and bilateral data controller agreements should be established.

The joint HBM4EU survey builds further on existing capacity and expertise of human biomonitoring programs. The individual data collections are not perfectly homogeneous. There are differences in sampling year, season and for some main characteristics like age, sex and educational level. Moreover, not all HBM data are representative for their country. This has implications for the derivation of European reference values and the geographical comparison of the exposure levels between regions. To provide an estimate of the internal exposure of the European population for a specific age group, we will calculate internal exposure values for each exposure biomarker from the HBM4EU population sample as it is recruited. We will refer to these values as 'European exposure values' for internal exposure of the HBM4EU population. To obtain European exposure values for internal exposure the weighted geometric mean and 95th percentile (P95), and their 95% confidence interval will be calculated for each exposure biomarker. The population that has been sampled in the joint HBM4EU survey will be clearly described for characteristics that are known to influence exposure levels, i.e. age, sex, sampling year, sampling season, smoking habit and

educational level. Descriptive characteristics will be presented for these parameters per participating data collection, per geographical region, and for the total population recruited. These main characteristics will be compared with Eurostat reference tables for the included countries and EU population to document observed differences between the HBM4EU sampled population and European population of the same age group (EUROSTAT, 2021). This information will allow comparison of the biomarker exposure values in HBM4EU with biomarker exposure values obtained in future monitoring campaigns in Europe or international monitoring programs, taking into account the characteristics of the sampled populations.

The joint HBM4EU survey results from a complex, stratified, multistage design survey. Due to that, data should be analysed using survey procedures that account for the complex survey design when calculating variance estimates. For the calculation of European exposure values, sample weights will be used to ensure that all geographical regions contribute proportionally to the number of inhabitants: North = 21%, East = 11%, South = 28% and West 40%. European exposure values will be calculated for the common set of analytically qualified exposure biomarkers (and sum-parameters) obtained in the contributing studies within one age group (i.e. children, teenagers, adults). For urinary biomarkers, geometric means and P95s will be calculated in μg/L and in μg/g creatinine, and μg/L corrected for specific gravity (SG) (only for teenagers); for lipid soluble blood biomarkers, geometric means and P95 will be calculated in μg/L and in μg/g lipid. The imputed biomarker data will be used, geometric means will only be estimated if at least 60% of the biomarker values are above the detection limits. For each biomarker, results will be given for the total population, and stratified by sex, educational level, degree of urbanisation and European region.

To formally test for geographical differences survey models will be fitted. Multiple linear regression models for the ln-transformed biomarkers will be built testing the fixed effect of EU region on the estimated geometric means, and quantile regression will be used to model the P95. Again, sample weights will be used to ensure that all geographical regions contribute proportionally to the number of inhabitants. As the samples of each PSU differ in population characteristics like age, sex, educational level, sampling year(s), sampling season, sampling type, etc., the models will be adjusted for those covariates that could influence the observed exposure values. Attention should be paid only to adjust for some basic covariates, and not for influencing factors that could possibly explain the differences observed between the regions. As a first step we will check if there are differences observed for the above mentioned covariates on the exposure biomarkers by regression models, per PSU and on the EU pooled database. If so, the models will be adjusted for these covariates. The overall p-value of region in the final model indicates if there is any significant difference observed for the estimated geometric mean/P95 levels of a biomarker between the EU regions. If the null hypothesis is not rejected (i.e. there were no significant differences between the regions at the 5% significance level), no further testing will be done. If the null hypothesis is rejected, further testing will be done comparing the geometric mean/P95 in each geographical region with the other geographical regions (all pairwise comparisons).

For the research questions on exposure determinants, associations between exposure and health effect, and pathway analysis, it will be evaluated whether pooled analysis of the data is suited, or if it is better to apply meta-analysis by first looking into the data per PSU and afterwards trying to combine the effect estimates. Regression models will be applied. Generalised additive models (GAMs) will be used to visualize the shape of the relationship between the exposure biomarkers and health effects. A directed acyclic graph (DAG) will be adopted for each exposure-effect association as a visual aid to check for relevant confounders and for completeness of the model. Mediation analysis will be considered to assess if a potential exposure-health effect association may be mediated by the selected molecular/clinical effect biomarkers.

2.11. Communication strategy

As HBM4EU is a collaborative research effort, at the science and policy interface, involving both the national- and the EU-level, respect for each other's role and needs is crucial, especially when communicating results. Communication of results to participants, and communication of country level results fall under the responsibility of the principle investigators of the contributing studies. For the interpretation and public communication of results on EU level a step-wise approach was developed. Key principles of the approach are: (i) results cannot be withheld or influenced, (ii) all actors involved must be informed in time and depending on their role have a say in the way results are communicated, (iii) national authorities are informed before public communication of results and (iv) respecting terms and conditions of national studies feeding their data into HBM4EU. The communication strategy should ensure that results and main key messages are supported by all partners which in turn can facilitate broad dissemination of results.

2.12. Ethics and data protection

All studies of the joint HBM4EU survey followed national and European ethics regulation. They all acquired approval from their country's ethics committees. Participation in all studies is voluntary, written informed consent was obtained from all participants and withdrawal from the study is possible at any time. Each study also confirmed that informed consent and approval were in place for secondary use of the collected data. New biomarker analysis in the frame of HBM4EU was covered by renewed ethics approvals in each country if the original approval did not cover surplus analyses. The project developed an inventory of all ethics and data protection approvals to ensure full compliance with EU requirements and also installed an Ethics Board. The data provider ensured legal and GDPR compliant use of data in pseudonymised format.

3. Results: a joint HBM4EU survey

The joint HBM4EU survey builds on the existing HBM capacity in Europe bringing together ongoing studies, that collected new samples and recently conducted studies, that provided biobanked samples. Because of these different scenarios of the participating HBM initiatives it was not feasible to implement a rigid scheme of mandatory protocols that would fit all participating countries. Instead the studies were aligned and post-harmonized as much as possible, to limit the effects on heterogeneity of the data, whilst respecting the countries individualities. Participating studies that were still in the planning/start-up phase were encouraged and supported to adhere as closely as possible to the recommendations for conducting a study developed in the frame of HBM4EU (HBM4EU D7.3 and D7.6) (Fiddicke et al., 2021).

3.1. Studies participating in the joint HBM4EU survey

An overview of the studies participating in the joint HBM survey is presented in Fig. 1. In total 34 studies from 21 countries took part in this initiative. Each participating study contributed with a maximum of 300 samples per age group. With the exception of a few studies that participated with a reduced number of subjects.

Under the joint HBM4EU survey 12 studies from 12 different countries targeting children between 6 and 11 years of age were brought

together. For Northern Europe, NEBII (Norwegian Environmental Biobank II; Norway) and OCC (Odense Child Cohort; Denmark) are included. For Eastern Europe, InAirQ (Transnational Adaption Actions for Integrated Indoor Air Quality Management; Hungary), PCB cohort (Endocrine disruptors and health in children and teenagers in Slovakia; Slovakia) and POLAES (Polish Aligned Environmental Study; Poland) are included. For Southern Europe SLO CRP (Exposure of children and adolescents to selected chemicals through their habitat environment; Slovenia), CROME (Cross-Mediterranean Environment and Health Network; Greece) and NACII (Northern Adriatic cohort II; Italy) and for Western Europe, ESTEBAN (Étude de santé sur l'environnement, la biosurveillance, l'activité physique et la nutrition; France), GerES V-sub, unweighted (German Environmental Survey 2014-2017 subsample; Germany), 3xG (Gezondheid, Gemeenten, Geboorte studie; Belgium) and SPECIMEn-NL (Survey on PEstiCide Mixtures in Europe, The Netherlands) are included. Together they result in a total study population of 3151 children distributed across Europe (Fig. 1A).

Another 11 studies targeted teenagers between 12 and 19 years of age. For Northern Europe NEBII (Norwegian Environmental Biobank II; Norway) and Riksmaten Adolescents 2016–17 (Sweden) are included. For Eastern Europe, POLAES (Polish Aligned Environmental Study; Poland), CELSPAC: Teenagers (Central European Longitudinal Studies of Parents and Children: Teenagers; Czech Republic) (Piler et al., 2017) and PCB cohort follow-up (Endocrine disruptors and health in children and teenagers in Slovakia; Slovakia) take part in the survey. For Southern Europe, SLO CRP (Exposure of children and adolescents to selected chemicals through their habitat environment; Slovenia), CROME (Cross-Mediterranean Environment and Health Network; Greece) and BEA (Biomonitorización en Adolescentes; Spain) are included and for Western Europe, ESTEBAN (Étude de santé sur l'environnement, la biosurveillance, l'activité physique et la nutrition; France), GerES V-sub, unweighted (German Environmental Survey 2014-2017 subsample; Germany) and FLEHS IV (Flemish Environment and Health Survey IV; Belgium) participate. This results in a total population of 2953 teenagers distributed across Europe (Fig. 1B).

In addition, the following 11 studies are targeting adults between 20 and 39 years of age. CPHMINIPUB/DYMS (Copenhagen Minipuberty study (parents)/Danish Young Men Study; Denmark), Diet_HBM (Icelandic National Dietary Survey; Iceland), and FinHealth (Finland) are included to represent Northern Europe. (C)ELSPAC: YA (Central European Longitudinal Studies of Parents and Children: Young Adults; Czech Republic) and POLAES (Polish Aligned Environmental Study; Poland) represent Eastern Europe. INSEF-ExpoQuim (Exposure of the Portuguese Population to Environmental Chemicals: a study nested in INSEF, 2015; Portugal) and HBM survey in adults in Croatia (Implementation of Human Biomonitoring Survey In Adults in Croatia Using HBM4EU Methodology; Croatia) are included to represent Southern Europe and ESTEBAN (Étude de santé sur l'environnement, la biosurveillance, l'activité physique et la nutrition; France), ESB (Environmental Specimen Bank; Germany), HBM4EU-study Switzerland (Human Biomonitoring for Europe Program for Switzerland; Switzerland) and Oriscav-Lux2 (Observation des Risques et de la Santé Cardiovasculaire au Luxembourg; Luxembourg) are included to represent Western Europe. Together these studies result in a total adult population of 3389 subjects distributed across Europe (Fig. 1C).

Across all three age groups, a total of 9493 participants were recruited from 21 European countries covering a time period from 2014 to 2020. In these subjects exposure to HBM4EU priority chemicals are assessed. Aligning HBM studies across Europe is a dynamic process influenced by many factors. As a result, some studies deviate to some extent from the proposed age ranges or time period. Due to unexpected delays in the execution of the study, part of the samples collected in Greece for children and teenagers were sampled in 2021, and for PCB cohort from Slovakia children were recruited at age 11 but turned 12 by the time actual sampling could take place. It was decided to accept these exceptional deviations and to not discard those samples and data from the joint HBM4EU survey.

4. Discussion

The project's main goal is to coordinate and advance human biomonitoring in Europe to provide evidence for chemical policy making and to supply data to inform the EU citizens about their exposure to chemicals and associated risks (Ganzleben et al., 2017). The importance of this work is highlighted by the announcement of the EU green deal and the European Commission's new chemicals strategy for a toxic-free environment (European Commission, 2020). Reliable and comparable HBM exposure data representative of the EU population is indispensable to feed into chemical risk assessment and support chemical policy making. HBM4EU aims to lay the foundation for a European HBM platform that serves as a framework for sustainable and harmonized HBM conduct in Europe. The resulting data of this joint HBM4EU survey provides a baseline for chemical exposure of EU citizens to evaluate existing and upcoming chemicals policies. From the perspective of establishing a sustainable framework, it was decided to adopt an approach that builds further on existing capacity in Europe, whilst improving comparability of the data. This has several advantages, but also brings challenges. By choosing this approach it was not feasible to implement a rigid scheme of mandatory protocols. Instead a sampling framework was developed that facilitates the integration of existing HBM initiatives in Europe, bringing them together in a centrally coordinated joint HBM4EU survey and aligning them to serve common goals on the EU level.

4.1. Improving comparability of HBM data across europe

When comparing and interpreting biomonitoring data several aspects should be carefully considered such as (i) overall study design including target population and sampling period, (ii) sample collection, handling, storage and transport (iii) chemical analysis and (iv) data handling and presentation. Most differences between the studies aligned under the joint HBM4EU survey originate from the early/initial stages of study conduct. Thereafter, all participating studies followed the same HBM4EU procedures from the point of transport of samples to conclusion.

There are many HBM data available within Europe. However, the data are often disparate in terms of sample collection period (years), biological matrices and targeted population (age and sex of participants). Smolders et al. (2010) did a case-study as part of the INTARESE project where they collected individual HBM data on blood-lead exposure from more than 20.000 subjects from 8 European countries to evaluate the comparability of the data. They found that it is difficult to use disparate data collections because of the inherent variability with respect to the sex and age of participants and the time period (years) of sample collection. They highlighted that the need to get data from comparable (sub-)populations is essential for appropriate use and interpretation of HBM data for environmental health impact assessment (Smolders et al., 2010). By integrating studies into the joint HBM4EU survey that fit the sampling frame and fulfill the inclusion and exclusion criteria, as defined in Table 1, we improved harmonization of those key aspects across all contributing studies. The selected age groups are also aligned with the age groups as defined in NHANES and CHMS which can facilitate international comparison of EU results. The current HBM capacity in Europe did not allow to select a more restricted time period for

the joint HBM4EU survey, therefore it was decided to include samples over a 7 year period (2014–2020). Towards future HBM surveys on European scale a more restricted time period aligned to international HBM cycles such as NHANES, CHMS should be strived for.

The present survey covers all 4 geographical areas of Europe with a minimum participation of 11 PSU (countries) per selected age group. Across all age groups we have 21 out of 31 different EU countries contributing to this study. Hence there is a significant EU appetite for such a work programme, especially considering countries had to secure 50% of the study budget via national funding channels. Aligning EU and national priorities in terms of target population and substances of interests are a key prerequisite for the success of this funding scheme. Collecting EU wide HBM data was feasible by including national representative as well as regional HBM initiatives. Geographical coverage was ensured by including PSUs from different geographical regions proportional to the % of EU inhabitants living in each region. When investigating and documenting HBM initiatives in Europe, eligible for inclusion into the joint HBM4EU survey, it became clear that HBM surveys with national representativity are scarce.

Moreover, different sample types are being used to assess pollutant levels in HBM initiatives. Longnecker et al. (2003) compared polychlorinated biphenyl (PCBs) levels across studies and pointed out that the use of different specimen types for analysis (i.e. serum, breast milk) complicates the comparability of HBM data (Longnecker et al., 2003). In the joint HBM4EU survey, the selected priority chemicals are all assessed in the same biological specimen (blood or urine) across the individual participating studies. The different PSU do have a mixture of blood-based matrices (serum, plasma) collected. Ehresman et al. demonstrated a 1:1 serum to plasma ratio for PFHS, PFOS, and PFOA (Ehresman et al., 2007). Given the lipophilic character of brominated flame retardants, they are associated with blood lipids, not with blood proteins, therefore results in serum and plasma are correlated, as blood lipid concentrations in serum/plasma are similar (Cholesterol and triglycer, 1977). The present study collects information of 60 exposure biomarkers from six chemical substance groups in urine and blood samples. When searching available HBM data different studies have often analysed a different set of biomarkers within a chemical substance group. Within the joint HBM survey we aimed to analyse a selected set of biomarkers for each substance group. Unfortunately, we could not identify a minimal set of biomarkers that could be analysed by all laboratories except for urinary cadmium and Hexamoll® DINCH metabolites (OH-MINCH and cx-MINCH). This is partly because the laboratories could choose for which metabolites they took part in the QA/QC programme and because not all laboratories had the capacity to measure all metabolites included in the HBM4EU QA/QC programme (Table 3). An important aspect of the joint HBM survey is to make full use of the expertise and experience available within the individual countries and to share this with each other. On the other hand, a major aim is to produce new results not only for the well-known markers but also for novel biomarkers. Balancing these 2 priorities, capacity building on one hand and project's ambition on the other hand, means we need to make compromises. To generate reliable biomonitoring data and reduce interlaboratory variability, samples for the joint HBM4EU survey were analysed in HBM4EU QA/QC laboratories, that successfully participated in the internal proficiency testing, only.

4.2. Establishing a sustainable HBM platform for europe built on existing capacity

By making use of existing HBM capacity within Europe, a bottom-up rather than top-down approach is used in developing a joint HBM4EU survey. Including existing HBM initiatives can ensure a more sustainable engagement of member states. Countries with a stronger tradition in HBM that have regular HBM campaigns can integrate and align their HBM initiative on EU level. They can continue their research activities and pursue goals on national level whilst expanding the study to meet specific objectives on EU level. This facilitates the comparison of their country results with EU results. The HBM4EU project provides the opportunity for countries without strong HBM tradition to initiate a new HBM study that fulfilled the inclusion criteria. They can make use of the HBM4EU developed guidelines and support of HBM experts. This is both time and budget wise a more efficient approach than initiating a completely new EU level HBM study as was done in the DEMOCOPHES project (Schindler et al., 2014). Moreover, this approach allows for the integration of HBM into existing Health Examination Surveys (HES) such as in Portugal (INSEF-ExpoQuim) or into nutritional surveys such as in Sweden (Riksmaten Adolescents, 2016–17) and Iceland (Diet_HBM) which can also be a cost effective method of implementing HBM (Moraeus et al., 2018).

As a result, the joint HBM4EU survey aligns a combination of (i) conducted studies with available biobanked samples, (ii) ongoing studies and (iii) studies in the planning phase. This poses some challenges: limited opportunities for upfront alignment and post-harmonization of the questionnaires is required. This is a very time-consuming process and has its limitations. Basic variables, required for each study participant, as listed in Supplementary Table 1 can be harmonized across all studies. More specific information e.g. consumption of canned food could not be harmonized for all studies since the information was not available in all studies. This is because the studies developed their questionnaire with the original study objectives in mind. When additional objectives such as analysing exposure to additional substance groups are set at a later stage, the questionnaires lack those more specific questions related to these new chemical exposures. This limits the possibilities for data analysis when studying for example, exposure determinants.

In addition, as a consequence of working with this combination of studies, the timelines of the different studies are not well synchronized at EU level. Getting results delivered by a common deadline is much more complicated when working with existing HBM initiatives as they all have their own priorities, timing and procedures in releasing/communicating results.

To date there are few EU countries that have a regular HBM survey at national level that can benefit from structured research funding. Most EU countries depend on non-recurrent project based funding for their HBM activities which complicates the development of a structured HBM monitoring system for Europe. Regular monitoring cycles every 2–3 years would be beneficial for evaluating time trends. EU support can help to boost HBM research in the member states. However, continued efforts are required to put HBM on the agenda of the EU member states, to ensure sustainable funding in order to safeguard success at both the national and EU level in providing current human exposure data to better protect citizens and the environment.

5. Conclusions

Although there are many HBM initiatives in Europe a harmonized, coordinated and sustainable European approach is currently lacking. There are several good practices of national HBM programs in Europe but they are aimed at national interests in terms of (sub-)populations included and measured biomarkers. However, as the responsibility for chemical policy lays at the EU level, national initiatives are less efficient to protect the respective citizens against chemical risks without cooperation at EU level. No overarching strategy exists, studies are not harmonized and aligned to common goals on EU level. To improve the

comparability of European HBM data in support of European and national environment and health policy and measures, ideally European studies should follow similar protocols. Within the HBM4EU project it was one of the priorities to combine EU and national interests and build on existing HBM knowledge and capacities present in the member states rather than initiating new HBM studies as was done in the COPHES/DEMOCOPHES project (Schindler et al., 2014). Therefore, within the joint HBM4EU survey ongoing European HBM initiatives are aligned and harmonized to determine internal exposure levels to priority chemicals in the European population. The HBM4EU project and in particular the joint HBM4EU survey provides a platform to exchange best practices and improve and align HBM in Europe including data exchange and analysis. It also enables additional aspects to be included in this framework such as exploring the added value of effect biomarkers and measuring additional exposure biomarkers in the same sampling frame. The newly collected HBM data can also be used to improve and validate exposure modelling. Several aspects such as target population (s), biomarker analysis, data handling, statistical analysis, transparency on ethics requirements and protocols for reporting of results are aligned thereby improving inter-study/inter-country comparability of the data and acceptability of the results by policy makers. Furthermore, this joint survey might convince national authorities to further expand human biomonitoring within their country from regional level to national level. In conclusion, with this first large scale joint HBM4EU survey ongoing HBM initiatives in Europe are aligned and harmonized in an effort to take the first steps towards a sustainable European HBM platform in support of environment and health chemicals policy. Via the current approach we sampled 9493 participants across three age groups, and a total of 60 individual biomarkers have been assessed in subsets of this population, the resulting data will provide a baseline for chemical exposure of EU citizens to evaluate existing and upcoming chemicals policies.

Declaration of competing interest

The Authors declare that there is no conflict of interest. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

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Appendix A. Supplementary data

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Internal exposure to perfluoroalkyl substances (PFAS) in vegans and omnivores

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ABSTRACT

Perfluoroalkyl substances (PFAS) are a complex group of anthropogenic compounds with exceptional properties. Due to their high persistence and mobility, they have caused ubiquitous environmental contamination and in part accumulate in the food chain. In the general population, diet is the main source of PFAS exposure, with the important sources fish and meat. As a vegan diet implies the complete exclusion of any animal products, it might be expected that vegans have lower blood levels of PFAS compared to omnivores. Furthermore, lower levels of cholesterol is one of the well-documented nutritional effects in vegans, but cholesterol levels were also found to be associated with higher PFAS levels in epidemiological studies.

To examine the relations of internal PFAS levels and the levels of cholesterol in vegans and omnivores, the cross-sectional "Risks and Benefits of a Vegan Diet" (RBVD) study was used involving 36 vegans and 36 omnivores from Berlin/Germany. Nine perfluoroalkyl substances were quantified in plasma using a triple-stage quadrupole mass spectrometer.

Lower median plasma concentrations were found in vegans compared to omnivores for perfluorooctane sulfonic acid (PFOS) (2.31 vs. 3.57 ng/ml, respectively; $p = 0.02$) and for perfluorononanoic acid (PFNA) (<0.25 vs. 0.41 ng/ml, respectively; $p < 0.0001$). No significant differences of the median concentrations were observed for perfluorooctanoic acid (PFOA) (1.69 vs. 1.44 ng/ml, respectively, $p = 0.26$) and perfluorohexane sulfonic acid (PFHxS) (1.96 vs. 1.79 ng/ml, respectively; $p = 0.70$). The strongest correlations with food groups, derived from a food frequency questionnaire, were observed between levels of PFOA and water consumption (in case of the total study population, $n = 72$), and between levels of PFOS as well as PFNA and the consumption of 'meat and meat products' (in case of the omnivores, $n = 36$). Levels of Low Density Lipoprotein (LDL) cholesterol were confirmed to be considerably lower in vegans compared to omnivores (86.5 vs. 115.5 mg/dl, respectively; $p = 0.001$), but no associations between the four main PFAS and LDL cholesterol were observed (all $p > 0.05$) at the low exposure level of this study.

According to the results of our study, a vegan diet may be related to lower PFAS levels in plasma. We highlight the importance of the adjustment of dietary factors like a vegan diet in case of epidemiological studies dealing with the impact of PFAS on the levels of blood lipids.

1. Introduction

Perfluoroalkyl substances (PFAS) are a complex group of man-made chemicals composed of a fluorinated carbon backbone of varying length, primarily terminated by a carboxylate (perfluoroalkyl carboxylic acids, PFCAs) or a sulfonate (perfluorooctane sulfonic acids, PFSAs) as

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functional group. The combination of the polar and non-polar structure makes PFAS 'amphiphilic' providing water and oil repellency, and the strength of their carbon-fluorine bonds results in extremely high chemical and thermal stability. Since decades, the compounds have been used for the production of many consumer products like nonstick cookware, breathable textiles or protective coatings for paper, food packing materials, and carpets. From these everyday objects, PFAS are released and have been found – due to their high persistence and mobility – to cause ubiquitous environmental contamination and in part to accumulate in the food chain (Sunderland et al., 2019).

Consumption of food and drinking water is the main route of background exposure in humans. Internal exposure to PFAS in individuals can easily be determined by an analysis of serum or plasma. Four compounds, namely the PFCAs perfluorooctanoic acid (PFOA) and perfluorononanoic acid (PFNA), and the PFSAs perfluorooctane sulfonic acid (PFOS) and perfluorohexane sulfonic acid (PFHxS), typically represent more than 90% of detectable PFAS in serum/plasma of adults in industrialized countries. In the European adult population, median concentrations of PFOS, PFOA, PFHxS, and PFNA were found to be 7.7, 1.9, 0.67 and 0.61 ng/ml, respectively, based on studies from 2007/2008 and onwards (EFSA Panel on Contaminants in the Food Chain, 2020). This pattern results from the occurrence in food and drinking water on the one hand, and from accumulation due to half-lives up to several years in humans on the other hand. The latter is due to missing metabolic degradation and low urinary excretion (EFSA Panel on Contaminants in the Food Chain, 2020).

According to the recent evaluation of the European Food Safety Authority (EFSA), 'Fish and other seafood' was the most important contributor to the mean Lower Bound (LB) exposure in case of PFOS and PFOA, followed by 'Eggs and egg products', 'Meat and meat products', and 'Fruit and fruit products' (EFSA Panel on Contaminants in the Food Chain, 2020). Therefore, internal background exposure to these two substances in humans can be expected to be influenced by their dietary habits. Over the last years, plant-based diets have become increasingly popular in Germany and many other western countries, not merely due to increasing awareness of suffering animals or environmental problems, but also because of expected health benefits (Janssen et al., 2016). As a vegan diet implies the complete exclusion of any animal products, it might be expected that vegans have lower blood levels of PFAS compared to omnivores. Studies on this issue are yet missing. Therefore, the first aim of this investigation was to compare the internal PFAS exposure of German vegans and omnivores. For this purpose, PFAS was analyzed in samples of the 'Risks and Benefits of a Vegan Diet' (RBVD) study in 36 vegans and 36 omnivores aged 30–60 years (Menzel et al., 2020, 2021; Weikert et al., 2020).

While a broad spectrum of toxic effects of different PFAS was observed in experimental animals primarily at higher doses, epidemiological studies conducted in recent years revealed associations of certain biological parameters and levels of PFAS in serum/plasma even in the higher background range (EFSA Panel on Contaminants in the Food Chain, 2020). Using data of reduced formation of vaccine antibodies in one-year old children (Abraham et al., 2020), EFSA derived a tolerable weekly intake (TWI) of 4.4 ng/kg body weight for the sum of PFOS, PFOA, PFHxS and PFNA. According to the modelling of EFSA, such an intake corresponds to an internal level of 6.9 ng/ml for the sum of these four PFAS in women at the age of 35 years (EFSA Panel on Contaminants in the Food Chain, 2020).

Regarding possible changes of lipid metabolism, positive associations have been observed especially between high background levels of PFOS/PFOA and levels of Low Density Lipoprotein (LDL) cholesterol (Frisbee et al., 2010; Steenland et al., 2009). In this context, a vegan diet may be an undervalued confounding factor: An on average lower level of LDL cholesterol is one of the well-documented nutritional effects in vegans compared to omnivores (Yokoyama et al., 2017), resulting from the missing intake of animals fats. As outlined above, vegans may concurrently have lower external and internal PFAS exposure, resulting from missing intake of foods of animal origin with relatively high PFAS content. Therefore, the second aim of this investigation was to compare the impact of internal PFAS exposure and of a vegan diet on blood lipid levels, especially with regard on levels of LDL cholesterol in the RBVD study.

2. Methods

2.1. Study population

Participants of the present RBVD study were recruited by announcement (flyer) in (organic/vegan) supermarkets and investigated between January 2017 and July 2017 at the German Federal Institute for Risk Assessment (BfR) in Berlin (Weikert et al., 2020). A phone screening was performed including a brief explanation of the study and checking inclusion criteria (age 30–60 years, following the diet at least one year) and exclusion criteria (BMI $\geq$30, cardiovascular disease, type 2 diabetes, cancer, pregnancy, breastfeeding, current infection). Hypercholesterolemia and taking lipid-lowering medications were no study exclusion criteria. The final study population comprises 36 vegans and 36 omnivores, who were matched by sex and age. In the present study, an omnivorous diet was defined as the consumption of at least three portions of meat per week or two portions of meat and two portions of processed meat (e.g. cold cuts, sausages) per week, whereas a vegan diet was defined as no consumption of any animal food products. Each participant visited the study center twice - on their first visit, participants gave their written informed consent, received instructions to document their diet, and got material to collect urine. At the second visit, a fasting blood sample was collected, anthropometric measurements were performed and lifestyle characteristics as well as a food frequency questionnaire were assessed. The time span was on average 2 weeks between the two visits in the study center (minimum 1 week to maximum 4 weeks). The study was approved by the Ethics Committee of Charité – Universitätsmedizin Berlin (No. EA4/121/16) and was conducted in accordance with the Declaration of Helsinki.

2.2. Assessment of lifestyle characteristics

Anthropometric measurements, i.e. weight, height, and waist circumference, were taken by trained and quality-monitored personnel on participants wearing only light underwear. Body weight was assessed by an electronic digital scale (Omron BF511, Omron Healthcare Ltd., Kyoto, Japan) and the height was measured using a flexible anthropometer (SECA 213, Hamburg, Germany). Waist circumference was defined as in the horizontal plane midway between the lowest ribs and the iliac crest. Information on physical activity, educational level and smoking status was assessed by computer-based questionnaires. In the RBVD study the physical activity has been determined by a physical activity questionnaire used in the European Prospective Investigation into Cancer and Nutrition (EPIC)-study, provided by the Human Study Center of the German Institute of Human Nutrition Potsdam-Rehbruecke (InterAct et al., 2012). Physical activity contains the sum of average hours in summer and winter per week spent on cycling, sports and gardening. Walking comprises the sum of average hours per week during summer and winter. Further, occupational activity was assessed in the RBVD study. The validated EPIC-Potsdam Study food frequency questionnaire (FFQ) collects semi-quantitatively for each food item information on the usual portion size and the average frequency of intake of 102 food items during the past 12 months (Nothlings et al., 2007). Portion size for each item was estimated via image of different portion sizes or with standard portion sizes e.g. a cup (150 ml). Food groups were derived from the FFQs and available as g/d. Individual food groups were summed up to derive food groups, as a basis serves the classification of EFSA: 'Fruits', 'Vegetables (including fungi)', 'Starchy roots and tubers', 'Waters', 'Grains and grain based products', 'Meat and meat products', 'Fish and other seafood' and 'Eggs' (supplemental Table 1).

2.3. Blood collection and laboratory analysis

About 60 ml of venous blood was collected from fasting participants at the BfR study center.

The accredited medical laboratory (Labor 28 GmbH, Berlin, Germany) measured routine biomarkers including plasma concentrations of total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides on the same day. Plasma samples used for PFAS assessment were stored at −80 °C in freezers until time of analysis. PFAS were measured at the Bavarian Health and Food Safety Authority. The following compounds were analyzed using 100 µl plasma: perfluorodecanoate (PFDA), PFNA, PFOA, perfluorohexanoic acid (PFHxA), PFOS, PFHxS, perfluorobutane sulfonate (PFBS), perfluorododecanoate (PFDoDA), and 3H-perfluoro-3-[(3-methoxy-propoxy) propanoate] (ADONA). Sample preparation, analysis and quality criteria have been previously described in detail by Mosch et al. (2010). In brief, an online extraction LC-MS/MS system was used, and the compounds were quantified with a triple-stage quadrupole mass spectrometer (API 5500 QTRAP™ Applied Biosystems, Darmstadt, Germany) equipped with a TurboIonSpray® interface. The perfluorinated substances and the corresponding isotope-labeled internal standards were purchased from Wellington Laboratories (Ontario, Canada). Limit of quantification (LOQ) in plasma was 0.25 ng/ml, based on a tenfold peak-to-noise ratio. Values below the LOQ were assigned the half value. The sum of PFAS was defined including PFOS, PFOA, PFHxS and PFNA ('PFAS sum'). Accuracy of the analysis was ensured by External Quality Assurance Schemes (EQUAS) for PFOS and PFOA (http://www.g-equas.de/).

2.4. Statistics

Normally distributed variables were reported as mean and standard derivation (SD). Skewed variables were reported as median and interquartile range (IQR). Categorical variables were reported as percentage. A Student's *t*-test or Mann-Whitney *U* test was used to compare continuous variables between vegans and omnivores, and a chi square test was used for categorical variables.

To investigate the association of veganism with biomarkers of the lipid metabolism, compared to omnivores, an analysis of variance (ANOVA) was performed for model 1 (unadjusted). Additionally, a multivariable adjusted analysis of covariance (ANCOVA) was conducted to detected differences between vegans and omnivores in model 2 (adjusted for several PFAS and the PFAS sum) and model 3 (additionally adjusted for age, sex, smoking status, education, waist circumference, and physical activity). The model was not adjusted for recent weight changes as the study did not assess data on weight changes. Blood lipid concentrations were skewed, thus variables were log-transformed for ANOVA or ANOVA, afterwards back-transformed and expressed as geometric means and 95%-confidence intervals (95%-CI).

To investigate a potential relationship between PFAS plasma levels and blood lipids, we used linear regression models and also a restricted cubic spline (RCS) regression analyses to investigate nonlinear associations. Three knots were used, located at the 5th, 50th and 95th percentiles. The RCS regression models fitted with generalized estimating equations were constructed using the SAS macro %RCS_Reg (v1.50) developed by Desquilbet and Mariotti (2010). Not only levels of blood lipids, but also those of PFAS were skewed distributed. Therefore, all variables were log-transformed for the analyses. The analyses were performed in unadjusted models (model 1), adjusted for type of diet (model 2) as well as additionally adjusted for age, sex, smoking status, education, waist circumference, and physical activity (model 3).

To investigate potential correlations between PFAS plasma levels and food groups, we calculated Spearman (partial) correlations for the total and the omnivorous sample. Correlation analyses between individual PFAS and individual food groups were performed in an unadjusted model (model 1) for omnivores, and for the total sample, model 1 was adjusted for type of diet. Model 2 was additionally adjusted for age, sex, smoking status, education, waist circumference, and physical activity.

The statistical analyses were performed using SAS software, version 9.4 (SAS institute, Cary, N.C., USA), IBM SPSS Statistics for Windows, Version 26.0 (Armonk, NY: IBM Corp) and R software (version 3.6.3). Test findings with p values of <0.05 were considered statistically significant.

3. Results

The general characteristics of the 72 sex- and age matched participants are shown in Table 1, according to vegan or omnivorous diet (n = 18 men and 18 women each). The median duration of veganism was 4.8 years (IQR: 3.1–8.7). Median age was 37.5 years (range 30–57) in vegans and 38.5 years (range 30–57) in omnivores, respectively. No relevant differences in anthropometric measurements, physical activity, smoking, education, were observed between the groups. Regarding occupational activity, 16.7% (n = 6) of vegans and 8.4% (n = 3) of omnivores reported a high level of intensive occupational activity. 61.1% (n = 22) of vegans and 77.8% (n = 28) of omnivores stated a high level of sedentary occupational activity. None of the participants took any lipid-lowering medications.

The following PFAS were analyzed: PFOS, PFOA, PFHxS, PFNA, PFDA, PFBS, PFHxA, PFDoDA and ADONA. Levels of the main four contaminants, PFOS, PFOA, PFHxS and PFNA are given in Table 2. PFOS and PFOA were quantifiable in all the 72 participants, whereas PFHxS and PFNA were below the LOQ in two samples and 22 samples, respectively. In case of PFDA, 58 samples were below the LOQ. Of the 14 samples quantifiable (range of 0.26–0.49 ng/ml), 13 were from omnivores. These values were not considered in the following evaluation. Levels of the other four compounds (PFBS, PFHxA, PFDoDA and ADONA) were not found above the LOQ.

Table 1
Characteristics of the study population according to vegan or omnivorous diet.

	Vegans (n = 36)	Omnivores (n = 36)	*p-value*
Duration vegan diet [years]	4.8 (3.1–8.7)		
Men	50.0% (18)	50.0% (18)	
Age [years]	37.5 (32.5–44.0)	38.5 (32.0–46.0)	*0.75*
Anthropometry			
BMI [kg/m^2]	22.9 ± 3.2	24.0 ± 2.1	*0.08*
Waist circumference [cm]			
Women	73.1 ± 6.9	77.2 ± 6.2	*0.07*
>80	8.3% (3)	13.9% (5)	
Men	84.5 ± 8.9	86.0 ± 6.1	*0.56*
>94	5.6% (2)	5.6% (2)	
Education [%]			*0.60*
Low	0.0% (0)	2.8% (1)	
Intermediate	30.6% (11)	30.6% (11)	
High	69.4% (25)	66.7% (24)	
Lifestyle			
Physical Activity [h/week]	2.8 (0.88–3.75)	2.3 (1.2–4.1)	*0.69*
Walking [h/week]	7.0 (5.0–12.0)	5.5 (3.5–11.8)	*0.15*
Smoking status			*0.30*
Non-smoker	66.7% (24)	58.3% (21)	
Ex-Smoker	22.2% (8)	16.7% (6)	
Smoker	11.1% (4)	25.0% (9)	
Blood lipids			
Total cholesterol [mg/dl]	157.0 (137.0–180.5)	203.5 (178.5–222.5)	<0.0001
HDL cholesterol [mg/dl]	56.5 (50.5–71.5)	61.5 (51.5–80.5)	0.21
LDL cholesterol [mg/dl]	86.5 (68.5–97.0)	115.5 (93.5–136.0)	0.001
Triglyceride [mg/dl]	71.0 (53.0–90.5)	85.0 (52.0–120.5)	0.26

Variables expressed as percentage (n), mean ± SD or median (IQR).

Table 2
PFAS according to a vegan or omnivorous diet (n = 72).

	Vegans (n = 36)		Omnivores (n = 36)		
	Median (IQR)	Min - Max	Median (IQR)	Min - Max	*p-value*
PFOS [ng/ml]	*n > LOQ: 36*		*n > LOQ: 36*		
All (n = 36)	2.31 (1.37–3.59)	0.34–6.70	3.57 (1.94–5.14)	0.84–11.1	*0.02*
Men (n = 18)	2.31 (1.37–4.47)	0.34–6.70	4.65 (3.28–5.86)	0.84–10.8	*0.04*
Women (n = 18)	2.31 (1.38–2.75)	0.59–6.36	2.62 (1.87–3.96)	1.38–11.1	*0.21*
PFOA [ng/ml]	*n > LOQ: 36*		*n > LOQ: 36*		
All (n = 36)	1.69 (1.35–2.75)	0.26–4.24	1.44 (0.98–2.61)	0.62–4.65	*0.26*
Men (n = 18)	1.66 (1.46–2.79)	0.26–4.24	1.68 (1.18–2.92)	0.62–4.65	*0.73*
Women (n = 18)	1.75 (1.28.2.10)	0.72–3.80	1.18 (0.92–2.04)	0.64–3.35	*0.23*
PFHxS [ng/ml]	*n > LOQ: 35*		*n > LOQ: 35*		
All (n = 36)	1.96 (0.88–3.75)	<LOQ –11.2	1.79 (0.92–2.74)	<LOQ – 6.09	*0.70*
Men (n = 18)	2.14 (1.06–3.76)	<LOQ – 8.97	1.93 (1.33–2.70)	0.38–5.08	*0.76*
Women (n = 18)	1.74 (0.69–3.74)	0.26–11.2	1.79 (0.85–3.11)	<LOQ– 6.09	*0.83*
PFNA [ng/ml]	*n > LOQ: 16*		*n > LOQ: 34*		
All (n = 36)	<LOQ (<LOQ -0.30)	<LOQ – 0.49	0.41 (0.33–0.58)	<LOQ - 1.05	*<0.0001*
Men (n = 18)	<LOQ (<LOQ -0.30)	<LOQ – 0.42	0.49 (0.39–0.65)	<LOQ– 1.05	*<0.0001*
Women (n = 18)	<LOQ (<LOQ -0.30)	<LOQ – 0.49	0.35 (0.29–0.48)	<LOQ– 0.92	*0.003*
PFAS sum [ng/ml]					
All (n = 36)	6.41 (4.08–9.38)	0.84–21.4	7.65 (5.02–11.1)	2.02–21.6	*0.33*
Men (n = 18)	6.93 (3.98–9.96)	0.84–18.5	8.85 (7.28–12.0)	2.02–21.6	*0.18*
Women (n = 18)	5.96 (4.18–8.25)	1.78–21.4	6.18 (4.70–8.55)	2.44–20.6	*0.80*

LOQ: limit of quantification. PFAS sum: PFOS + PFOA + PFHxS + PFNA.

In the total study population (n = 72), median concentrations (IQR) of PFOS: 2.71 ng/ml (1.64–4.67), PFOA: 1.62 ng/ml (1.14–2.71), PFHxS: 1.84 ng/ml (0.91–3.19) and PFNA: 0.32 ng/ml (<0.25–0.44) were observed. The median concentration (IQR) of the PFAS sum was 7.05 ng/ml (4.72–9.85). The distributions of plasma levels of PFAS were skewed, as depicted in Fig. 1 for the lead compounds PFOS and PFOA in vegans and omnivores. According to the dietary group, data on plasma levels of the all four PFAS evaluated are compiled in Table 2 for the whole study group and separately for men and women. Regarding PFOA, no significant differences were observed between vegans and omnivores (p = 0.26), however, vegans were with tendency more likely to have higher PFOA concentrations compared to omnivores. In case of PFOS, the levels were significantly higher in omnivores (median 3.57 ng/ml, IQR: 1.94–5.14) compared to vegans (median 2.31 ng/ml, IQR: 1.37–3.59) (p = 0.02). The strongest difference was seen for PFNA with median values below the LOQ of 0.25 ng/ml (IQR: <0.25–0.30) for vegans compared to 0.41 ng/ml (IQR: 0.33–0.58) for omnivores (p < 0.0001). Of the 22 PFNA measurements below the LOQ, two were from omnivores, and 20 from vegans. No significant differences between vegans and omnivores were seen for PFHxS (p = 0.70) and the PFAS sum (p = 0.33). Considering the duration of the vegan diet, long-time vegans were with tendency more likely of lower level of the PFAS sum (PFOS + PFOA + PFHxS + PFNA, Fig. 2).

Regarding the level of the PFAS sum in women of childbearing age (≤45 years of age), a median value of 5.82 ng/ml (IQR: 4.44–7.76, range 1.78–21.4) was observed (n = 28, 15 vegans and 13 omnivores, median PFAS sum 5.74 vs. 5.91 ng/ml, respectively; p = 0.53). Ten of these 28 women (36%) exceeded the EFSA derived plasma level of 6.9 ng/ml corresponding to the TWI in women at the age of 35 years.

Correlations between PFAS and eight selected food groups are visually summarized in Fig. 3, and specific correlation coefficients are presented in supplemental Table 2. In the total population (n = 72), the strongest correlations were observed between PFOA and water consumption (model 2 correlation coefficient 0.34, p = 0.01, supplemental Table 2). Regarding the consumption of 'meat and meat products' in omnivores (n = 36), the strongest correlations were observed with the concentrations of PFOS (model 2 correlation coefficient 0.38, p = 0.04) and PFNA (model 2 correlation coefficient 0.50, p = 0.01, supplemental Table 2). Supplemental Table 3 shows the intake of the food groups based on FFQ data in vegans and omnivores.

Levels of LDL and total cholesterol were considerably lower in vegans compared to omnivores (Tables 1 and 3). As shown in Table 3, after adjustment of several PFAS (model 2), none of the investigated PFAS, neither the PFAS sum, alter the differences of LDL cholesterol between vegans and omnivores (Table 3) indicating no relevant impact of PFAS on the association between vegans/omnivores and LDL cholesterol. After further adjustment for lifestyle factors (model 3), the difference between both diet groups got smaller, especially omnivores had lower concentrations of LDL cholesterol. Nevertheless, further adjustment of PFAS sum (model 3, Table 3) as well as for PFOS, PFOA, PFHxS or PFNA did not alter the difference of blood lipid concentrations. We observed no relevant linear or non-linear associations between LDL cholesterol and different PFAS (on the log-scale), as depicted by splines in the supplemental Figure 1. Concerning other blood lipids, concentrations of HDL and total cholesterol as well as of triglycerides in vegans and omnivores also did not change after adjustment for different PFAS (data not shown). In line, linear regression analyses detected no associations between PFAS and blood lipids (supplemental Table 4).

4. Discussion

The present cross-sectional study is the first study investigating differences in PFAS levels in vegans compared to omnivores. Significantly lower concentrations of PFOS and PFNA were observed in vegans compared to omnivores. Accordingly, we observed correlations with food groups expected to contribute most to the internal exposure with PFAS. At the present level of internal PFAS exposure, we did not observe relevant associations between PFAS and blood lipids in particular under consideration of the large differences in LDL and total cholesterol levels between vegans and omnivores.

Only very few data of recent years on internal exposure to PFAS are available for German adults. The internal plasma levels of PFAS measured in our total study group (residing in Berlin) were found to be – despite the high proportion of vegans – relatively high. The best

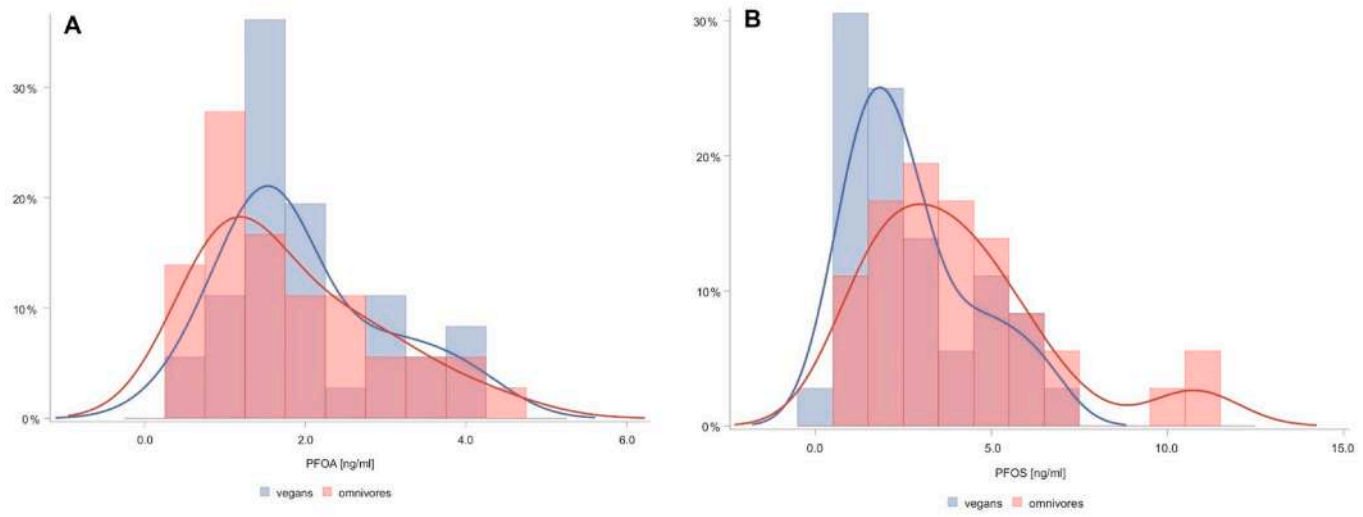

Fig. 1. Distributions of plasma PFOA and PFOS levels according to vegans and omnivores. Distribution of plasma PFOA levels [ng/ml] (A) and PFOS levels [ng/ml] (B) of the study population. Histograms depicted vegans in blue and omnivores in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

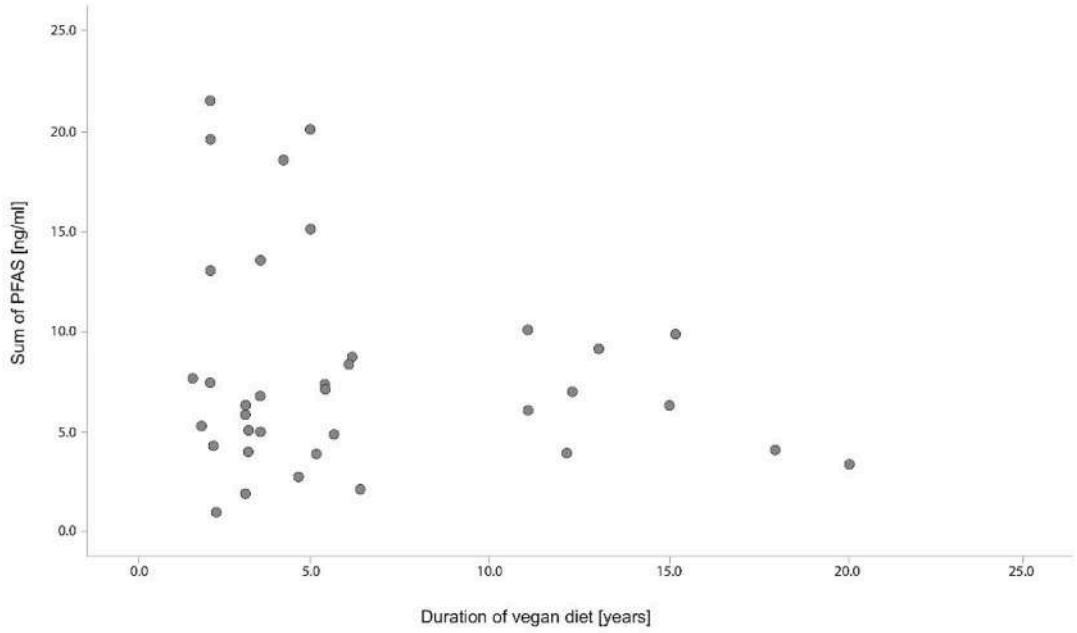

Fig. 2. Scatter plot of sum of PFAS (PFOS + PFOA + PFHxS + PFNA) according to the duration following a vegan diet. Scatter plot of sum of PFAS levels [ng/ml] to the duration of vegan diet [years] in vegans (n = 36).

comparison within Germany is possible with a group from Munich ("Side C", n = 158 blood donors, median age 39.5 years) investigated 2016 (Fromme et al., 2017), serving as a control for a contaminated region in Bavaria/Germany. Results revealed median levels of PFOS, PFOA, PFHxS, PFNA and the PFAS sum to be 2.1, 1.1, 0.5, 0.4 and 4.1 ng/ml, respectively (PFAS sum: personal communication of Prof. Fromme). Furthermore, data are available from the German Environmental Specimen Bank (n = 40 students aged 20–29 years from Münster), with median levels for the 2017/2019 sampling of PFOS, PFOA, PFHxS, PFNA and the PFAS sum of 2.6, 1.7, 0.5, 0.4 and 5.8 ng/ml, respectively (Gockener et al., 2020). The main difference between these investigations and our study are the surprising high levels of PFHxS, with a 3.7-fold higher median in Berlin (PFHxS median: 0.5 vs. 1.8 ng/ml). This may be due to regionally different nutritional habits or to higher concentrations in drinking water as well as due to differences in the socio-economic status. However, sample sizes of all studies were rather small and therefore also a chance finding cannot be excluded.

In the three investigations mentioned from Munich, Münster and Berlin, the sex ratios were exactly or roughly 1:1, and confirmed the higher levels of PFAS in men compared to women observed in many studies. As presented by EFSA, this is due to several factors. Besides a higher external exposure (e.g. due to a higher meat consumption), physiological differences including urinary elimination, menses, and use of oral contraceptives (2 vegans, 6 omnivores) have to be considered, as well as pregnancy and lactation (EFSA Panel on Contaminants in the Food Chain, 2020). In our study, the median levels of the PFAS sum were 8.2 ng/ml in men and 6.0 ng/ml in women. According to the recent risk assessment of EFSA, an internal level of the PFAS sum of up to 6.9 ng/ml corresponds to an external intake below the TWI in women of the childbearing age (EFSA Panel on Contaminants in the Food Chain,

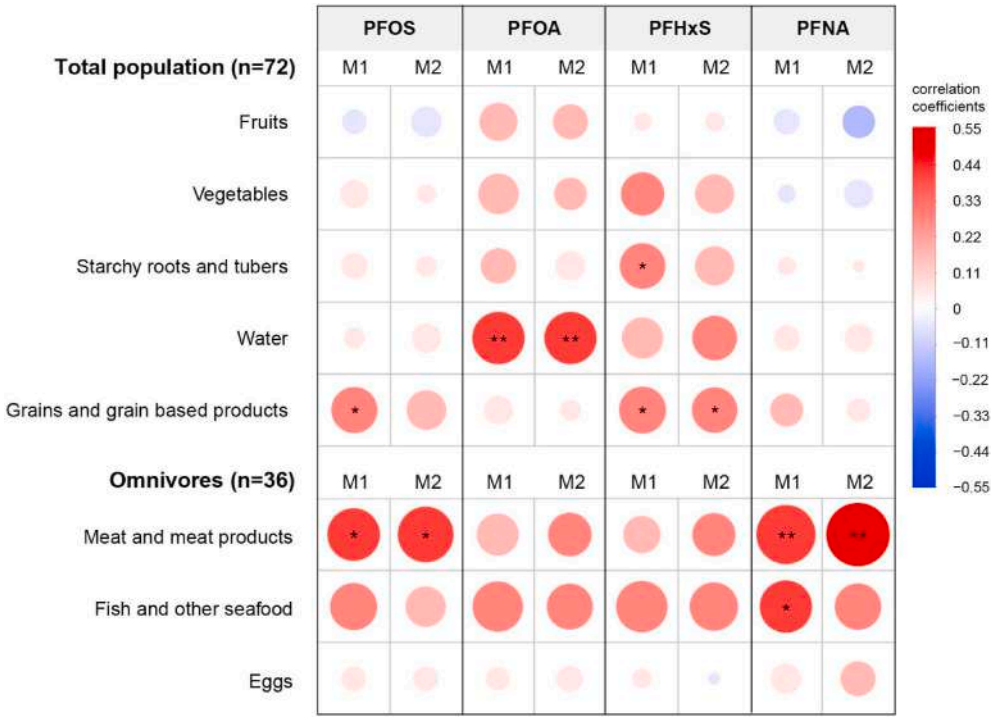

Fig. 3. Heatmap on correlations of 8 relevant food groups with PFAS plasma concentrations. Total population (n = 72) M1: adjusted for type of diet (vegan or omnivores), M2: additional adjustment for age, sex, smoking status, education, waist circumference, and physical activity; Omnivores (n = 36) M1: unadjusted model, M2: additional adjustment for age, sex, smoking status, education, waist circumference, and physical activity; **p < 0.01, *p < 0.05; Water includes drinking water, coffee, tea, and herbal tea.

Table 3
LDL cholesterol concentrations according to a vegan or omnivorous diet (n = 72).

	Vegans (n = 36)	Omnivores (n = 36)	*p-value*
LDL cholesterol [mg/dl]			
Model 1			
Unadjusted	86.3 (77.7–95.9)	110.3 (99.3–122.5)	*0.002*
Model 2			
PFOS	85.9 (77.1–95.8)	110.8 (99.4–123.5)	*0.002*
PFOA	86.2 (77.6–95.9)	110.4 (99.3–122.7)	*0.002*
PFHxS	86.3 (77.7–96.0)	110.3 (99.2–122.6)	*0.002*
PFNA	84.2 (74.6–95.1)	113.1 (100.1–127.7)	*0.003*
PFAS Sum	86.2 (77.6–95.9)	110.4 (99.3–122.8)	*0.002*
Model 3			
Lifestyle factors	86.1 (68.4–108.5)	103.6 (83.0–129.2)	*0.02*
Lifestyle factors + PFOS	85.7 (67.8–108.3)	104.0 (83.2–130.1)	*0.02*
Lifestyle factors + PFOA	86.3 (68.4–109.0)	103.5 (82.8–128.3)	*0.02*
Lifestyle factors + PFHxS	85.6 (67.8–108.0)	102.1 (81.5–128.0)	*0.02*
Lifestyle factors + PFNA	83.3 (65.5–106.0)	106.4 (84.7–133.6)	*0.01*
Lifestyle factors + PFAS Sum	85.7 (68.0–108.2)	103.4 (82.8–129.1)	*0.01*

expressed as geometric mean (95%-CI); Model 1: unadjusted, Model 2: adjusted for several PFAS, Model 3: additional adjusted for lifestyle factors i.e. age, sex, smoking status, education, waist circumference, physical activity; PFAS sum: PFOS + PFOA + PFHxS + PFNA.

2020). For this group (women between 18 and 45 years of age), 10 of 28 women (36%, maximum level 21.4 ng/ml) exceeded the level on our study, while 1 of 52 women exceeded it in Munich 2016 (2%, maximum level 7.2 ng/ml (Fromme et al., 2017)) and 6 of 20 women in Münster 2017/2019 (30%, maximum level 16.3 ng/ml (Gockener et al., 2020). These numbers demonstrate large regional differences in internal exposure to PFAS, and representative studies are necessary to get a more reliable picture of the proportion of women in the German population exceeding EFSA's internal level for the PFAS sum of 6.9 ng/ml corresponding the TWI.

The study detected significant differences in PFOS and PFNA concentrations between both diet groups, showing 54% and 240% higher median concentrations in omnivores compared to vegans, respectively. Obviously, this is due to the relatively high PFAS concentrations in food products of animals. According to the exposure assessment of EFSA (Annex A) for German Adults (EFSA Panel on Contaminants in the Food Chain, 2020), 'Fish and other seafood' was the most important contributor to the mean LB exposure, followed by 'Meat and meat products', 'Fruit and fruit products' and 'Eggs and egg products' in case of PFOS. In case of PFNA, 'Fruit and fruit products' and 'Fish and other seafood' were the most important contributors to the mean LB exposure. Therefore, the differences observed between vegans and omnivores are obviously better to explain by the diet in case of PFOS than in case of PFNA. Regarding the heat map generated from the FFQ data (Fig. 3), the pattern of PFOS and PFNA in omnivores seem comparable, with highest correlations for 'Meat and meat products' and 'Fish and other seafood'. However, due to the small number of participants of the study, the nutritional data from the FFQ with respect to the internal exposure to PFAS should be interpreted with caution. Nevertheless, our results are in line with another study. Lin et al. noticed that participants (n = 941 adults with pre-diabetes) with high consumption of meat, fried fish, and other fish/shellfish (but not omega-3 rich fish) had higher plasma concentrations of PFOS, PFHxS and PFNA (Lin et al., 2020).

Currently, PFAS levels in many food groups are found to be nearly completely below the presently available LOQs (EFSA Panel on Contaminants in the Food Chain, 2020). Therefore, more sensitive analytical methods are needed for the quantification of PFAS in foods. A higher proportion of quantified PFAS levels in food groups may lead to a better estimation of the contributions of different food groups to the exposure of different PFAS via food consumption and possibly to a change of the pattern of these contributions especially in case of PFNA.

Regarding PFOA and PFHxS in the two diet groups, no relevant differences were detected in vegans compared to omnivores. In case of PFOA, this is surprising, as EFSA identified 'Fish and other seafood', 'Eggs and egg products' as well as 'Meat and meat products' as most

important contributors to the mean LB exposure of PFOA (and PFOS) (EFSA Panel on Contaminants in the Food Chain, 2020). Interestingly, besides the above mentioned food categories, 'Alcoholic beverages' and 'Drinking water' were reported to be also important contributors to the mean LB exposure in case of PFOA in Germany (EFSA Panel on Contaminants in the Food Chain, 2020). Indeed, PFOA levels were found to have the highest correlations with the consumption of 'Water' in the total study group (see heat map, Fig. 3). In case of PFHxS, 'Fruit and fruit products', 'Alcoholic beverages' and 'Drinking water' were the only contributors to the mean LB exposure in Germany (EFSA Panel on Contaminants in the Food Chain, 2020). This is reflected in the heat map in case of 'Water' only, but as in case of PFNA, more sensitive analytical methods may change the pattern of the contributors to external exposure.

Epidemiological studies provided consistent findings of associations between serum levels of PFOS/PFOA and levels of cholesterol in populations with relatively high exposure (EFSA Panel on Contaminants in the Food Chain, 2020). In 2018, EFSA even used these associations with serum cholesterol levels to derive TWIs for both PFOS and PFOA (EFSA Panel on Contaminants in the Food Chain, 2018). However, a clear mode of action is missing. Since lower LDL cholesterol levels in vegans are one of the most highlighted health benefits of this diet (Benatar and Stewart, 2018), we thought to also analyze the relation between different PFAS and LDL cholesterol in our small study sample. Although a wide range of concentrations of LDL cholesterol was observed in our study population, we did not find any relevant relation between PFAS and LDL cholesterol. This may be due to the relatively low concentrations of PFAS in comparison to previous studies (EFSA Panel on Contaminants in the Food Chain, 2020). The distinctly lower levels of LDL cholesterol in vegans with concurrently lower levels of PFOS and PFNA strikingly raises the issue of confounding by diet in case of the above-mentioned epidemiological studies, and the extent of necessary adjustment to avoid false interpretations. Interestingly, in most studies investigating associations between PFAS and blood lipids, statistical models were adjusted – beside age, sex, smoking, alcohol intake and education – only for body mass index or waist circumference (EFSA Panel on Contaminants in the Food Chain, 2018). Only very few studies adjusted for further dietary variables such as saturated or animal fat intake (Eriksen et al., 2013; Nelson et al., 2010) or food groups such as meat or fish intake (Canova et al., 2020; Lin et al., 2020; Skuladottir et al., 2015) or healthy diet score (Donat-Vargas et al., 2019). Taking altogether, evidence for a causal relationship between PFAS and blood lipids is still not convincing and residual confounding still cannot be completely excluded to explain the observed associations between PFAS and blood lipids in many but not all studies. Further studies are necessary to clarify at one hand possible mechanisms and to investigate associations with improved statistical models including more potential confounders in particular dietary factors on the other hand.

Different methods exist for dietary assessments, and each has its advantages and disadvantages. Because of the long half-life time of PFAS, FFQ might be a good method assessing the past long-term diet. The EPIC-FFQ (Nothlings et al., 2007) captured the time span of the previous 12 months. We see the limitation of the present FFQ, which did not cover all variety of a vegan diet, as some food groups will not be fully assessed, thus the diet of vegans might be underestimated. However, in the present evaluation we analyzed only predetermined food groups, eaten by both omnivores and vegans, for example fruits, vegetables or water. Therefore, the use of the FFQ for our study purpose is acceptable. For the food groups of meat, fish or eggs, the analyses were only performed in omnivores to avoid bias due to non-consumption of the vegan population. Nevertheless, this study underlines the need for assessment tools in science for a past long-term diet also for participants following a plant-based diet (e.g. vegan, vegetarian) or the modification of already existing tools.

Limitations of our study deserve to be mentioned. The present RBVD study is relatively small (n = 72), including middle aged vegans and omnivores from a relatively small area (Berlin, Germany); therefore, the results may not be generalizable to other populations. Nevertheless, the RBVD study provides comprehensive high-quality data as a result of the standardized procedures in combination with extensive information from computer-based questionnaires and anthropometric measurements. Regarding FFQ, we see also some limitations concerning recall bias and under/over-reporting, attributed to reliance on participant's memory, inability to accurately estimate portion sizes and misinterpretation of the questions, or social desirability bias (Hooson Jzh et al., 2020). Other routes of exposure than diet such as ingestion of house dust, inhalation of indoor air, and dermal absorption may substantially contribute to the exposure to PFAS on an individual basis (Poothong et al., 2020), but these routes could not be considered in our study. Further, some packing materials and take-away food, as well as kitchen utensils might be potential sources of exposure to PFAS.

5. Conclusion

Lower levels of PFOS and PFNA, but not of PFOA and PFHxS were observed in vegans compared to omnivores. FFQ data allowed the identification of relevant food groups contributing to the levels of these four PFAS. The strong impact of a vegan diet on levels of blood lipids, especially on LDL cholesterol, was confirmed in our study. In contrast, the association of PFAS and LDL cholesterol was found to be negligible, possibly due to the relatively low levels of PFAS observed. However, we highlight the importance of the adjustment of dietary factors like a vegan diet in case of epidemiological studies dealing with the impact of PFAS on the levels of blood lipids.

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.2021.113808.

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Lead, cadmium, mercury, and chromium in urine and blood of children and adolescents in Germany – Human biomonitoring results of the German Environmental Survey 2014–2017 (GerES V)

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ABSTRACT

Metals reach humans through food and drinking water intake and inhalation of airborne particles and can have detrimental health effects in particular for children. The metals presented here (lead, cadmium, chromium, and mercury) could lead to toxic effects such as neurotoxicity, mutagenicity, and have been classified as (possible) carcinogens. Using population representative data from the German Environmental Survey 2014–2017 (GerES V) from 3- to 17-year-old children on lead and cadmium in blood (n = 720) and on cadmium, chromium, and mercury in urine (n = 2250) we describe current internal exposure levels, and socio-demographic and substance-specific exposure determinants. Average internal exposure (geometric means) in blood was 9.47 µg/L for lead and below 0.06 µg/L (limit of quantification) for cadmium, and in urine 0.072 µg/L for cadmium, 0.067 µg/L for mercury, and 0.393 µg/L for chromium, respectively. Younger children have higher concentrations of lead and chromium compared to 14-17-year-old adolescents, and boys have slightly higher mercury concentrations than girls. With respect to substance specific determinants, higher lead concentrations emerged in participants with domestic fuel and in non-smoking children with smokers in the household, higher levels of cadmium were associated with smoking and vegetarian diet and higher levels of mercury with the consumption of seafood and amalgam teeth fillings. No specific exposure determinants emerged for chromium. The health based guidance value HBM-I was not exceeded for mercury and for cadmium in urine it was exceeded by 0.6% of the study population. None of the exceedances was related to substantial tobacco smoke exposure. Comparisons to previous GerES cycles (GerES II, 1990–1992; GerES IV, 2003–2006) indicate continuously lower levels.

1. Introduction

Human exposure to metals is ubiquitous due to their presence in soil and dust and the resulting uptake via food and drinking water (Becker et al., 2008; Järup, 2003; Nordberg et al., 2015; WHO, 2016). Exposure via inhalation of airborne particles has, however, decreased in the past decades (Becker et al., 2008), leaving ingestion as the main exposure pathway, after inhalation through active smoking (Basu et al., 2018; German Human Biomonitoring Commission, 1996; 1998; Krause et al., 1996; Lermen et al., 2021). According to WHO (2016) dirt, dust, and food account for more than 80% of the daily lead intake. Here, exposure to four metals – lead in blood, cadmium in blood and urine, urinary mercury, and urinary chromium – in 3–17-year olds is presented.

Lead (Pb) is classified as "possibly carcinogenic to humans" (group 2B) by the IARC, inorganic lead compounds even "probably carcinogenic to humans" (group 2A; IARC, 1987, 2006). Furthermore, it is classified as toxic to reproduction (ECHA, 2020c) and neurotoxic (German Human Biomonitoring Commission, 2002). Lead enters the environment primarily through combustion of fossil fuels, production of lead-acid batteries, and its use in the manufacturing industries (ATSDR (Agency for Toxic Substances and Disease Registry), 2020; United Nations Environment Programme, 2010; World Health Organization, 2017). Due to the elimination of lead from gasoline and tighter restrictions on the production and application of many commodities (e.g., paints, plumbing and solder), lead exposure in the general population

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Abbreviations

AM	arithmetic mean
ANOVA	analysis of variance
CAS	Chemical Abstract Service
Cd	Cadmium
Cr	chromium
CHMS	Canadian Health Measures Survey
crea	creatinine
CV-AAS	cold vapour atomic absorption spectrometry
CZ-HBM	Environmental Health Monitoring System in the Czech Republic
EC	European Commission
EEA	European Environment Agency
FLEHS	Flemish Environment and Health Study
G-EQUAS	German External Quality Assessment Scheme
GerES	German Environmental Survey
GM	geometric mean
HBM	human biomonitoring
HBM-I value	human biomonitoring value I
HBM-II value	human biomonitoring value II
HBM4EU	European Human Biomonitoring Initiative
Hg	mercury
IARC	International Agency for Research on Cancer
ICP-MS	inductively coupled plasma mass spectrometry
IPASUM	Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, Erlangen, Germany
KiGGS Wave 2	German Health Interview and Examination Survey for Children and Adolescents
KorEHS-C	Korean Environmental Health Survey in Children and Adolescents
LOQ	limit of quantification
MAX	maximum value
MOCEH	Korean Mothers' and Children's Environmental Health
n	sample size
NHANES	Health and Nutrition Examination Survey
P	percentiles
Pb	lead
QC	quality control
RKI	Robert Koch Institute
Sd	standard deviation
SES	socio-economic status
UBA	German Environment Agency
95CI GM	95% confidence interval for the geometric mean

has declined substantially in recent decades (Timothy and Tagui Williams, 2019; World Health Organization, 2019). Nevertheless, some relevant sources of human lead exposure still exist, such as exposure via food, drinking water, tobacco smoke and polluted dust particles (ATSDR (Agency for Toxic Substances and Disease Registry), 2020; European Food Safety Authority, 2013; German Human Biomonitoring Commission, 1996). Given that neurotoxic effects were observed even at the lowest concentration investigated, no threshold for safe lead exposure could be derived (Etchevers et al., 2014; German Human Biomonitoring Commission, 2002; 2009; UNICEF and Pure Earth, 2020; WHO, 1995).

Cadmium (Cd) is carcinogenic to humans (group 1; IARC, 2012) and suspected to be mutagenic and toxic to reproduction (ECHA, 2020a). It is inter alia used for anti-corrosive coating for iron and steel, in batteries and metal alloys, and is a by-product of zinc mining (German Human Biomonitoring Commission, 2011; Jaishankar et al., 2014). Cadmium is readily taken up by various plants such as vegetables, cereals, and tobacco. Tobacco smoke is volatile, effectively resorbed after inhalation, and a major source of exposure. For non-smokers, ingestion of vegetables is the main exposure pathway (Becker et al., 2008; European Food Safety Authority, 2009). Cadmium is mainly bound to blood cells and only after chronic exposure also stored in the cortex of kidney. Therefore, blood cadmium levels represent recent cadmium exposure (days and weeks before sampling), while urinary cadmium is rather a measure of chronic and cumulative life-long exposure (German Human Biomonitoring Commission, 1998; Wang et al., 2017). The German Human Biomonitoring (HBM) Commission derived health based guidance values (HBM-I and HBM-II values) on a level as to avoid kidney-damaging cadmium accumulation (German Human Biomonitoring Commission, 2011). The HBM-I value for urinary cadmium levels is 0.5 μg/L and the HBM-II value is 2 μg/L for children and adolescents.

Mercury (Hg) is toxic to reproduction (ECHA, 2020d) and methylmercury compounds are classified as possible carcinogens (group 2B; IARC, 1993). Mercury mainly damages the central nervous system (German Human Biomonitoring Commission, 1999). It is released into the environment by burning of fossil fuels, smelting, cement production, and waste incineration (German Human Biomonitoring Commission, 1999). Many usages and applications of mercury have already been eliminated in the past, such as mercury-containing pesticides, catalysers, or paint and also medical applications (including amalgam teeth fillings) have been reduced (German Human Biomonitoring Commission, 1999). Still, dental amalgam fillings, especially in combination with chewing gum, and broken fluorescent light bulbs might be sources of human mercury exposure (Berglund et al., 2005; Dunn et al., 2008; Sällsten et al., 1996; SCHER (Scientific Committee on Health and Environmental Risks), 2012). Another source is the consumption of fish and other seafood (Basu et al., 2018; German Human Biomonitoring Commission, 1999; Tratnik et al., 2019). Urinary mercury is an indicator mainly of inorganic mercury exposure, represents long-term exposure (Berglund et al., 2005; Ruggieri et al., 2017), and is increased also on account of seafood consumption because of demethylation of methylmercury from seafood (SCHER (Scientific Committee on Health and Environmental Risks), 2012). The German HBM Commission derived a HBM-I value of 7 μg/L and a HBM-II value of 25 μg/L for mercury in urine of children (German Human Biomonitoring Commission, 1999).

Chromium exists in various oxidation states, of which trivalent Cr (III) and hexavalent Cr(VI) are the most abundant, i.e. stable. Cr(III) is considered to have essential effects on nutrition and in glucose metabolism (Jaishankar et al., 2014). Cr(VI) is carcinogenic to humans (group 1; IARC, 2012), genotoxic (European Commission, 2008), and suspected to be skin sensitising (ECHA, 2020b). Chromium is used in alloys, e.g. for stainless steel, for electroplating, in dyes, pigments, cement, and wood preservation (Fréry et al., 2010). Exposure of the general population occurs mainly via ingestion while occupational exposure also includes inhalation. Cr(VI) easily penetrates biological membranes, contrary to Cr(III) (Leng, 2012). In urine it is not possible to differentiate between Cr(III) and Cr(IV). However, as most of the bioavailable chromium is excreted via urine, the determination of total chromium in urine is an acceptable biomarker for chromium exposure (Kerger et al., 1996; Langård and Costa, 2015; Leng, 2012).

The importance of all four metals – lead, cadmium, mercury, and chromium(VI) – for the health of European citizens has also been emphasized by their identification as substances of priority concern by the European Human Biomonitoring Initiative HBM4EU (www.hbm4eu.eu), a joint effort of 30 partner countries, the European Environment Agency (EEA) and the European Commission (EC), co-funded under Horizon 2020 (Ganzleben et al., 2017).

The present study aims at describing the current internal exposure of children and adolescents to lead, cadmium, mercury, and chromium, and testing possible exposure determinants for internal exposure in

bivariate analyses with a national population representative, cross-sectional data on human internal exposure to environmental chemicals and its sources. The German Environmental Survey has repeatedly been conducted since the 1980s (Kolossa-Gehring et al., 2012a, 2012b). The field work of the last cycle – GerES V for Children and Adolescents – was conducted from 2014 to 2017, investigating 2294 children and adolescents in Germany aged 3–17 years (Schulz et al., 2017). GerES combines HBM with ambient monitoring and collection of information on exposure-relevant behaviour and the living environment.

2. Material and methods

2.1. Study population and sample collection

In GerES V, a subsample (n = 2294) of the 3- to 17-year-old participants of the German Health Interview and Examination Survey for Children and Adolescents (KiGGS Wave 2) of the Robert Koch Institute (RKI) was examined (Mauz et al., 2017; Schulz et al., 2017). Participants of GerES V were visited by a trained interviewer, conducting an interview on exposure-relevant behaviour and collecting information on the living environment with the participants and their parents or legal guardians, and collecting inter alia samples of first-morning void urine and tap water, among other media. The sample collection for GerES V was conducted between 2015 and 2017. Samples of whole blood (n = 720) were collected during the KiGGS Wave 2 examination which was on average 104 days (sd = 21, range = 48–163) prior to the GerES V examination. KiGGS Wave 2 sampling was running from 2014 to 2017. For more details on both studies see Murawski et al. (2020) and Hoffmann et al. (2018).

The Ethics Committee of the Berlin Chamber of Physicians (Eth-14/14) and the Federal Officer for Data Protection and Freedom of Information (III-425/009#0018) had approved the project.

2.2. Chemical analysis

In urine samples, mercury, chromium, and cadmium were determined. Additionally, lead and cadmium were determined in samples of whole blood. All elements were analysed by the Institute and Outpatient Clinic of Occupational, Social and Environmental Medicine, Erlangen, Germany (IPASUM). For lead, cadmium, and chromium, inductively coupled plasma mass spectrometry (ICP-MS) was applied. Prior to analysis blood samples were diluted with an ammoniacal solution, while nitric acid was used for dilution of urine samples. Terbium and rhodium were used as internal standards for ICP-MS. Moreover, for all samples with a molybdenum concentration above 10 µg/L the concentration of cadmium had been corrected to minimise the influence of the molybdenum oxide ion ($^{98}Mo^{16}O^{+}$) on the cadmium ion ($^{114}Cd^{+}$). Mercury concentration was determined in urine samples by using cold vapour atomic absorption spectrometry (CV-AAS). More details on the analytical method can be found in Schramel et al. (1999) and Heitland and Köster (2006).

Creatinine content of the urine samples was photometrically determined by a validated standard method based on the Jaffé method (Blaszkiewicz and Liesenhoff-Henze, 2010). Creatinine was analysed by the Analytisch-Biologisches Forschungslabor (ABF GmbH), Munich (Germany). The ABF also determined urinary cotinine using liquid chromatography coupled to mass spectrometry (Piller et al., 2014). Cotinine is considered the objective measurement of smoking status when dichotomized at 50 µg/L (Gruber and Schuurmans, 2018: ≥50 µg/L indication for active smoking). All analytical methods were validated according to DFG (German Research Foundation) guideline (Bader et al., 2012).

Internal quality control was ensured by analysis of quality control (QC) samples with known concentrations. Additionally, blinded control samples were randomly included in all analytical cycles. As blinded control samples a native urine from one individual was used. After sample collection over several hours, urine was homogenized, aliquoted into pre-cleaned tubes and deep-frozen. The validity of internal QCs and blinded QCs was evaluated based on the acceptance criteria for internal QCs (±3σ). For internal QCs and blinded QCs, the acceptance criterion was fulfilled by at least 94% and 79% of cases, respectively. For additional quality control, field blanks of ultrapure water were used for mercury, cadmium, and chromium analysed in urine. Neither cadmium nor chromium could be detected in field blanks. 2% of field blanks used for mercury analysis showed levels slightly above the limit of quantification (LOQ).

External quality control was ensured for urinary mercury, cadmium, and chromium as well as lead and cadmium in blood by successful participation in the regular biannual ring trials German External Quality Assessment Scheme for analyses in biological materials (G-EQUAS) from 2015 to 2017 as well as by participation in the HBM4EU quality assurance programme (Nübler et al., 2021), see also Supplementary Tables S8 and S9. Table 1 summarises all elements, the respective acronyms, the CAS number, the analysed matrix, and LOQ.

To quantify lead and chromium in tap water samples, ICP-MS (x-series from Thermo Fisher Scientific equipped with an SC 2 DX autosampler from ESI Elemental Service & Instruments GmbH, Germany) was applied. All samples were acidified to a final concentration of 2% with nitric acid (Suprapur® purchased from Merck, Germany). An internal standard (2 ppb rhodium in 2% nitric acid) was continuously added online to the samples and determined simultaneously. If the internal standard concentration deviated from the control range (less than 80% or greater than 120% compared to the reference/blank), the measurement was repeated. Three QCs with different concentrations along the measurement range were included and measured regularly after about 30 measurements. The QC evaluation corresponds to the guidelines of the US Food and Drug Administration (FDA) and the limit of quantification complies with the DIN 32645 (2008-11).

2.3. Statistical analysis

First, we calculated basic statistical measures for all metals including analysed sample size (n), number of samples below LOQ, sample proportion equal to or exceeding LOQ, selected percentiles, maximum value, arithmetic mean, and geometric mean (GM) with its 95% confidence interval. Case weights, provided by the RKI (Hoffmann et al., 2018), are applied to account for minor deviations of the sample from the general 3- to 17-year-old population in Germany. Next, each variable was tested for differences in mean internal exposure levels (operationalized as GMs) of subgroups (sex, socioeconomic status (SES), migration background etc.) by applying a one-way ANOVA in SPSS 20 and 25. If 50% or more of the measured concentrations were below LOQ, the biomarker concentration was dichotomized and the difference between proportions of (volume-based) values above and below LOQ was checked with a χ^2 test of independence. Further information on statistical analyses and the classification of population subgroups are given in Murawski et al. (2020). SES is a multi-dimensional score summarising parent's income, education, and profession (Lampert et al., 2018).

Metals in blood and urine were already analysed in previous cycles of GerES. For lead, cadmium, and mercury the applied analytical methods

Table 1
Metals analysed in urine and blood of GerES V participants. Name, acronym, CAS number of the substances, matrix, and respective limit of quantification (LOQ).

Substance	Acronym	CAS Number	Matrix	LOQ (µg/L)
lead	Pb	7439-92-1	whole blood	2.1
cadmium	Cd	7440-43-9	whole blood	0.06
			first-morning void urine	0.05
mercury	Hg	7439-97-6	first-morning void urine	0.02
chromium	Cr	7440-47-3	first-morning void urine	0.2

CAS – Chemical Abstract Service.

allow for comparison of the present data to those of GerES II (1990–1992) and GerES IV (2003–2006). Therefore, the case weights of the previous cycles were adjusted to reflect the population structure of GerES V regarding age and sex. Data were re-evaluated according to a harmonized LOQ, i.e. taking the highest LOQ of the three surveys and re-assigning LOQ/2 for all values below the joint LOQ.

3. Results and discussion

3.1. Concentrations in blood and urine

General characteristics of the sample are given in Table 2. Statistical characteristics are described for the total sample, socio-demographic subgroups (sex, age, community size, SES, regions of residence (former East or West Germany), and migration background), and further variables regarding personal characteristics, behaviour, the living environment, and dietary patterns. Metals in first-morning void urine were measured in 2242–2250 samples and metals in blood in samples of 720 participants, respectively. In 100, 95, and 91% of the samples, lead, mercury, and chromium levels were above the LOQ. Cadmium was quantified in 76% of the urine and 44% of the blood samples. GMs for lead in blood were 9.47 µg/L, for cadmium in urine 0.072 µg/L (0.062 $\mu g/g_{crea}$) and in blood below LOQ, respectively, for mercury in urine 0.067 µg/L (0.057 $\mu g/g_{crea}$), and for chromium in urine 0.393 µg/L (0.335 $\mu g/g_{crea}$). Statistical characteristics for various subgroups are presented in Table 3 exemplarily for lead in blood and in Supplementary Tables S1–S7 for cadmium, mercury, and chromium. Results with respect to stratified subgroups are presented based on the significance level of 0.05. The p-value is shown in the respective tables.

Sex, age, and ethnicity/migrating background are common internal exposure determinants for describing exposure to lead and other metals. GM lead concentrations in blood were slightly, but significantly ($p < .05$), higher in males than in females, which was also reported by several other authors for children, adolescents and young adults (Baeyens et al., 2014; Burm et al., 2016; Etchevers et al., 2010; Lermen et al., 2021) and attributed partly to higher haematocrit levels of males, as lead binds to the proteins of erythrocytes in blood (ATSDR, 2007). Children aged 3–10 years had around 25% higher blood lead concentrations than 11-17-year-old adolescents (Fig. 1 & Table 3), which is in line with Burm et al. (2016), who discussed higher internal exposure of young children due to more frequent hand-to-mouth contact and hence more uptake of soil and dust and also goes hand in hand with EFSA Panel on Contaminants in the Food Chain (2010) who report a peak in blood lead in children aged 1–13. Children living in small communities or in former East Germany had significantly higher blood lead levels compared to those living in medium-sized or large communities or in former West Germany. In contrast to Burm et al. (2016), no differences regarding SES were found in the GerES V sample. Children with no migration background had 24–30% higher lead concentrations than those with migration background. Among the group of lead-specific determinants, the fuel used for heating – a rather little investigated factor – and exposure to tobacco smoke – a previously often included but differently measured factor – were analysed in relation to blood lead levels. Domestic fuel was associated with 21% higher lead concentration in blood (Fig. 1 & Table 3). Heating with oil, coal, pellet or other wood, however, cannot explain the observed difference in internal lead exposure between former East and West Germany, as the abundance of domestic fuel in GerES V was actually higher in former West Germany (Murawski et al., 2020). GM lead concentrations between smoking and non-smoking participants did not differ significantly (both self-reported and in urinary cotinine levels), but the GerES V sample contained only 2% of self-reported active smokers and 3% of active smokers identified by urinary cotinine. Non-smoking children and adolescents living with more than one smoker in the same household, however, had around 20% higher blood lead levels than those living in a non-smoking household, which is in line with Mannino et al. (2003) who operationalized second-hand smoke with categories of cotinine level in serum. The frequency of staying in a room where people smoke was not found to be associated with blood lead levels. However, only few participants reported on such passive smoke exposure.

Lead water pipes, where still in use, are another substantial source of lead exposure. Regardless of the remaining, rare cases of lead water pipes, lead as a component of alloys is still of relevance. The GerES V subsample analysed for lead in blood comprised only three individuals whose parents stated to have lead water pipes at home. None of them exceeded the reference value of 35 µg/L. Despite the low Pearson correlation coefficient between lead in blood and tap water lead concentration, a thorough investigation of the influence of the latter on the former with multivariate methods is currently in preparation.

Cadmium concentrations in both, blood and urine, were about the same for boys and girls, but increased significantly with increasing age (Fig. 1), as also reported by Burm et al. (2016). 14- to 17-year-old adolescents had 71% higher GM urinary cadmium levels than 3- to 5-year-old children. The age gradient was not observed in creatinine-adjusted results due to increasing creatinine concentrations with increasing age levelling off this effect (Barr et al., 2005). Children and adolescents living in large communities had 14–18% higher GM urinary cadmium concentrations (volume-based and creatinine-adjusted) and 1.4 times as often blood cadmium levels at or above LOQ compared to those living in small communities. No differences in urinary or blood cadmium concentrations were found regarding the SES of participants. Baeyens et al. (2014) discussed blood cadmium in relation to educational level, pointing towards higher levels associated with lower education due to higher proportions of smokers, but simultaneously increased cadmium

Table 2
Summary of metal concentrations in urine and blood in GerES V participants.

	n	n < LOQ	% ≥ LOQ	P10	P50	P90	P95	P98	MAX	AM	GM	95CI GM
in urine, volume-based (µg/L)												
Cadmium	2250	533	76	< LOQ	0.08	0.18	0.24	0.34	1.85	0.096	0.072	0.070–0.075
Mercury	2242	122	95	0.03	0.07	0.17	0.25	0.40	6.21	0.104	0.067	0.065–0.069
Chromium	2250	203	91	0.20	0.43	0.71	0.81	0.96	2.62	0.450	0.393	0.384–0.402
in urine, creatinine-adjusted ($\mu g/g_{creatinine}$)												
Cadmium	2250	–	–	*0.03*[a]	0.06	0.13	0.17	0.22	4.22	0.075	0.062	0.060–0.063
Mercury	2242	–	–	0.02	0.06	0.17	0.24	0.37	3.79	0.088	0.057	0.055–0.059
Chromium	2250	–	–	0.17	0.34	0.62	0.77	0.98	2.41	0.383	0.335	0.328–0.343
in blood (µg/L)												
Lead	720	0	100	5.3	9.4	16.8	19.9	24.0	129	10.62	9.47	9.16–9.80
Cadmium	720	406	44	< LOQ	< LOQ	0.19	0.23	0.30	2.74	< LOQ	< LOQ	–

The sample and subgroup sizes were calculated as the sum of case weights. Due to rounding to nearest whole numbers, the sum of stratified sample sizes does not always exactly match the total sample size. Further discrepancies may be due to missing values in stratification criteria.
n = sample size; % of values ≥ LOQ (limit of quantification); P10, P50, P90, P95, P98 = percentiles; MAX = maximum value; AM = arithmetic mean; GM = geometric mean; 95CI GM = approximate 95% confidence interval for the GM; – = not calculated (95CI GM not given, if lower limit < LOQ); [a] = corresponding volume-based value is <LOQ.

Table 3
Concentration of lead in blood (µg/L) in subpopulations of the GerES V participants.

	n	n < LOQ	P10	P50	P90	P95	P98	MAX	AM	GM	95CI GM
Sex (p = .039)											
male	371 (52%)	0	5.7	9.6	17.9	20.9	24.7	36.9	10.87	9.80	9.36–10.28
female	349 (48%)	0	5.1	9.2	16.0	19.2	22.8	129	10.36	9.13	8.71–9.58
Age group (p = .000)											
3–5 years	138 (19%)	0	6.3	9.6	19.4	23.1	31.4	32.1	11.52	10.43	9.69–11.23
6–10 years	231 (32%)	0	6.2	10.8	17.5	20.3	29.8	48.4	11.72	10.63	10.04–11.26
11–13 years	143 (20%)	0	4.6	8.3	16.1	17.5	22.0	23.0	9.23	8.36	7.76–9.01
14–17 years	208 (29%)	0	5.2	7.9	14.4	15.6	22.9	129	9.77	8.52	8.01–9.06
Community size (inhabitants) (p = .004)											
<50,000	194 (27%)	0	5.7	9.8	18.4	22.6	32.1	48.4	11.54	10.34	9.69–11.04
50,000 - < 100,000	45 (6%)	0	5.2	8.3	16.4	20.0	–	21.8	9.34	8.46	7.40–9.67
≥100,000	481 (67%)	0	5.2	9.2	16.1	19.8	22.2	129	10.37	9.24	8.87–9.63
Socio-economic status (p = .157)											
low	149 (21%)	0	5.7	9.2	16.4	21.3	32.1	129	11.29	9.58	8.85–10.37
medium	420 (60%)	0	5.3	9.6	17.4	20.0	22.0	48.4	10.63	9.66	9.26–10.07
high	135 (19%)	0	4.8	9.0	16.5	19.8	30.5	36.9	10.02	8.85	8.13–9.64
Region of residence (p = .000)											
former West Germany (including West Berlin)	602 (84%)	0	5.2	9.2	16.1	19.4	22.6	129	10.28	9.18	8.85–9.52
former East Germany (including East Berlin)	118 (16%)	0	6.5	11.1	19.7	23.3	38.1	48.4	12.38	11.12	10.23–12.08
Migration background (p = .000)											
no migration background	493 (70%)	0	5.7	10.1	19.2	21.0	25.6	48.4	11.31	10.22	9.82–10.64
one-sided migration background	66 (9%)	0	4.5	9.1	14.1	14.1	20.1	36.9	9.00	8.24	7.42–9.16
two-sided migration background	146 (21%)	0	4.7	7.6	13.1	14.7	19.0	129	9.20	7.86	7.30–8.46
Fuel for heating (p = .000)											
oil, coal, pellet or other wood	209 (30%)	0	5.3	11.1	19.2	22.6	32.1	129	12.64	10.85	10.12–11.64
other fuels	496 (70%)	0	5.2	9.0	14.8	19.4	21.6	36.9	9.84	9.00	8.67–9.34
Smoking status (p = .654)											
non-smoker	707 (98%)	0	5.3	9.4	16.6	19.9	24.0	129	10.62	9.46	9.15–9.79
smoker	13 (2%)	0	6.3	10.6	19.6	–	–	19.6	10.86	10.03	7.61–13.23
Cotinine concentration in urine (p = .494)											
<50 µg/L	690 (97%)	0	5.2	9.4	17.0	20.1	24.1	129	10.7	9.5	9.17–9.83
≥50 µg/L	22(3%)	0	5.8	9.4	16.5	19.6	–	19.6	9.5	8.9	7.44–10.58
Number of smokers in household (non-smoking children) (p = .000)											
no smoker	416 (59%)	0	4.8	9.3	16.5	19.8	22.8	36.9	10.24	9.23	8.82–9.65
one smoker	181 (26%)	0	5.5	9.1	14.9	18.0	25.2	129	10.55	9.10	8.52–9.73
more than one smoker	110 (16%)	0	7.0	10.7	19.6	22.6	32.1	32.1	12.13	11.09	10.24–12.00
Staying in rooms at home where people smoke (non-smoking children) (p = .112)											
never	628 (89%)	0	5.2	9.4	16.5	20.1	23.4	48.4	10.38	9.36	9.03–9.70
not daily	56 (8%)	0	5.8	9.4	19.2	19.2	129	129	13.36	10.69	9.27–12.33
daily	22 (3%)	0	6.7	9.2	19.3	26.9	–	26.9	10.34	9.60	8.22–11.23
Staying in rooms at friends' where people smoke (non-smoking children) (p = .466)											
never	550 (78%)	0	5.1	9.4	16.7	19.6	25.1	129	10.64	9.37	9.01–9.76
not daily	156 (22%)	0	6.6	9.3	17.0	20.3	22.8	26.9	10.55	9.78	9.20–10.39
daily	1 (0%)	0	–	–	–	–	–	17.0	–	–	–

The sample and subgroup sizes were calculated as the sum of case weights. Due to rounding to nearest whole numbers, the sum of stratified sample sizes does not always exactly match the total sample size. Further discrepancies may be due to missing values in stratification criteria.
n = sample size; P10, P50, P90, P95, P98 = percentiles; MAX = maximum value; AM = arithmetic mean; GM = geometric mean; 95CI GM = approximate 95% confidence interval for the GM; – = not calculated.
Significance test: One-way ANOVA (differences of GM) with the p-value given in brackets.

levels associated with high education due to increased consumption of vegetables. Urinary GM cadmium concentrations were 11–12% higher in participants living in former West Germany than in those living in former East Germany. Children and adolescents with two-sided migration background had 17–24% higher GM urinary cadmium concentrations and 42% more blood cadmium concentrations at or above LOQ than those with no migration background.

As tobacco smoke and vegetable consumption are important and previously investigated sources of internal cadmium exposure, analyses were extended to cover these aspects as well. Active smoking was found to be associated with 28% higher volume-based GM urinary concentrations when operationalized via self-report (n = 48; mean age = 16.1 years; sd age = 1.12 years) and 36% higher levels when measured with the urinary cotinine cut-off (n = 84, mean age = 15.3 years; sd age = 2.4 years) compared to non-smokers (self-report: n = 2201; mean age = 10.2 years; sd age = 4.26 years; cotinine: n = 2166; mean age = 10.1 years; sd age = 4.24 years). In blood, higher cadmium levels of smokers (self-report: n = 13; mean age = 15.6 years; sd age = 1.75 years; cotinine: n = 22; mean age = 15.5 years; sd age = 2.02 years) compared to non-smokers (self-report: n = 707; mean age = 10.1 years; sd age = 4.34 years; cotinine: n = 690; mean age = 10.1 years; sd age = 4.27 years) were reflected by more individuals with quantifiable concentrations (both in self-reported and in urinary cotinine levels), namely twice as many measurements at or above LOQ (Fig. 1). In contrast, passive smoke exposure of non-smoking children (mean age = 10.1; sd age = 4.3) was not found to be associated with increased urinary cadmium levels. However, staying daily in rooms at home where people smoke was associated with a significantly increased percentage of blood cadmium measurements at or above LOQ, indicating higher cadmium concentrations of passive smoking compared to non-exposed participants. Vegetarian diet was related to 35–41% increased average urinary cadmium concentration and about 1.5 times as many blood cadmium measurements at or above LOQ (Fig. 1). The European Food Safety Authority suggested that cereals, nuts, oilseeds, and pulses are the components of a vegetarian diet leading to vegetarian's higher internal exposure to cadmium (European Food Safety Authority, 2012). In addition, EFSA (2009) suggested that higher cadmium uptake is related to iron deficiency. A follow-up check of the levels of ferritin – an

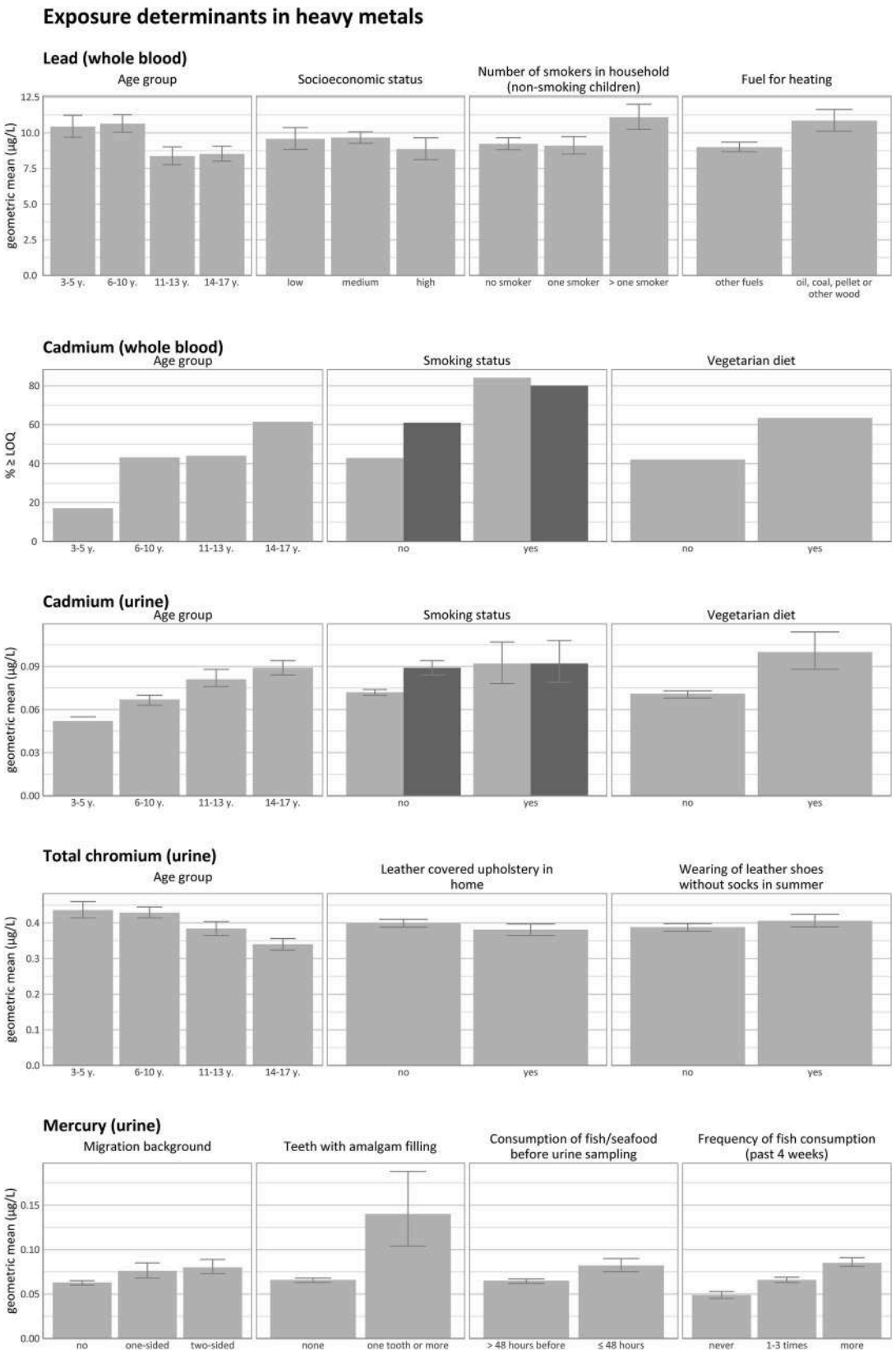

Fig. 1. Geometric mean (GM) or proportion of samples above or equal to LOQ (% ≥ LOQ) of metal concentrations in urine and blood for subgroups of selected exposure-relevant determinants. Bars represent the geometric mean of urinary concentrations (µg/L) or % ≥ LOQ, error bars (presented for GM only) indicate the 95% confidence interval of the GM. Within the factor smoking status light grey reflects the whole sample and dark grey bars show the subgroup of 14–17-year-olds who have about 10% more smokers (cotinine levels >50 µg/L) than other age groups which have less than 1% of smokers.

iron-storage protein – indicated that participants with a vegetarian diet had a significantly lower ferritin GM (23.2, CI = 19.9–27.1) than those with a non-vegetarian diet (GM = 30.5, CI = 29.4–31.6). Low iron levels might be an explanation for higher levels of cadmium in vegetarian children. Subgroup differences of internal exposure to cadmium were rather similar for both matrices. Albeit urinary and blood cadmium levels being considered measures for different internal exposure periods – blood reflecting the short-term internal exposure and urine being a measure for chronic internal exposure – our result might suggest continuously unchanged internal exposure of GerES V participants. Unfortunately, data on the physiological maturation on the cadmium kinetics are not available. Current approaches for estimating the lifetime risk by cadmium exposure consider the different dietary intake during different stages of life, but do not deal with different kinetics (Pruvost-Couvreur et al., 2020).

Mercury concentrations in urine were found to be significantly higher in boys than in girls (GM of 0.072 µg/L vs. 0.062 µg/L), but constant across age-groups when considering volume-based results in µg/L. Children and adolescents living in communities with ≥100,000 inhabitants and those with high SES had volume-based mercury concentrations significantly elevated by about 15% compared to their peers. The positive association with SES might be attributed to the higher consumption of fish and seafood by GerES V participants with high SES. Further checks revealed that the consumption of any seafood within two days before the urine sampling and the frequency of fish consumption within the last four weeks were significantly and positively associated with GM urinary mercury concentrations. Children and adolescents with migration background had significantly elevated (by 21–27%) volume-based mercury concentrations compared to those with no migration background.

With respect to previously reported mercury-specific determinants, results indicate that participants with at least one amalgam dental filling (n = 50; 2% of participants) had GM mercury concentrations more than twice as high as those without such tooth fillings. The effect of the ban by the EU commission on amalgam tooth fillings for children under 15 – effective as of 2017 (European Commission, 2017) – is yet to be seen in the upcoming decade. Consumption of chewing gum – a rather less investigated factor, if the participants also had teeth with amalgam filling, was not associated with elevated urinary mercury concentrations. Neither was the breaking of a fluorescent lamp (n = 196; 9% of participants) in the household within the last year associated with higher mercury levels in urine. Differences in internal mercury exposure found for migration background, teeth with amalgam fillings, and consumption of seafood are shown in Fig. 1.

Young children had significantly higher internal chromium exposure than adolescents. This effect was evident in both, volume-based and creatinine-adjusted GM urinary concentrations and amounted to 1.3 times higher levels in 3- to 5-year-olds than in 14- to 17-year-olds (Fig. 1). Apart from that, no differences in GM chromium concentrations were found regarding sex, community size, SES, region of residence, or migration background. Neither was exposure to leather upholstery at home nor skin contact to leather by wearing leather shoes without socks – rather little investigated factors – associated with increased urinary chromium concentrations.

As drinking water might contain chromium predominantly as a result of geological prerequisites (Allendorf et al., 2016; Kaprara et al., 2015; Mahringer et al., 2020), its concentration has been monitored. Tap water samples taken in the households of all participants were analysed for chromium which was found in quantities above the LOQ of 0.06 µg/L in slightly more than half of the samples. As shown in studies total chromium concentration in drinking water in Germany is represented by the concentration of Cr(VI) (Allendorf et al., 2016; Mertineit et al., 2013), this species has been detected by means of IC-ICP-MS. There was, however, no correlation between the chromium concentrations in urine and the concentrations of Cr(VI) in tap water (Pearson correlation coefficient < 0.1).

Since no specific source of internal chromium exposure could be identified here, the cause for the relatively higher internal exposure of young children remains unrevealed. Possibly, their higher uptake of soil and dust as discussed by Burm et al. (2016) regarding internal lead exposure is also the cause for increased internal chromium exposure. Multivariate analyses are needed in future to shed more light on exposure determinants in chromium concentrations.

The tests were made on individual metals but since the toxicity of their mixtures may depend on several interdependent factors (Wu et al., 2016) a mixture exposure approach is needed in future.

3.2. Comparison with previous cycles of GerES

Lead and cadmium in blood as well as cadmium and mercury in urine of children and adolescents were already analysed in the two previous cycles GerES II (sampled 1990–1991 in former West Germany and 1991–1992 in former East Germany; Krause et al., 1996) and GerES IV (2003–2006; Becker et al., 2008). For comparisons between former GerES cycles and GerES V all data sets were harmonized with the same LOQ, and trimmed and weighted to the intersecting age group of 6- to 14-year-olds. As a result, GerES II data are available for 715 participants' blood samples and 716 participants' urine samples, GerES IV data from 1225 and 1330, and GerES V from 424 up to 1349 blood and urine samples, respectively. Since the separation of Germany in 1949 is considered a (quasi-) natural experiment creating two states with different political systems and living conditions among other features which lasted for a long time beyond the reunification in 1990, we stratified the samples in addition by East and West Germany.

GMs for the full sample and for subsamples of former East and West Germany for each GerES cycle are shown in Fig. 2. The GMs for internal lead (blood) and mercury (urine) exposure indicate that concentrations were considerably lower in recent GerES cycles with a decrease of 74% and 80% from the earliest (GerES II) to the newest cycle (GerES V), respectively. The difference was more pronounced between GerES II to GerES IV with 58% decline for lead and 75% for mercury. Levels in mercury, however, strongly differed between East and West Germany in GerES II (1990–1992) and participants from East Germany contributed heavier to the observed overall decrease (85% in East vs. 68% in West German participants). Children and adolescents in GerES V had 38% and 18% lower lead and mercury levels than those in GerES IV with comparable levels of mercury in the two former parts in Germany in the recent cycle.

Declining trends for lead since the 1990s have been reported for young adults by the German time trend study Environmental Specimen Bank (ESB) which the authors interpreted as legal lead regulations having been effective (Lermen et al., 2021; Wiesmüller et al., 2007). For mercury, too, a decrease can be seen for young adults (Göen et al., 2018).

Comparisons of cadmium levels in blood and urine samples between different GerES cycles also suggest a considerable but smaller decrease than observed for lead and mercury. Overall, participants in GerES V had about 15% lower cadmium concentrations than GerES II children and adolescents. Participants from the former East Germany had considerably stronger disparities between the first two cycles (~40% in urine and ~25% in blood) and participants from the former West Germany almost none. Between the GerES IV and V cycle, cadmium urine levels are similar whereas cadmium blood concentrations are somewhat lower in the most recent cycle.

The smaller internal cadmium exposure in 6-year-old German children is also confirmed by the data of the DEMOCOPHES study, which samples were reanalysed in 2014 and showed a geometric mean of 0.051 µg/L urine (Schwedler et al., 2017). For young adults, the German time trend study ESB reported that cadmium levels in blood have remained unchanged between 2000 and 2005 (Wiesmüller et al., 2007) and publicly available data of the ESB indicate that cadmium in 24-hour-urine has decreased between 1990 and 2019 (UPB, 2021).

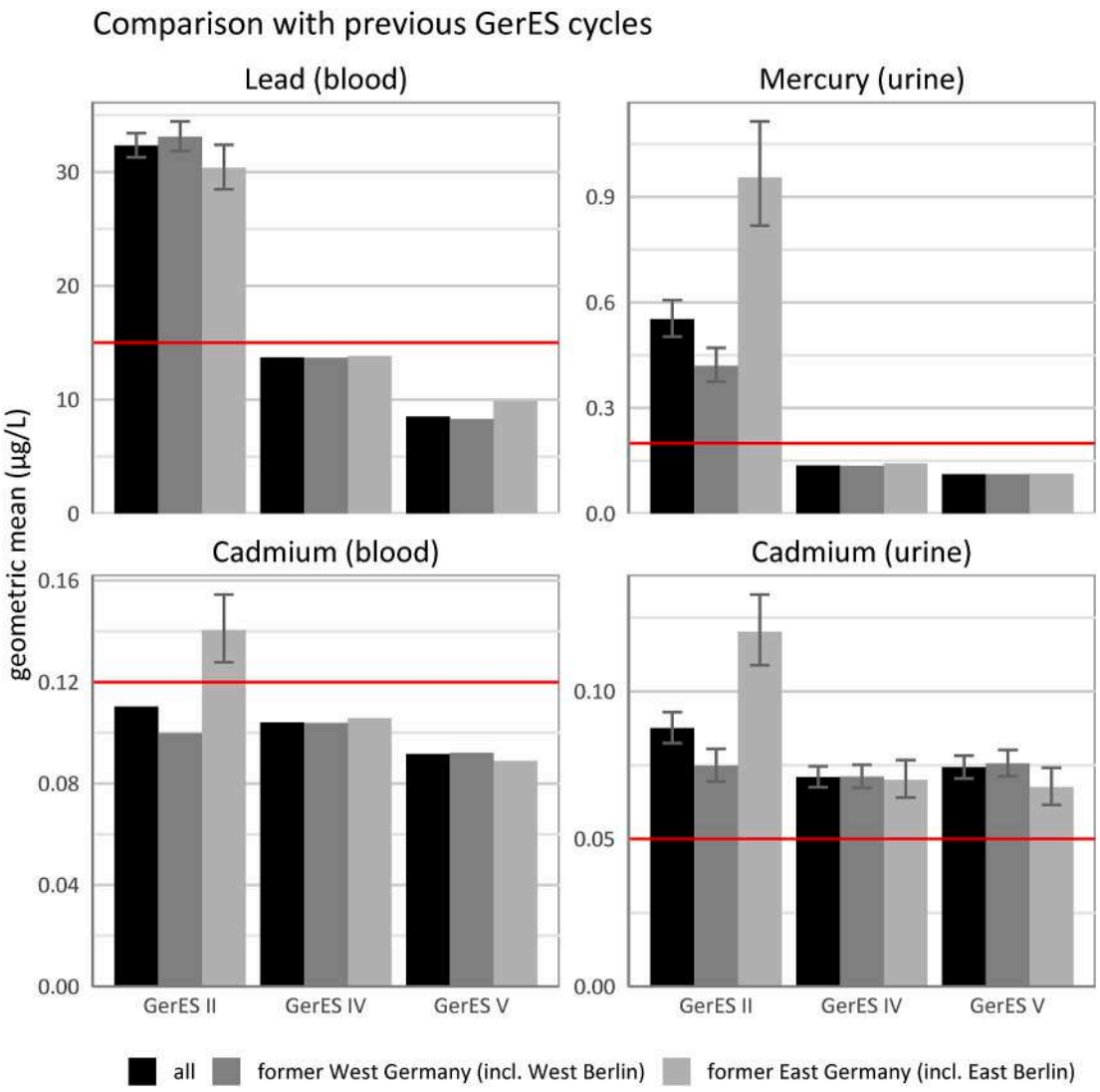

Fig. 2. Comparison of heavy metal concentrations in blood and urine for GerES II, GerES IV, and GerES V on the joint subset of 6- to 14-year-olds with case weights adjusted according to the GerES V population structure. Bars represent geometric mean urinary concentrations (µg/L) and error bars indicate its 95% confidence interval (only presented if above LOQ). The red horizontal lines denote the joint LOQ. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

While the approach of using the same LOQ for all cycles aimed at enhancing comparability with the most recent cycle GerES V, it is important to note that it might also have slightly influenced the total concentrations due to varying LOQs.

In sum, all four metals show lower levels in recent GerES cycles. A progressive approximation of internal exposure levels in the former East and West Germany with passing time since reunification has also been observed in levels of other chemical substances (e.g. polychlorinated biphenyls and organochlorine pesticides; Bandow et al., 2020; polycyclic aromatic hydrocarbons; Murawski et al., 2020).

3.3. Comparison with other national surveys

To enhance comparability between GerES V and other national HBM programs running around 2014 to 2017 we recalculated GMs, and P50s in GerES V for comparable age groups. While such posterior adaptations improve comparability between international studies, interpretations still need to be drawn with caution due to heterogeneity in study design, analytical methodologies, sampling years, analysed matrices etc. As shown in Table 4, we compared concentrations from GerES V to those obtained in a similar time period in the United States' National Health and Nutrition Examination Survey ("NHANES 2015–2016", CDC, 2019), the Canadian Health Measures Surveys ("CHMS 2014–2015" & "CHMS 2016–2017", Health Canada, 2019), the Korean Mothers' and Children's Environmental Health study ("MOCEH 2016", Jeong et al., 2017), the Korean Environmental Health Survey in Children and Adolescents ("KorEHS-C 2012–2014", Burm et al., 2016), the Environmental Health Monitoring System in the Czech Republic ("CZ-HBM 2016", SZÚ, 2017), the Swedish Riksmaten Adolescents study (2016-2017, Almerud et al., 2021), and the Flemish Environment and Health Study ("FLEHS III 2012–2015", De Craemer et al., 2017).

As can be seen in Table 4, in all age groups lead levels (GM or P50) of participants in GerES V were higher than in NHANES and CHMS, but similar to the other European studies CZ-HBM, Riksmaten, and FLEHS III, and lower than in MOCEH and KorEHS-C for 3-5-year-olds. The higher internal exposure of Korean children might be due to higher levels of metals in seafood and/or higher food intakes of rice and seafood (Ha et al., 2014; Lim et al., 2015). Interestingly, the age effect of younger children having relatively higher concentrations than their older counterparts (see section lead results in GerES V) is also visible in the other studies with more than one age group – NHANES, CHMS, and KorEHS-S – supporting Burm et al.'s (2016) explanation of more frequent

Table 4
Comparison of median (P50) and geometric mean (GM) concentrations (μg/L) of blood lead and cadmium in different national surveys in a similar time period. Values presented as "<" refer to concentrations below the respective LOQ or LOD (limit of detection).

survey/country	sampling year	age	medium	n	Lead		Cadmium	
					GM	P50	GM	P50
GerES V, Germany	2014–2017	3–5	whole blood	138	10.43	9.6	–	<0.12
NHANES, USA (CDC, 2019)	2015–2016	1–5	blood	790	7.58	6.90	–	<0.1
CHMS, Canada (Health Canada, 2019)	2014–2015	3–5	whole blood	479	6.7	6.4	0.082	0.093
CHMS, Canada (Health Canada, 2019)	2016–2017	3–5	whole blood	473	5.6	5.2	–	<0.097
MOCEH, Republic of Korea (Jeong et al., 2017)	2016	3	whole blood	555	15.3	–	0.90	–
		5		347	14.3	–	0.97	–
KorEHS-C, Republic of Korea (Burm et al., 2016)	2012–2014	3–5	whole blood	427	–	13.4	–	–
GerES V, Germany	2014–2017	5–9	whole blood	231	10.73	10.9	<0.12	<0.12
CZ-HBM, Czech Republic (SZÚ, 2017)	2016	5–9	whole blood	418	12.3	12.4	0.304	0.317
GerES V, Germany	2014–2017	6–11	whole blood	279	10.36	10.2	–	<0.12
NHANES, USA (CDC, 2019)	2015–2016	6–11	blood	1023	5.71	5.50	–	0.100
CHMS, Canada (Health Canada, 2019)	2014–2015	6–11	whole blood	925	5.9	5.6	0.094	0.10
CHMS, Canada (Health Canada, 2019)	2016–2017	6–11	whole blood	511	5.4	5.1	–	<0.097
KorEHS-C, Republic of Korea (Burm et al., 2016)	2012–2014	6–11	whole blood	958	–	12.6	–	–
GerES V, Germany	2014–2017	12	whole blood	48	8.89	8.5	<0.12	<0.12
Riksmaten Adolescents, Sweden (Almerud et al., 2021)	2016–2017	12	whole blood	331	–	7.3	–	0.10
GerES V, Germany	2014–2017	14–15	whole blood	101	8.56	8.07	<0.12	0.13
FLEHS III, Flanders, Belgium (De Craemer et al., 2017)	2012–2015	14–15	blood	406	9.26	–	0.185	–
GerES V, Germany	2014–2017	15	whole blood	52	8.51	7.6	0.120	0.13
Riksmaten Adolescents, Sweden (Almerud et al., 2021)	2016–2017	15	whole blood	410	–	7.0	–	0.13
GerES V, Germany	2014–2017	12–17	whole blood	304	8.35	8.1	<0.12	0.12
NHANES, USA (CDC, 2019)	2015–2016	12–19	blood	565	4.67	4.50	0.133	0.130
CHMS, Canada (Health Canada, 2019)	2014–2015	12–19	whole blood	974	5.4	5.1	0.14	0.12
CHMS, Canada (Health Canada, 2019)	2016–2017	12–19	whole blood	521	4.8	4.6	0.110	0.110
KorEHS-C, Republic of Korea (Burm et al., 2016)	2012–2014	12–18	whole blood	1003	–	11.4	–	–

hand-to-mouth contact in younger children.

Concentrations for cadmium in blood were very similar between GerES V and NHANES, CHMS, or Riksmaten (see Table 4 for details), slightly higher in FLEHS III, almost three times higher in the CZ-HBM and tenfold in the Korean MOCEH study (<0.12 μg/L in GerES V vs. 0.19 μg/L in FLEHS III, 0.30 μg/L in CZ-HBM, and 0.90 μg/L in MOCEH). A similar relative order of studies' concentrations can be found in cadmium concentrations in urine (Table 5). Those of GerES V were above reported LOQs of NHANES and CHMS, concentrations were higher in CZ-HBM (0.1 μg/L vs. 0.07 μg/L in GerES V for 5-9-year olds), and considerably higher in KorEHS-C (0.39 μg/L vs. 0.05 in GerES V for 3-5-year olds). In MOCEH, Riksmaten, and FLEHS III publications urinary cadmium was not reported.

Concentrations for urinary mercury were below LOQ in NHANES and available in CZ-HBM, with 5-9-year olds in the Czech Republic having been exposed to four times higher levels than the German participants (0.07 μg/L in GerES V vs. 0.28 μg/L in CZ-HBM). The observed overall higher concentrations of cadmium and mercury in CZ-HBM might be due to a higher environmental contamination and differences in the nutrition (Puklová et al., 2005, 2010).

Observed differences in internal exposure levels between national HBM programs can be influenced by various factors. To name a few, first, concentrations can depend on analysed matrix with morning urine (e.g. in GerES V) having higher levels of the analysed substance while spot urine (e.g. NHANES) having lower concentrations and being strongly influenced by the diurnal variations. In addition, due to differences in chemical analyses, low level differences such as between NHANES and GerES V (similar in cadmium in blood but GerES V has higher urinary cadmium concentrations) are very uncertain. However, the applied analytical procedure and its performance were comparable to the methods used in other national surveys. Comparisons between countries can also depend on local environmental conditions (e.g. levels in tap water, degree of urbanisation) or could be confounded by different sampling years underlying time dependent differences such as, for example, decreasing concentrations over time. Although age groups were adjusted where possible in GerES V to be comparable to those in the other studies, participants of the age 1, 2, 18 and 19 were not recruited in GerES V. The lack of national programs investigating

Table 5
Comparison of median (P50) and geometric mean (GM) concentrations (μg/L) of urinary cadmium and mercury in different national surveys in a similar time period. Values presented as "<" refer to concentrations below the respective LOQ or LOD (limit of detection).

survey/country	sampling year	age	medium	n	Cadmium		Mercury	
					GM	P50	GM	P50
GerES V, Germany	2015–2017	3–5	first-morning void	394–399	–	0.05	–	0.07
NHANES, USA (CDC, 2019)	2015–2016	3–5	spot urine	486–496	–	<0.036	–	<0.13
CHMS, Canada (Health Canada, 2019)	2016–2017	3–5	spot urine	553	–	<0.066	–	–
KorEHS-C, Republic of Korea (Burm et al., 2016)	2012–2014	3–5	12-h urine	427	–	0.39	–	–
GerES V, Germany	2015–2017	5–9	first-morning void	735–736	0.063	0.07	0.068	0.07
CZ-HBM, Czech Republic (SZÚ, 2017)	2016	5–9	urine	400	0.096	0.096	0.276	0.28
GerES V, Germany	2015–2017	6–11	first-morning void	884–886	–	0.07	–	0.07
NHANES, USA (CDC, 2019)	2015–2016	6–11	spot urine	379–380	–	<0.036	–	<0.13
CHMS, Canada (Health Canada, 2019)	2016–2017	6–11	spot urine	538	–	<0.066	–	–
KorEHS-C, Republic of Korea (Burm et al., 2016)	2012–2014	6–11	first-morning void	958	–	0.37	–	–
GerES V, Germany	2015–2017	12–17	first-morning void	965	0.087	0.09	0.068	0.07
NHANES, USA (CDC, 2019)	2015–2016	12–19	spot urine	402	0.055	0.049	–	<0.13
CHMS, Canada (Health Canada, 2019)	2016–2017	12–19	spot urine	534	–	<0.066	–	–
KorEHS-C, Republic of Korea (Burm et al., 2016)	2012–2014	12–18	first-morning void	1003	–	0.44	–	–

background internal exposure goes hand in hand with underrepresented countries and continents where priority is often given to high exposure in hot spots, but studies investigating various contamination conditions are emerging (e.g. Zambia, Yabe et al., 2020). In addition, more harmonising efforts between national studies such as the German External Quality Assessment Scheme (G-EQUAS) in which KorES-C and GerES V have participated, or the European initiative HBM4EU (Ganzleben et al., 2017) are needed to embed results from national HBM programs into international internal exposure to pollutants.

3.4. Comparison with human biomonitoring assessment values

The German Human Biomonitoring Commission derived health based guidance values (HBM values) for cadmium in urine and mercury in urine and blood (German Human Biomonitoring Commission, 1999; 2011). The HBM-I value is defined as the concentration of a substance in human biological material at and below which, according to current knowledge, no risk for adverse health effects is to be expected (Apel et al., 2017). The HBM-II value "describes the concentration of a substance in human biological material at which and above which adverse health effects are possible and, consequently, an acute need for the reduction of exposure and the provision of biomedical advice is given" (Apel et al., 2017).

The HBM-I value for urinary cadmium was defined on a level as to avoid kidney-damaging cadmium accumulation (German Human Biomonitoring Commission, 2011) and set to 0.5 µg/L for children and adolescents, the HBM-II value was set to 2 µg/L. 0.6% of the sample reached or exceeded the HBM-I value, which amounts to 67,740 children and adolescents in Germany. None of those exceeding the HBM-I value was an active smoker or considerably exposed to environmental tobacco smoke, which suggests that even non-tobacco exposure sources can amount to health-relevant internal cadmium exposure. The HBM-II value was not exceeded by any of the participants.

The HBM-I value for mercury in urine was exceeded by none of the GerES V participants – the MAX observed concentration was 6.21 µg/L (3.79 µg/gcrea) and the HBM-I value is 7 µg/L (5 µg/gcrea) (German Human Biomonitoring Commission, 1999). However, as additional information on the impact of mercury on health has been generated (Bellanger et al., 2013), the HBM values might benefit from a revision.

4. Conclusion

In the population representative GerES V (2014–2017), cadmium and lead were measured in blood, and chromium, cadmium, and mercury in urine of 3- to 17-year-old children and adolescents living in Germany. Results from bivariate analyses indicate that younger children had higher levels of lead and chromium than older children, and boys showed higher concentrations of mercury than girls. Among substance-specific determinants of internal exposure, using domestic fuel was associated with higher concentrations of lead and smoking and vegetarian diet were associated with higher internal cadmium exposure. While we did not identify specific sources for chromium, mercury concentrations were higher when children consumed more fish and seafood or had amalgam tooth fillings. Multivariate analyses are needed in future to shed light onto possibly confounding factors (e.g., age and sex). Comparison with data on internal lead, cadmium and mercury exposure collected in previous GerES cycles conducted from 1990 to 1992 and 2003 to 2006, concentrations were clearly lower the later the study was conducted. The comparison of the GerES V data with the data of other national surveys showed internal exposure in the same magnitude, but also revealed differences, which were almost reasonable in view of the specific environmental contamination and life-style factors.

Declaration of competing interest

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

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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.2021.113822.

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Measuring household hygiene access and handwashing behaviors: Findings from 14 low- and middle-income countries

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Handwashing with soap (HWWS) is critical for preventing diarrheal and respiratory infections and is an important policy priority to achieve the Sustainable Development Goals (SDGs). We analyzed hygiene data from 36,860 household surveys from rural areas in India, Honduras, and twelve countries in sub-Saharan Africa (SSA). We report descriptive statistics and compare and critique three indicators: (1) access to basic hygiene services, defined as a reported designated handwashing area with observed water and soap at the time of the survey; (2) use of both soap and water during demonstrated handwashing; and (3) reported handwashing both after defecation and before preparing food. Overall, 10% of surveyed households (4% in SSA) had access to basic hygiene services and 48% of respondents (45% in SSA) used both soap and water during demonstrated handwashing. Inconsistencies between these indicators suggest no single indicator can provide a holistic picture of household hygiene; reporting on handwashing infrastructure alone may underestimate household access to soap and water and HWWS behaviors. Across the 14 countries, there was an average 22 percentage point (p.p.) gap (median 20 p.p.) in use of both water and soap during demonstrated handwashing between respondents in the wealthiest and poorest quintiles surveyed. This finding highlights the continued need to emphasize inclusivity aspects of the SDGs. Data around respondents' reported exposure to hygiene promotion showed that respondents rarely heard messaging about cleanliness from faith leaders, revealing an overlooked opportunity to empower faith leaders to promote handwashing in low- and middle-income countries.

Abstract

1. Introduction

Handwashing with soap (HWWS) is critical for infection prevention and is recognized as a global policy priority under Sustainable Development Goal (SDG) 6.2, "By 2030, achieve access to adequate and equitable sanitation and hygiene for all" (United Nations General Assembly, 2015). Hands can transport pathogens that use airborne, fomite, and fecal-oral routes. HWWS can reduce diarrheal disease by an estimated 23–47% and is especially important in preventing diarrheal disease in settings lacking adequate water and sanitation services (Aiello et al., 2008; Curtis and Cairncross, 2003; Ejemot et al., 2008; Fewtrell et al., 2005; Freeman et al., 2014; GBD Diarrhoeal Diseases Collaborators, 2018; Wolf et al., 2018). HWWS can reduce acute respiratory infections by 16–21% (Aiello et al., 2008; Rabie and Curtis, 2006) and has been central in efforts to control COVID-19 transmission, especially in communities facing inadequate healthcare infrastructure, a shortage of personal protective equipment, and challenges implementing social distancing (Bong et al., 2020; Brauer et al., 2020; WHO and UNICEF, 2020a).

The WHO and UNICEF Joint Monitoring Programme for Water Supply, Sanitation, and Hygiene (JMP) monitors progress towards SDG 6.2 through the target, "proportion of population with a handwashing facility with soap and water available at home" (United Nations General Assembly, 2015; WHO and UNICEF, 2019), also called access to "basic hygiene services." The JMP reports 40% of the global population and 75% of the sub-Saharan African (SSA) population lack access to a household handwashing facility with water and soap (WHO and

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UNICEF, 2019). This estimate includes 78 countries and represents 52% of the global population (WHO and UNICEF, 2019). Other studies have estimated 26–27% of the global population (Brauer et al., 2020; Wolf et al., 2019) and 82–91% of the population in Africa (Roche et al., 2017; Wolf et al., 2019) lack access to a household handwashing facility with soap and water.

To measure access to basic hygiene services, the JMP recommends the survey enumerator ask to be shown where members of the household most often wash their hands, and then observe the availability of water and soap at that area (WHO and UNICEF, 2018). According to the JMP definitions, "handwashing facilities can be fixed or mobile and include a sink with tap water, buckets with taps, tippy-taps, and jugs or basins designated for handwashing. Soap includes bar soap, liquid soap, powder detergent, and soapy water but does not include ash, soil, sand or other handwashing agents" (WHO and UNICEF, 2018). Prior to the release of this guidance by the JMP in 2018, household surveys often used disparate questions or methods for monitoring hygiene access, and may not have defined handwashing facility as broadly (Loughnan et al., 2015).

While access to basic hygiene services is a reliable and cost-efficient indicator for HWWS behaviors when consistently defined (Ram, 2013; WHO and UNICEF, 2018), it does not directly relay information about HWWS technique or frequency of HWWS at critical times for infection prevention. Sub-national, national, and global estimates of HWWS behaviors are limited. Rates of HWWS at critical times as measured through structured observations are generally associated with – but lower than – the proportion of people with a household handwashing facility with soap and water (Biran et al., 2008; Ram et al., 2014; Wichaidit et al., 2019; Wolf et al., 2019). In this study, we analyzed hygiene access and reported and demonstrated handwashing indicators from a large-scale, multi-country household survey to understand the relationships between such indicators and offer additional data to monitor progress towards SDG 6.2.

HWWS in the home is determined both by an enabling household environment and an individual's motivations and habits (White et al., 2020). Where enabling infrastructure and psychological determinants are lacking, actors including government officials, community health workers, and non-governmental organizations (NGOs) promote HWWS through behavior change campaigns. At-scale hygiene promotion interventions are moderately or inconsistently effective (Biran et al., 2009; Briceno et al., 2015; Chase and Do, 2012; De Buck et al., 2017; Galiani et al., 2012; Wichaidit et al., 2019). Inconsistent intervention outcomes are often blamed on insufficient handwashing infrastructure (Biran et al., 2012; De Buck et al., 2017; Hoque, 2003); overemphasis on increasing people's knowledge about HWWS and under-emphasis on altering attitudes and norms (Wichaidit et al., 2019; Wilson et al., 2011); and the short-term or infrequent nature of promotional campaigns (Naikoba and Hayward, 2001; Pickering et al., 2019). Channels for HWWS messaging vary widely, from national mass media communication to community-based interpersonal efforts (De Buck et al., 2017). While evidence suggests that trusting or admiring the person delivering hygiene messages is linked to their effectiveness (De Buck et al., 2017; White et al., 2020), there is little evidence around which community actors are most involved with or suitable for community-based hygiene promotion efforts. We present data on where and from whom respondents report hearing hygiene messaging to add to this conversation.

In this study, we analyzed hygiene data from 36,860 household surveys across 14 countries, including twelve in SSA, to quantify and compare three hygiene monitoring indicators: (1) household access to basic hygiene services, defined as a reported designated handwashing area with observed water and soap at the time of the survey; (2) respondents' use of both soap and water during demonstrated handwashing; and (3) reported handwashing both after defecation and before preparing food, the two most critical times for preventing diarrheal disease according to the literature (Adane et al., 2018; Luby et al., 2011). We disaggregate these indicators between the wealthiest and poorest quintiles of respondents, and identify household characteristics associated with each indicator using Honduras and India as case studies to identify drivers of hygiene access and HWWS behaviors. We explore data around what actors and places are involved in household hygiene messaging to highlight opportunities for HWWS promotion.

2. Methods

2.1. Study population and sampling

36,860 household surveys were conducted from July to December 2017 in rural areas of 14 countries: Ethiopia, Ghana, Honduras, India, Kenya, Malawi, Mali, Mozambique, Niger, Rwanda, Tanzania, Uganda, Zambia, and Zimbabwe. The international non-governmental organization (iNGO) World Vision commissioned data collection as part of a water, sanitation, and hygiene evaluation of its program areas and randomly selected non-program areas. Deidentified datasets from the evaluation are publicly available through the Water Institute at the University of North Carolina (UNC) (The Water Institute at UNC; World Vision Inc., 2020).

The full sampling methodology is described by The Water Institute at UNC (2020). In short, the study employed multi-stage, population-based sampling. Rural clusters were identified through a national census bureau or statistics office and randomly selected with probability proportional to size allocation from two strata: areas where World Vision has active water, sanitation, and hygiene programs and areas where World Vision does not work. Within each stratum, 56 clusters were identified. All households in selected clusters were mapped and 25 households were randomly selected for the survey.

2.2. Data collection

The survey instruments included interview questions and direct observations (see Table 1 for questions used to generate the three hygiene indicators used in this study). The survey included questions to determine household water and sanitation service level definitions according to the JMP definitions (WHO and UNICEF, 2019). The survey instrument was translated into local languages and verified by research consultants or World Vision staff. Data were collected electronically using the mobile survey tool mWater (New York, NY, USA) by local research supervisors and enumerators who were experienced administering mobile surveys and trained to use the instrument. Surveys were administered to the female head of household. If she was not available, another adult household member was surveyed. If no one was available or a household declined to respond, an alternative household was randomly selected from the cluster.

The survey instrument was developed prior to the JMP's 2018 publication on core hygiene monitoring questions (WHO and UNICEF, 2018), which recommends the enumerator ask to be shown where members of the household most often wash their hands. The JMP question is more inclusive than the corresponding question used in the present survey, which asked about a "designated area for handwashing." The latter implies the handwashing facility is used solely for that purpose.

2.3. Analysis

All data were exported from mWater into Stata/SE 14.2 (College Station, TX, USA) for cleaning and analysis. Analysis consisted of descriptive statistics and cross-tabulations. We report the median values from each of the 14 countries due to outliers in the data. We present aggregate descriptive statistics for all 36,860 households. Our analysis centers around three hygiene indicators: (1) household access to basic hygiene services, defined as a reported designated handwashing area with observed water and soap at the time of the survey; (2) respondents' use of both soap and water during demonstrated handwashing; and (3)

Table 1
Survey questions and observations used to generate three hygiene indicators.

Hygiene indicator	Question	Question type	Coded responses
Household access to basic hygiene services, defined as a reported designated handwashing area with observed soap and water at time of survey	Does your household have a designated area for handwashing?	Reported; select-one	Yes No Decline to state
	If yes, can you show me your handwashing area? *[Observe] which of the following are present at the handwashing station?*	Observation; select all that apply	Water Soap Ash Alcohol-based hand rub Other cleanser Hygienic drying material
Respondents' use of both soap and water during demonstrated handwashing	Can you show me how you wash your hands? *[Observe] what actions do they make?*	Observation; select all that apply	Use of water Use of soap Use of ash Makes contact between both hands Makes rubbing motion Hygienic hand drying
Reported handwashing both after defecation and before preparing food	When do you wash your hands? *[Note: This question did not specify whether respondent washes hands with both soap and water.]*	Reported; do not read answer choices; select all that apply	After defecation After cleaning or changing a baby/child Before food preparation Before eating Before feeding a child After eating After working outside After greeting people After coming home from places or functions After cleaning Respondent doesn't wash hands ever Other

reported handwashing both after defecation and before preparing food. During demonstrated handwashing, respondents were not provided any materials or instructions for handwashing; any water and soap used was already present.

Wealth quintiles were calculated in accordance with protocols developed by the Demographic and Health Surveys (DHS) Program (Rutstein, 2015). We conducted multivariable logistic regression to explore factors associated with the three binary hygiene indicator outcomes in Honduras and India, as all other countries in this study had very low levels of access to basic hygiene services. Independent variables were the same for both countries and included: the education level of the male head-of-household; household wealth quintile relative to other households surveyed; household's water and sanitation service levels according to the JMP definitions (WHO and UNICEF, 2019); whether the household's water point is on the plot; whether the household pays for water; whether the household has one or more members under the age of five or with a disability. To assess model validity, regression diagnostics were conducted to examine specification errors, goodness-of-fit, and multicollinearity. For all regressions, statistical significance was evaluated with a p-value of 0.05; odds-ratios and 95% confidence intervals are also reported.

2.4. Ethical approval

Informed consent was obtained from all participants in their own language before beginning the survey. This study was approved by the UNC-Chapel Hill Institutional Review Board (IRB #17–0663) and by the appropriate agencies within each country including the National Regional Government of Oromia Planning and Economic Development Commission in Ethiopia (reference: WVE/ORO/0393/2017); the Ministry of Water Resources in Ghana (reference: TJMSW); the Secretary of Energy and Natural Resources in Honduras (reference: DMA-0220-2017); the SRM University School of Public Health in India (reference: SRMSPH/IEC001/2017/July 24, 2017); the Ministry of Water and Irrigation in Kenya (reference: MWI/PARAS/10/62/(31)); the Director of Irrigation and Water Development in Malawi (reference: IWD/CONF/1/1); the University of Bamako Medical School in Mali (reference: 2017/105/CE/FMPOS); the National Institute of Statistics in Mozambique (reference: 2/DICRE/INE/900/2017); the Ministry of Water Resources in Niger (reference: 000008/MH/A/DGH); the Ministry of Infrastructure in Rwanda (reference: ND/JOBD/WASH/IPD/20/03/17); the National Institute for Medical Research in Tanzania (NIMR/HQ/R.8a/Vol. IX/2386); the Makarere University School of Biomedical Sciences ethics committee in Uganda (SBS-HDREC-437); the Ministry of Local Government and Housing in Zambia (MLGH/101/18/102); and the Medical Research Council in Zimbabwe (reference: MRCZ/A/2223).

3. Results

3.1. Access to basic hygiene services

Household access to hygiene services was defined in the survey using two survey questions (Table 1). Respondents were first asked whether they had a designated handwashing area; if yes, then enumerators observed the designated area for the presence of soap and water. By this definition, a median of 5% of households surveyed in each country (10% of all 36,860 households) had access to basic hygiene services (Fig. 1). Access to basic hygiene services was highest in Honduras (60%) and India (25%).

A median of 3% of households (5% all households) had limited hygiene services, defined as having a designated handwashing area without observed water and/or soap. Limited service was more often due to the absence of soap than the absence of water. A median of 62% of handwashing areas had observed soap at the time of the survey (Table S1); among the twelve SSA countries, a median of 53% of handwashing areas had observed soap. Almost all designated handwashing areas (median of 94% across countries) had observed water at the time of survey. Designated handwashing areas rarely had observed hygienic drying materials, defined as disposable paper towels or a clean cloth towel (median of <1%).

The majority of observed designated handwashing areas (72% total) were within 5 meters of the household's food preparation area. In addition to observing designated handwashing areas, enumerators observed respondents' closest latrine. Of the 60% of respondents with observed latrines, a median of 14% had a handwashing station within 5 meters of the latrine. Almost half (46%, n = 1986) of those respondents

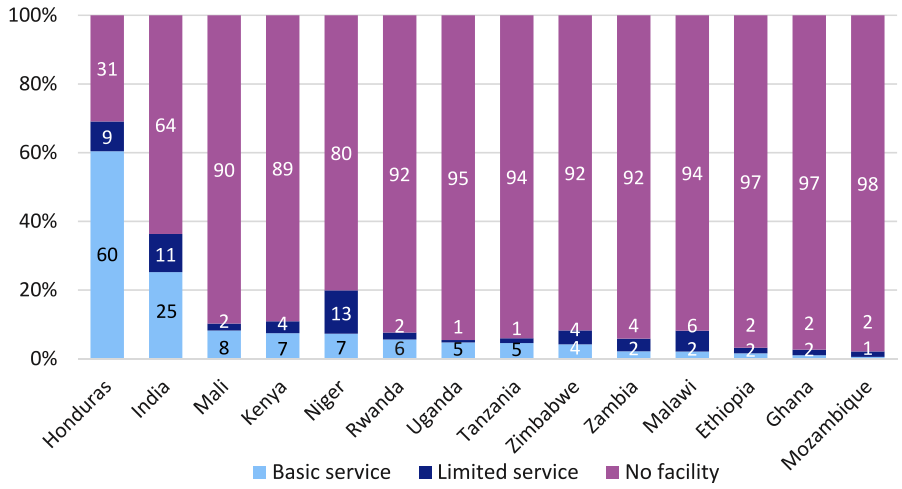

Fig. 1. Household hygiene service levels by country. Basic service is defined as having a designated handwashing area with observed water and soap at the time of the survey. Limited service is defined as having a designated handwashing area without observed water and/or soap.

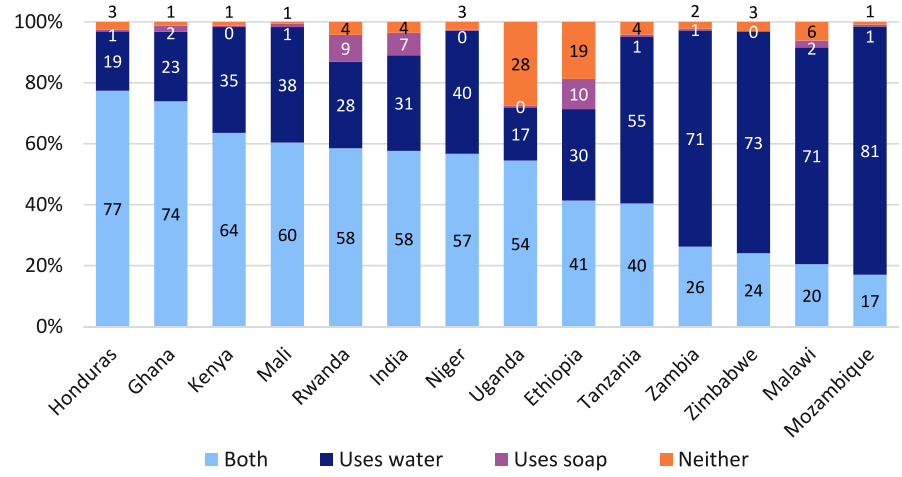

Fig. 2. Percentage of respondents who use water, soap, both, or neither during demonstrated handwashing by country.

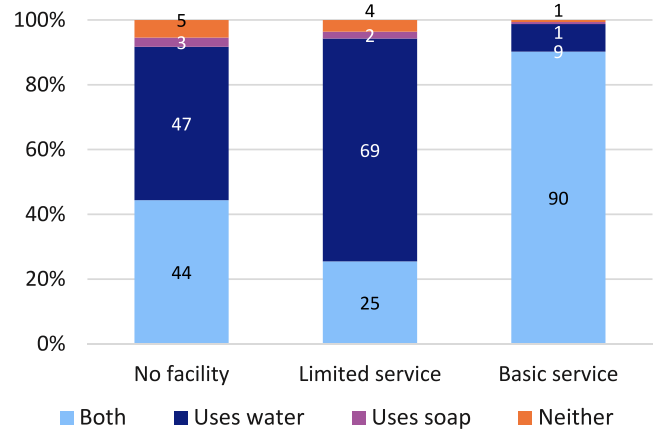

Fig. 3. Percentage of respondents who use water, soap, both, or neither during demonstrated handwashing by hygiene service level for all countries.

with a handwashing station near their observed latrine conflictingly answered that they did not have a designated handwashing area in their household when asked earlier in the survey. Overall, 17% (n = 889) of respondents with a designated handwashing area had a handwashing station that was not observed to be near a food preparation area nor near a latrine.

3.2. *Demonstrated handwashing*

When the enumerator asked respondents to show how they wash their hands, most respondents used water (median 97%) and about half used both water and soap (median 56%) (Fig. 2). Few respondents substituted soap with ash, except in Ethiopia (18%) and India (33%). The majority (90%) of respondents with access to basic hygiene services used both soap and water, while the majority (69%) of respondents with limited hygiene service used only water without soap (Fig. 3). Of the respondents without a reported designated handwashing area, 44% used both water and soap, and 47% used water without soap. Use of hygienic drying materials was rare at a median of 4% (Table S2).

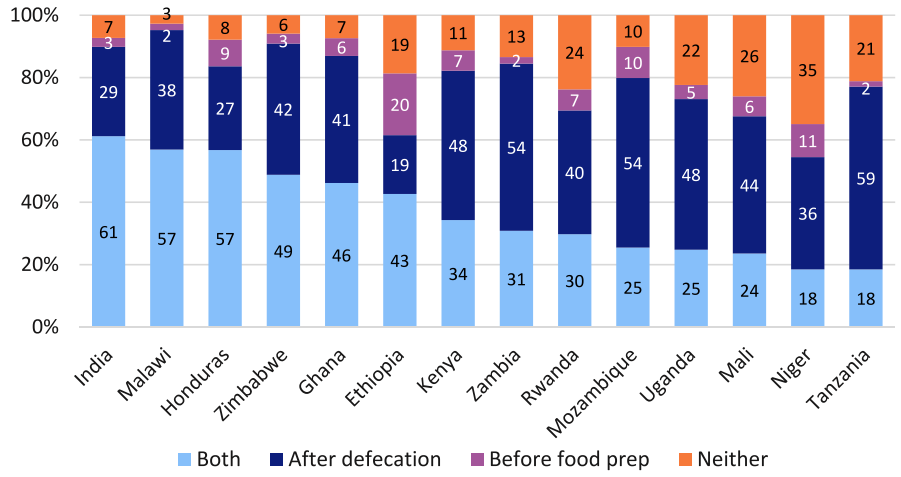

Fig. 4. Percentage of respondents who report handwashing after defecation, before preparing food, both, or neither by country.

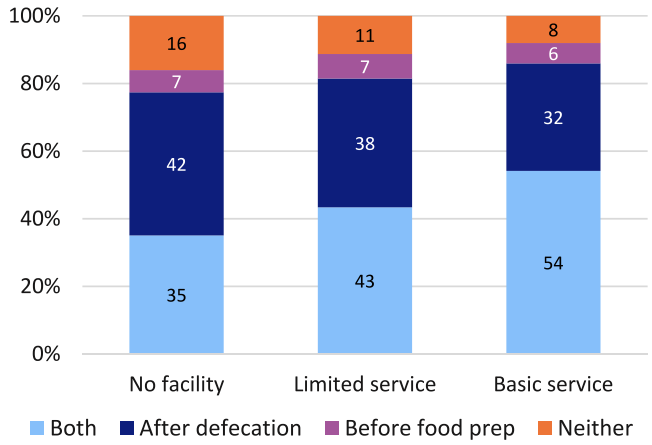

Fig. 5. Percentage of respondents who report handwashing after defecation, before preparing food, both, or neither by hygiene service level for all countries.

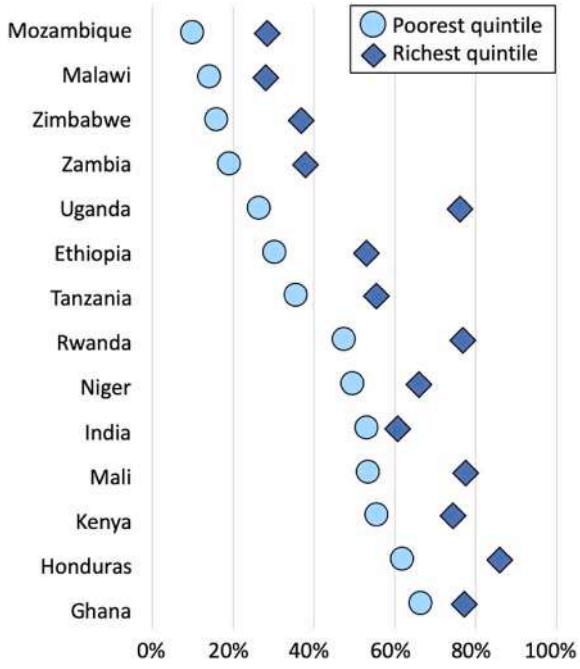

Fig. 6. Percent of respondents in wealthiest and poorest quintiles who use both water and soap during demonstrated handwashing.

3.3. Reported handwashing times

Enumerators asked respondents to list the times they wash their hands (and enumerators did not read aloud any potential answer choices). This question did not specify whether the respondent uses soap and water. The most frequently reported handwashing times were before eating (median 90%), after defecating (median 81%), and after eating (median 72%) (Table S3). Fig. 4 shows the overlap between handwashing after defecation and before preparing food, the two most important events for preventing diarrheal disease according to the literature (Adane et al., 2018; Luby et al., 2011). Handwashing both after defecation and before preparing food was higher among respondents with access to basic hygiene services (54%) than among those with limited services (43%) or without a designated handwashing area (35%) (Fig. 5). Handwashing both after defecation and before preparing food was highest among respondents who demonstrated handwashing with both soap and water (57%) and lowest among respondents who demonstrated handwashing with neither soap nor water (33%) (Fig. S1).

3.4. Factors associated with hygiene access and handwashing behaviors

The wealthiest quintile of respondents consistently had higher outcomes than the poorest quintile of respondents across countries and hygiene indicators. The greatest disparity was seen using the demonstrated HWWS indicator (Fig. 6). There was an average 22 percentage point (p.p.) difference (median 20 p.p.) in demonstrated handwashing with both soap and water between the wealthiest and poorest quintile of respondents, with the largest gap seen in Uganda (50 p.p.). Gaps in access to basic hygiene services between the poorest and wealthiest quintiles averaged 11 p.p. (median 7 p.p.), ranging from 2 p.p. in Ghana to 35 p.p. in India (Fig. S2). Gaps in reported handwashing at the two critical times averaged 12 p.p. (median 13 p.p.) and were evident in every country except India (Fig. S3).

Results from multivariable logistic regressions using data from Honduras (Table S4) and India (Table S5), the two countries with the highest access to basic hygiene services in this study, are included in the Supplemental Materials. In brief, wealthier households were more likely to have access to basic hygiene services in both Honduras and India. In Honduras, wealth quintiles were also associated with demonstrated handwashing with soap and water and with reported handwashing at the two critical times. There were mixed findings around associations between the hygiene indicators and the sanitation service level and

water supply variables in the regression analyses. There were mixed findings around associations between the hygiene indicators and household demographic variables including education levels, presence of a child under five, and presence of a household member with a disability.

3.5. Information about cleanliness

Enumerators asked respondents several questions about their access to information about cleanliness and selected all answers that applied (Table S6).[1] About half of respondents (median 51%) could recall the last time someone spoke to them about cleanliness. Respondents most frequently reported hearing about cleanliness from government personnel (median 49%) and NGO workers (median 21%). Such conversations most frequently took place in the respondent's home (median 59%) or at a community gathering (median 52%). Respondents rarely reported hearing cleanliness messages from faith leaders (median 4%), from trained religious volunteers (median 1%), or in places of worship (median 3%).

4. Discussion

We organized our discussion into three sections. First, we propose explanations for inconsistencies between the hygiene access and handwashing behavior indicators analyzed, as well as between our findings and the literature. This discussion examines each indicator's benefits and disadvantages and explores the relationship between hygiene access as commonly reported in the literature and actual hygiene behaviors. Next, we discuss wealth disparities and elaborate on household characteristics associated with hygiene indicators to illustrate constraints and drivers of hygiene access and handwashing. Finally, we propose recommendations around hygiene monitoring and programming based on our findings and briefly highlight opportunities for faith leaders to engage in hygiene promotion.

4.1. Comparison of hygiene access and handwashing behaviors indicators

The data from our study suggest that access to basic hygiene services – defined as a reported designated handwashing area with observed water and soap – was less than 10% across all surveyed households, lower than other published estimates (Brauer et al., 2020; Roche et al., 2017; WHO and UNICEF, 2019; Wolf et al., 2019). Estimates of low hygiene access in this study are due in part to intentional sampling bias; the survey was part of an NGO program evaluation which was not nationally representative and may disproportionately include low-resource areas. This may explain why our estimates were generally closest to the JMP's hygiene access estimates for each country's poorest quintile (WHO and UNICEF, 2019).

Our low estimates may also be explained by systemic measurement bias. The survey was developed before the JMP published guidance around core hygiene monitoring questions, which recommends asking to be shown where household members most often wash their hands and observing the availability of soap and water there (WHO and UNICEF, 2018). The survey analyzed in the present article asked whether the household has "a designated area for handwashing," and observed the availability of soap and water there only if the respondent answered yes. It is possible enumerators and/or respondents may not have interpreted "a designated area for handwashing" as broadly as the JMP, which classifies mobile units, buckets with taps, tippy-taps, and designated jugs or basins, both inside and outside of the dwelling as "handwashing facilities" (WHO and UNICEF, 2018). Of the 12% of total respondents (n = 4318) with a handwashing station near their observed latrine, almost half (46%, n = 1986) conflictingly answered they did not have a designated handwashing area in their household when asked earlier in the survey. This contradiction points to a misinterpretation.[2] Nearly 20% (n = 889) of respondents with a designated handwashing area had a handwashing station that was not near a food preparation area nor near a latrine. We hypothesize some surveyed households may use a piped water supply, tippy tap, or designated bucket somewhere in the yard for handwashing; such set-ups may not have been consistently characterized as a "designated area for handwashing."

The access to basic hygiene services indicator in the data far underestimated demonstrated HWWS and reported handwashing times. While less than 10% of total households had access to basic hygiene services, 48% of respondents could demonstrate HWWS when asked, and 37% of respondents reported handwashing both after defecation and before preparing food. Demonstrated HWWS and reported handwashing at the two critical times were highest among respondents with access to basic hygiene services. Interestingly, 25% of respondents with limited hygiene access – defined as having a designated handwashing area without soap and/or water – used both soap and water during demonstrated handwashing, presumably having retrieved the materials from a location other than the handwashing area. Similarly, 44% of respondents without a reported designated handwashing facility used both water and soap during demonstrated handwashing. This inconsistency between hygiene service level and demonstrated handwashing findings may in part be due to misinterpretation of "designated handwashing area" as discussed. It also appears likely that in rural areas where informal or makeshift alternatives to piped water and handwashing stations are the norm, people can and do HWWS without a designated facility.

Evidence generally suggests that HWWS rates are associated with, but lower than, access to basic handwashing facilities (Biran et al., 2008; Ram et al., 2014; Wichaidit et al., 2019; Wolf et al., 2019). Wolf et al. (2019) estimated across 77 countries that HWWS after potential fecal contact and before food contact is at least two times higher in the presence of a handwashing facility with soap and water. The authors assert the presence of a handwashing facility "grossly overestimates actual handwashing prevalence" (Wolf et al., 2019). Our findings add an additional complication to this assertion by suggesting that, depending on the definitions and survey questions used, the presence of a designated handwashing facility may actually underestimate households' HWWS ability or prevalence in some cases.

Inconsistent trends and magnitudes between three analyzed hygiene indicators suggests no single indicator can give a holistic picture of household hygiene access and behaviors. Each of the analyzed indicators has limitations. Access to basic hygiene services is a commonly used proxy for handwashing as it is cost-efficient, reproducible, and overcomes social desirability bias. However, it does not consistently match prevalence of HWWS behaviors in magnitude (Biran et al., 2008; Danquah, 2010; Halder et al., 2010; Wichaidit et al., 2019; Wolf et al., 2019) and vague or varying definitions of "handwashing facility" can cloud its meaning. Self-reported handwashing times tend to be influenced by social desirability bias, resulting in overestimates, or recall errors, leading to underestimates (Biran et al., 2008; Contzen et al., 2015; Ram, 2013). In this study, the self-reported handwashing times survey question did not specify whether respondents used both soap and water, which makes the indicator in this dataset imprecise. Demonstrated HWWS is similarly subject to social desirability bias, may be a better indicator of knowledge and ability than routine practices, and may not reflect HWWS at critical times as measured through structured

[1] We interpret the term "cleanliness" here as a general word encompassing household and personal hygiene. The term "cleanliness" was not fully defined in the questionnaire and it is unclear how it may have been translated differently into local languages.

[2] The majority of the observed latrines with nearby handwashing stations in question were located on the household plot and not shared with other households, eliminating those possible reasons for the widespread discrepancy.

observations (Biran et al., 2008; Danquah, 2010; Halder et al., 2010; Ram, 2013; Wichaidit et al., 2019).

4.2. Household characteristics associated with hygiene

Descriptive statistics and regression analysis suggest that wealthier households had higher outcomes across all three hygiene indicators, a trend commonly described in the literature (Kumar et al., 2017; Luby et al., 2011; White et al., 2020; WHO and UNICEF, 2019). White et al. (2020) notes that the causal route for the association between wealth and hygiene is difficult to identify due to the likely interactions with other determinants. Our data suggest material barriers to HWWS may be a limiting factor for many households in the rural study areas. Access to basic hygiene services and access to or use of hygienic drying materials during demonstrated handwashing was consistently very low. Only about half of respondents used soap during demonstrated handwashing, likely implying soap was not available in the house or only sparingly used for handwashing. On the other hand, the vast majority of households had water available at designated handwashing areas and for demonstrations. Material barriers alone likely do not fully explain the disparities in reported handwashing at critical times and other hygiene indicators between the wealthiest and poorest households; handwashing behaviors among the poorest may also be constrained by habitual or motivation factors.

Associations between household hygiene and water supply and sanitation indicators were inconsistent across countries and indicators in our regression analysis, which generally agrees with the mixed or positive associations reported in the literature (White et al., 2020). While evidence elsewhere suggests piped water or a water source close to the household is positively associated with handwashing outcomes (White et al., 2020), a lack of basic water services is not the only limiting factor for having basic hygiene access (WHO and UNICEF, 2020b). Adequate water access alone is insufficient for HWWS behaviors if individuals lack soap, knowledge, or motivation, and implementers should not assume increased HWWS will naturally follow water service provision. Meanwhile, households that are lacking adequate water quantities may require more context-specific information on how or when to prioritize HWWS over other domestic water uses.

4.3. Implications for hygiene monitoring and programming

Where survey time and resources allow, such as for local-level monitoring or implementer-driven evaluation efforts, behavioral indicators around demonstrated handwashing technique and reported handwashing events can complement the standard access to basic hygiene services proxy to build a more holistic picture of household HWWS. For example, a study in Indonesia by Hirai et al. (2016) found that relying on a composite indicator of presence of both soap and water at a handwashing place as well as use of water and soap during demonstrated handwashing more conservatively estimated HWWS behavior than either indicator alone by 15–20 p.p. (Hirai et al., 2016). When an indicator for access to basic hygiene services is used longitudinally, such as in international multi-indicator survey efforts (e.g., Demographic and Health Surveys), actors must clearly communicate the indicator's limitations. The growing body of data associating hygiene service level and observed HWWS behaviors can help inform how to mathematically adjust the basic hygiene access proxy to more accurately reflect HWWS behaviors (Wolf et al., 2019).

Our finding that a substantial proportion of the study population had both soap and water during demonstrated handwashing without a reported designated handwashing facility as interpreted by enumerators and respondents warrants further discussion on the suitability of focusing household hygiene monitoring solely on handwashing facilities. This is particularly true for low-income households where makeshift alternatives to stationary handwashing facilities are the norm. Although the observed presence of soap designated for handwashing in the household is likely an overestimate for handwashing behavior (Kumar et al., 2017), the presence of soap for handwashing combined with a separate indicator about water availability may be a more informative upper bound for the proportion of the population capable of HWWS than the presence of a designated household handwashing facility. The observed presence of household soap for handwashing has been included in past Multiple Indicator Cluster Surveys (MICS) and Demographic and Health Surveys (DHS). Analysis of the 2010–2013 MICS and DHS suggests that across eleven SSA countries, there was a median of 47 p.p. disparity between the proportion of households with soap in the dwelling and proportion with access to a handwashing facility with soap and water, ranging from 0.6 p.p. in Senegal to 83 p.p. in Kenya's Nyanza region (Kumar et al., 2017). Global reporting on the observed presence of household soap as a primary indicator can better communicate the extent to which HWWS behaviors are constrained by soap availability or affordability relative to the current limited hygiene service level indicator, while simultaneously eliminating any survey confusion or cultural differences regarding the definition of a designated handwashing facility.

Household wealth, more so than other household characteristics, was predictive of both hygiene access and handwashing behaviors, highlighting the continued need to emphasize the universal and inclusivity aspects of SDG 6, "equitable hygiene for all" (United Nations General Assembly, 2015). Continued reporting of the hygiene gap between the wealthiest and poorest can help hold governments and practitioners accountable for equitably monitoring and improving hygiene. Additionally, this finding suggests governments and practitioners should intentionally invest time and resources in targeting the poorest households with promotion campaigns and that inexpensive, high-quality handwashing products are needed. The barriers for these households are likely multi-faceted and context specific. Poorer households may require assistance to overcome affordability-related hygiene barriers as well as longer-term and more frequent HWWS promotion efforts to facilitate sustainable behavior change.

Lastly, our analysis explores the reported actors and places involved in hygiene promotion. Through analysis of the UN-Water Global Analysis and Assessment of Sanitation and Drinking-Water (GLAAS) report, Jiménez et al. (2014) reports "there are barely enough hygiene promoters to reach 10% of the people" in GLAAS countries, and that government officials' hygiene promotion concerns center around the lack of human resources and funds. Strengthening the work of traditional hygiene promoters such as community health workers is critical and exploring additional actors to spread HWWS messages may also be necessary. Our analysis found that respondents rarely heard cleanliness messages from faith leaders, from trained religious volunteers, or in places of worship. Religious actors have played important roles in Ebola response, HIV prevention, and other health efforts globally (Marshall and Smith, 2015; Maurice, 2015), but there remains limited evidence of their engagement in hygiene promotion.

Engaging faith leaders may be an opportunity to increase the effectiveness, reach, and sustainability of hygiene promotion work. First, faith leaders are well-placed to deliver influential and locally-appropriate hygiene messages (Greaves et al., 2009). People are more likely to comply with health prevention messages when they trust the authority delivering the message (Blair et al., 2017; White et al., 2020). Second, hygiene messages are already aligned with the texts and messages of the major religions in the countries studied – including Islam and Christianity – that discuss cleanliness and caring for one's body. Although faith leaders are traditionally focused on transmitting messages of spiritual well-being, many would likely welcome the opportunity to engage in furthering the holistic well-being of their community members. Lastly, faith communities can provide the frequent and sustained promotion required for hygiene behavior change (Pickering et al., 2019) and may be able to communicate in a way that raises both the empirical and normative expectations required to create a social norm (Bicchieri, 2005). Faith communities often gather on a regular basis, will

remain in a community even after other actors exit, and may exist as one of the few social institutions in remote, hard-to-reach areas (Greaves et al., 2009).

4.4. Limitations

Survey sampling was not representative of countries or regions, as data collection was restricted to rural areas and stratified by areas where World Vision does and does not conduct water, sanitation, and hygiene programming. Therefore, the data can best be interpreted when combined with other JMP datasets with the use of geographic analysis (Local Burden of Disease WaSH Collaborators, 2020) or used to assess potential associations between outcome measures and covariates. The reported results are cross-sectional, and associations identified in the regression analysis should not be taken to indicate causality.

While enumerators were instructed to prioritize interviewing female heads of households and to only interview another adult if she was not available, the analyzed data did not record who ultimately answered the survey. Respondents' sex, as well as other factors such as age and position within the household may influence their HWWS behaviors. Future research and monitoring efforts should disaggregate individual-level data by respondents' sex, age, and other notable characteristics. Furthermore, reported or observed behaviors by a single individual in a household may not be representative of the behaviors of the entire household, though for some similar behaviors, adult females in the household can accurately report the behaviors of others in the household (Jenkins et al., 2014). Comparing behaviors of all family members can help inform the extent to which hygiene promotion programming should be differentiated by sex, age, or other sub-populations.

5. Conclusion

We analyzed hygiene data from 36,860 household surveys in 14 LMICs to compare and critique household handwashing indicators, identify household characteristics associated with handwashing indicators, and generate monitoring and implementation insights. Our findings suggest that no single indicator can give a complete picture of household hygiene access and behaviors. Rather, local, regional, and global hygiene monitoring indicators should be tailored to the needs of implementers and policymakers. Whenever practical, multiple questions or demonstrations around HWWS behaviors should be used to complement the common access to basic hygiene services indicator, which may not accurately capture prevalence of HWWS. Access to household soap designated for handwashing should be emphasized in hygiene monitoring and programming efforts as rural households in LMICs, especially those in the poorest quintile of their communities, face both material and behavioral constraints to HWWS.

While the data analyzed in this study was restricted to hygiene access and handwashing behaviors in respondents' households, further research is required to understand how these findings can be extrapolated to the various settings respondents spend time in throughout the day. Because HWWS at critical times must be consistent across locations to achieve health outcomes, additional research, monitoring, and programming is needed to understand and influence how access to soap and water as well as HWWS motivations, perceived abilities, or habits do or do not change across individuals' frequented public spaces or work settings.

An exploration of the actors and places involved with household hygiene messaging reported by respondents reveals a largely overlooked opportunity to empower faith leaders as trusted community members to sustainably promote hygiene messages. More informative and holistic HWWS monitoring efforts coupled with targeted, context-specific, and sustainable work to overcome HWWS barriers can prevent diarrheal and respiratory infection transmission in LMICs.

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.2021.113810.

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Mega festivals like MahaKumbh, a largest mass congregation, facilitated the transmission of SARS-CoV-2 to humans and endangered animals via contaminated water

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ABSTRACT

Our surrounding environment has been influenced by the COVID-19 pandemic situation. The second wave of COVID-19 in India has proven to be more devastating and aggressive than the first wave of the pandemic, which led to recognizing India as one of the world's topmost worst-hit nations considering >4000 fatalities reported in a single day in May 2021. Such "resurgence and acceleration" of COVID-19 transmission has been fuelled by the MahaKumbh festival and political mass gathering (elections rallies) events, where the COVID-19 protocols have been ignored by millions of pilgrims/followers. The present review discusses only the consequences of this year's MahaKumbh festivals, the largest religious mass gathering on earth, which was held during the COVID-19 pandemic in India, and its impact on both the spread of SARS-CoV-2 among participants and their families and its influence on the quality of the river Ganga. This article tries to give readers outside of India an overview of how much impact of any such single large gathering of any relgion in any part of the world can drive coronavirus infections and effectively commence the second/third wave outbreak with this case study. Furthermore, the religious large scale celebration are widely accepted through out the world that have played a significant role in the spread of the pandemic into remote villages and towns all over the subcontinent/world, thus affecting many areas with insufficient healthcare facilities that have been relatively spared. This review also highlights the potential risk of transmission from infected humans into the aquatic environment of the river Ganga. Besides the obvious relevance of SARS-CoV-2, a large variety of other water-related disease vectors (bacteria, viruses, and protozoa) stemming from visitors to the religious congregation were introduced into the upstream regions of the Ganga river. Their sheer number is assumed to have had a severe influence on its delicate ecosystem, including endangered mammals such as the river Dolphins. The detailed epidemiological and clinical study on transmission routes of SARS-CoV-2 is the need of the hour to understand the pathogenesis of RNA virus infection and prevent the massive spreading of such infectious respiratory diseases. An interdisciplinary approach, rooted in evidence-based efficient learning, contextual strategies, and a streamlined unified approach should be adopted to help in the development of a proactive prevention model during future MahaKumbh festival (and similar religious gatherings) instead of just "picking up the pieces" in a conventional post-event model.

The emergence of the severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) and its associated COVID-19 disease in January 2019 lead to the outbreak of a global pandemic resulting in severe health concerns with nearly 197 million people affected (and >4.0 million deaths) in >200 countries around the globe by the 3rd August 2021 and posed environmental, economic, and social challenges (WHO, 2021). To impede the spread of infection, non-pharmaceutical precautions such as local lockdowns, social distancing, increased hand hygiene, and the use

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of personal protection equipment (PPE), such as facemasks, are being practiced and proved to be successful (Askitas et al., 2021). In India, an overall improving situation became grimmer in April 2021 due to the fatal outbreak of the second wave of COVID-19, which is reported to cause average daily deaths of more than 2,000 (Singh et al., 2021). The super spreading of the second wave of COVID-19 across the country has created a formidable challenge leading to the recognition of India (~11 million active cases) as one of the top five worst-hit nations after the USA (~24 million cases) as of 21st January 2021 (Ganguly and Chakraborty, 2021). COVID-19 transmission has been notably accelerated by several religious events (MahaKumbh festival) and political mass gatherings, where COVID-19 containment protocols at all times have been tossed away (The Lancet, 2021). The pivotal role of SARS-CoV-2 B.1.617 (or Delta) lineage and other variants of concern (e.g., B.1.1.7 or Alpha) along with the massive congregation of people have been identified as potential and likely contributing factors towards the rapid rising of the second wave of COVID-19 (Singh et al., 2021).

The MahaKumbh festival is considered the biggest religious human mass gathering on earth every 12 years. In 2021, more than 5 million devotees from different parts of India assembled for such a religious festival at Haridwar, an ancient Hindu pilgrimage city located at the river bank Ganga in the northern state of Uttarakhand (Quadri and Padala, 2021). It has been estimated that nearly 7 million pilgrims have converged (and flouted COVID-19 norms) at Har Ki Pauri ghat in Haridwar for the holy bath in the Ganga from January 14 to April 30, 2021. As of April 12, 2021, around 0.2 million saffron-clad seers and ash-smeared Nagas have contributed to the rapidly moving upward trajectory of COVID-19 cases (>35,000 in one week period) (Oestigaard, 2021). As expected, the upsurge of COVID-19 during the gigantic event of mass bathing resulted in the potential "super-spreading" of aerosols and airborne SARS-CoV-2 virions among the mass of devotees and seers (Fig. 1). The likelihood of rapid virus dispersal throughout the crowd and subsequently (upon their return to villages and cities all over the Indian subcontinent) the country was very high. Amidst the ongoing MahaKumbh festival in April, an increase of nearly 89-fold in COVID-19 cases has been documented in Uttarakhand compared to February (the initial period of the festival) (Upadhyay, 2021), with scores of transmissions that have not been picked up by clinical diagnostics (Inbaraj et al., 2021). The number of confirmed positive cases that have been found among MahaKumbh returnees across the country (~0.4 million cases on May 8, 2021, a global record), clearly indicates to what immense extend congregated religious gathering has fuelled India's coronavirus outbreak and led to the spread of the pandemic into even remote villages (Bhutta et al., 2021). As mentioned earlier, political mass gathering (happening at proximity/extensive physical contact) for elections in different states has also lasted for over a month and due to frequent traveling of the political leaders to different states, the incident of COVID-19 has risen exponentially.

The complex nature of SARS-CoV-2 and the multitude of symptoms mentioned in a plethora of studies that report possible transmission routes in various environmental mediums (Kumar et al., 2020; Shao et al., 2021; de Oliveira et al., 2021). While some like air (aerosols, secretions, respiratory droplets), biota (minks, bats, pangolins), and fomites, as well as surfaces (food transportation, medical treatment, social gatherings), could be proven with little doubt (Shao et al., 2021), others like contaminated soil and water (via fecal-oral transmission) are more elusive but should not be ignored (Maal-Bared et al., 2020). Different clinical symptoms (lungs/intestine/eye infection) have been reported with the various primary and secondary transmission routes (respiratory, ocular, nasal, fecal-oral, blood vessels/lymphatic/hematogenous) of SARS-CoV-2 (Falahi and Kenarkoohi, 2020; Li et al., 2020). The vertical/transfusion transmission of SARS-CoV-2 has been contradictorily reported in the literature and flow physics of such disease transmission routes have been discussed thoroughly through the Susceptible-Exposed-Infectious-Recovered-Deceased (SEIRD) model (Chaudhuri et al., 2020; Falahi and Kenarkoohi, 2020). Although the airborne transmission of such highly transmissible etiological agents has been considered as the primary pathway of the pandemic outbreak, the

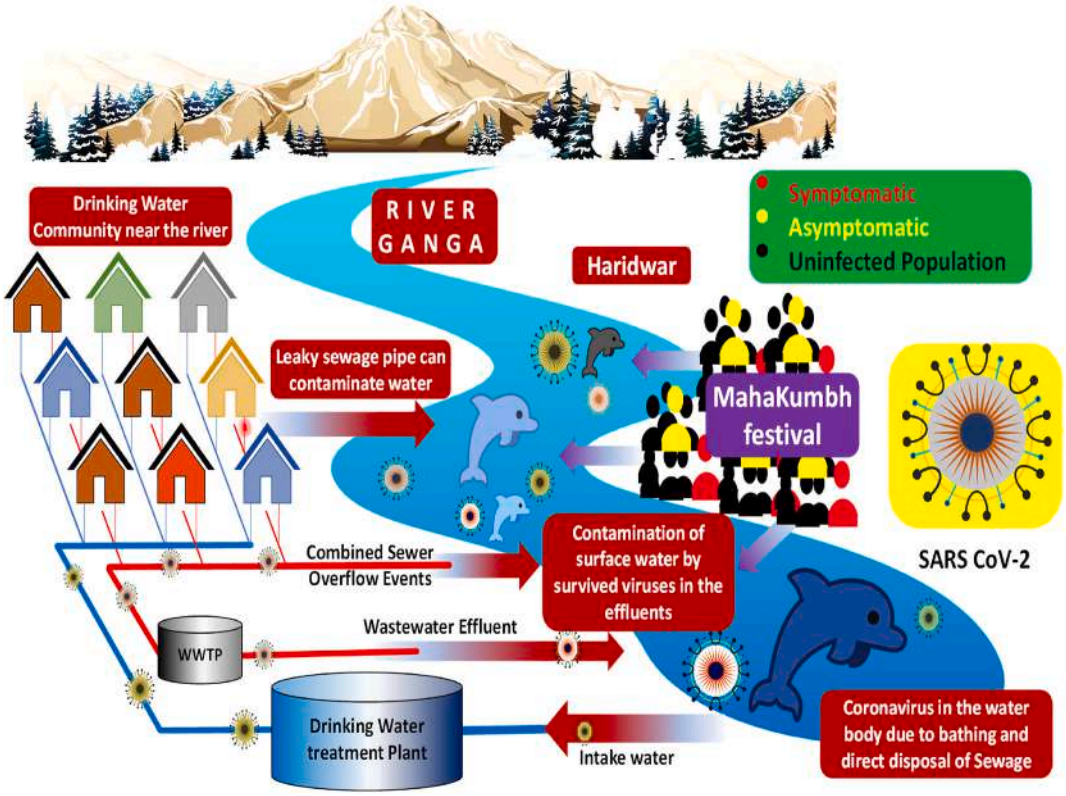

Fig. 1. Conceptual formulation of probable transmission of SARS-CoV-2 through the River Ganga during the Mahakumbh festival held at Haridwar.

waterborne transmission of these viruses should not be ruled out. Recent studies such as the one by de Oliveira et al. (2021) have documented the possible fecal-oral transmission and survival potential of SARS-CoV-2 in the water cycle, where such RNA viruses have been detected in untreated sewage/wastewater and agricultural run-off. Li et al. (2020) and Guo et al. (2021) have documented the association of gastrointestinal/enteric symptoms (through the infection of enterocyte lineage cells) for many other respiratory viruses although the fecal-oral transmission contributed a small proportion (≤20%) of respiratory virus transmission. Some researchers have reported the persistence of novel coronavirus in such a dissemination medium (untreated wastewater/fecal contamination) between 8 (≥35 °C) −27 days (≥4 °C) (Shao et al., 2021; Guo et al., 2021; Giacobbo et al., 2021). Although there is scanty information related to possible ways of virus migration through water sources, a group of the researcher through comparative genome analysis focused on the reverse zoonotic transmission of SARS-CoV-2 infection on aquatic vertebrates (Akinsorotan et al., 2021; Audino et al., 2021; Khan et al., 2020). Zhang et al. (2020) have reported that fecally contaminated water (stemming from wastewater discharges) can not be excluded as contributing to infections in the aquatic biodiversity of Ganga as SARS-CoV-2 can be detected with molecular methods targeting its RNA genome in fecal samples after an estimated 22 days compared to 16 days and 18 days in serum and respiratory specimens. To have a clear insight on viral occurrence and persistence in aquatic and marine ecosystems (and to study their possible negative impact?), it is of utmost importance to provide emphasis on the immunohistochemistry of sea mammals (Audino et al., 2021; Tiwari et al., 2020). Recently, wastewater surveillance has been globally recognized for tracking several emerging contaminants, the pattern of antibiotic use, and providing a weak signal of virus transmission at a community scale (Thompson et al., 2020). This approach of detecting the RNA of viral pathogens could also be exploited at the national level as a useful monitoring tool in early detection (and prevalence) of local clusters and outbreaks as well as asymptomatic carriers of SARS-CoV-2 in India, where a huge population and lack of resources often lead to under-detected cases (Kumar et al., 2020).

In addition to the individual-to-individual transmission of SARS-CoV-2, the MahaKumbh festival very likely resulted in contamination of the river Ganga by mucosal, intestinal, fecal, and skin microbes and thus increase bacterial loads during such holy dip, as recently reported by Upadhyay et al. (2021). Therefore, the potential risk of transmission of the infection to the aquatic environment from a variety of water-related diseases vectors (etiological agents causing, among others, typhoid, dysentery, and cholera) should not be ignored. In addition to the consensus that human, environmental, and animal health should not be viewed separately but as a whole (the “One Health” concept) (Yasobant et al., 2019), the existence of SARS-CoV-2 in cetaceans (whales and dolphins) raised a further dimension of the ongoing pandemic (Mathavarajah et al., 2021; Tiwari et al., 2020). COVID-19 infections of marine mammals have been confirmed through structural analysis of the protein sequences of the Angiotensin-converting enzyme 2 receptors (ACE2), the main point of attack of the SARS-CoV-2 spike protein (Damas et al., 2020; Delahay et al., 2021). Although there is not enough evidence in Indian perspectives to suggest the threat to aquatic biodiversity because of SARS-CoV-2, several epidemiologists/marine researchers have identified that captive marine mammals (such as cetaceans, fissipeds, and pinnipeds) could get infected with SARS CoV-2 through wastewater (Delahay et al., 2021; Guo et al., 2021; Mathavarajah et al., 2021). They have hypothesized that the social behavior of marine mammals, the transmission of the virus to mucous membranes from wastewater discharge sites (from fecal shedding), and close contact with asymptomatically infected caretakers may induce the risk of infection through water (Larsen and Wigginton, 2020; Khan et al., 2020). The South Asian River dolphins (*Platanista gangetica*) are regarded as the national aquatic animal of India, and wildlife experts have long warned about possible threats to their existence due to excessive water pollution, anthropogenic activities, and commercial water transportation services (Delahay et al., 2021; Paudel and Koprowski, 2020; Tiwari et al., 2020). According to the International Union for Conservation of Nature (IUCN), Gangetic river dolphins and Gharials (*Gavialis gangeticus*) were already listed under the “endangered” and “critically endangered” categories before the COVID-19 pandemic (Sinha et al., 2014). As several studies have reported the occurrence of SARS-CoV-2 in pets (dogs and cats) and wildlife (lions and tigers), the possibility of COVID-19 infections to aquatic mammals through horizontal (interspecies) and vertical (mammary gland infection/bodily fluids) transmission pathways (US-CDC, 2021) as observed in the case of Cetacean morbillivirus (CeMV) infections seem realistic (Jo et al., 2018; Quadri and Padala, 2021; Usui et al., 2021). Guo et al. (2021) have reported the impact of SARS-CoV-2 on marine animals through analysis of the biophysical hydrodynamic model and justified that in winter due to long persistence and long-distance migration there is a high chance of pathogenic contamination. Audino et al. (2021) and Charlie-Silva et al. (2021) have supported the hypothesis of potential susceptibility of different aquatic animals infection by SARS-CoV-2 through binding strength analysis of ACE-2 receptor and evaluation of biomarkers (synthesized SARS-CoV-2 spike protein peptides). Thus, after the MahaKumbh festival, the contaminated water from the ceremonial sites may prove even more detrimental to the Dolphin population than before. Hence, there is a significant potential threat to the Ganga’s aquatic biodiversity and fishing industries due to the presence of functional SARS-CoV-2 receptors in them.

Keeping in mind the public health risks and possible significant threats to aquatic (and marine) ecosystems it is advisable to avoid public interaction with fragile aquatic environments such as the river Ganga during this pandemic and that proper healthcare measures need to be addressed as early as possible before facilitating large (e.g. religious) gatherings along its shores shortly. However, the matter of food chain contamination is yet untouched by the concerned research community. The issue draws inevitable importance since marine species as well as a human population are involved in the food chain, it’s high time to invest attention to study this aspect. To minimize the possibility of a health crisis during such religious gatherings, some socio-administrative measures (i.e. online registration and GPS tracking) of pilgrims/participants, the screening of most susceptible (i.e. older and sick) people in gatherings, the scaling up of healthcare infrastructure, adhering to non-pharmaceutical measures such as face masks, hand hygiene and social distancing (where possible) as well as promoting advanced public health facilities after meeting with organizing committee and religious leaders can be adopted. Additionally, some other preventive measures i.e. the infrastructure of hand-sanitizing stations, clean and hygienic quarantine facilities, utilizing the concept of “Digital India” (digital infographics) before and during the festival, should be recommended and included in planning to obviate complex public health challenges. However, it is of utmost importance to build up a multi-disciplinary consortium of event managers, administrators, public health experts, and academics (at national and international levels) to draft and design robust recommendations and clear guidelines. This will also provide context-specific solutions so that improved policies and practices can be implemented to mitigate the potential risk factors during congregated events such as the MahaKumbh festival, the Hajj etc., and any other religious gatherings of such scale globally. Such interdisciplinary approaches based on evidence-based efficient learning, contextual strategies, and a streamlined, unified approach will help develop a proactive prevention model during such religious mass congregation instead of a conventional post-event model.

Declaration of competing interest

The authors declare no competing financial interest.

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Microbiological survey and occurrence of bacterial foodborne pathogens in raw and ready-to-eat green leafy vegetables marketed in Tehran, Iran

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ABSTRACT

Fresh leafy (FL) and ready-to-eat (RTE) vegetables are recognized as an important source of foodborne disease outbreaks worldwide. Currently, there are no data available for the prevalnce of bacterial foodborne pathogens (FBPs) in raw vegetables consumed in Iran. Here, we evalated the presence of common bacterial FBPs among 366 samples of raw vegetables including 274 FL and 92 RTE collected from 21 districts of Tehran. The presence of FBPs were screened using conventional microbiological culture methods and real-time PCR assays. Overall, a higher rate of bacterial contamination was detected in FL compared to RTE samples using both detection methods. The results obtained by microbiological methods showed that *Staphylococcus aureus* (134/366, 36.6%), followed by *Escherichia coli* (85/366, 23.2%) and *Clostridium perfringens* (66/366, 18%) were detetcted as the most prevalent pathogens in this study. *Vibrio cholerae* was not detected in any of the samples either by microbiological methods or by the real-time PCR assays. There was a noticeable reduction in the proportion of *Campylobacter* positive samples using conventional microbiological methods (3.5%) compared to the real-time PCR assay (20.7%). The proportion of FL and RTE positive samples obtained by conventional microbiological methods was significantly different ($P < 0.05$) for *C. perfringens*, *Campylobacter* spp. and *S. aureus*. The proportion of positive samples in FL and RTE vegetables obtained by the real-time PCR assays was significantly different ($P < 0.05$) for *C. perfringens*, *S. aureus*, *Helicobacter pylori* and STEC/EHEC, the last one was found more frequently in RTE than in FL samples. Our findings indicated a contamination of FL and RTE vegetables in Iran with a range of well-known and emerging FBPs. Positivity and the distribution of bacterial species from the current data indicated different contamination sources, and overall a lack of effective decontamination steps during the production chain. Moreover, further information about the quality of the water, the hygiene measures implemented during the processing, storage and marketing are required to better identify the critical points and define the proper measures.

1. Introduction

Vegetables are the major part of any healthy diet and balanced meal owing to their high nutritional compounds including vitamins, minerals, and phytonutrients, and contribute in the metabolic functions of the body (WHO 2003; Losio et al., 2015; Pezzuto et al., 2016). Regarding the nutritional value and health benefits of vegetables, there has been an increasing demand for fresh leafy (FL) vegetables and other ready-to-eat

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(RTE) products over the last decade (Losio et al., 2015). FL vegetables are usually sold untreated, unwashed and unpacked, whereas RTE vegetables are packaged and minimally processed products intended to be consumed without additional preparation (Losio et al., 2015; Arienzo et al., 2020). Both FL and RTE vegetables are mostly eaten raw. Therefore, they can harbor complex microbial flora and are well recognized as a potential source of bacterial infections which have caused outbreaks worldwide (Denis et al., 2016). Moreover, raw vegetables can be contaminated by a broad range of enteric viruses, such as hepatitis A virus (HAV) and noroviruses. These viruses which may originate from virus-infected food handlers and virus-contaminated irrigation water are highly assosiated with viral foodborne illnesses (Cheong et al., 2009; Bosch et al., 2018).

Fresh vegetables are recommended as major component of human diet since they present excellent nutritional value (WHO:https://www.who.int/news-room/fact-sheets/detail/healthy-diet). However, increasing numbers of foodborne infections are connected to the consumption of fresh produce (ECDC/EFSA/EMA 2017), and FL green vegetables and their RTE salads are recognized as a source of foodborne pathogens (FBPs) in many parts of the world (Taban and Halkman, 2011; Kotzekidou, 2016). Soil, water, air, contaminated equipment, and workers handling the product can be a potential source of contamination for fresh crops (Nguz et al., 2005). The contamination of the fields by manure used as fertilizer or from the grazing of animals, or the use of contaminated water for irrigation are two main factors that affect the microbiological quality of the vegetables in pre-harvesting (Taban and Halkman, 2011; Das et al., 2016; Iwu and Okoh, 2019). Harvesting and post-harvest processing are also potential contamination sources by human or indirect contact (Brackett, 1994; Brackett, 1999; Taban and Halkman, 2011; Hou et al., 2013; Gil et al., 2015; Losio et al., 2015). The contamination of raw produce, especially FL vegetables, with FBPs is of particular concerns because they have poor or no decontamination steps during the processing (Losio et al., 2015). Raw leafy green vegetable products can become contaminated with a range of potential hazards such as bacteria, enteric viruses, helminths, and protozoa during pre-harvest and post-harvest processing. However, bacteria are reported to be the most serious issue in terms of microbiological hazards on an international scale (Taban and Halkman 2011). The most frequently encountered FBPs include *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes*, *Shigella* spp., *Yersinia* spp., parasites such as *Cryptosporidium*, *Cyclospora*, helminths and viruses such as hepatitis A and noroviruses (Park et al., 2012; Ferrario et al., 2017). Various studies in Europe and the USA revealed that there has been a close association between foodborne disease outbreaks and consumption of raw and fresh vegetable products (Harris et al., 2003; ECDC/EFSA 2014). For instance, 9.2% of the foodborne disease occurred in the USA from 2004 to 2010 were linked to consumption of fresh produce, and this number increased to 12.7% from 2010 to 2017 (Carstens et al., 2019). It has been reported that *Salmonella* spp., *E. coli* O157:H7, *Vibrio cholera*, *Staphylococcus aureus*, *Campylobacter* spp. and *L. monocytogenes* could be related to the increase in the number of foodborne outbreaks linked to the consumption of fresh produce worldwide (Wadamori et al., 2017).

Dietary habits in Iran include consumption of raw vegetables as an important part of the diet. Since they are considred as the potential sources of FBPs, the importance to ensure safety of these fresh produce should be highly appraised (Iwu et al., 2019). Based on our knowledge there is no previously published study on the microbial contamination of fresh and RTE vegetables in Iran. The purpose of this study was to evaluate the presence of FBPs in FL and RTE vegetables available on the farms, vegetable markets, greengrocer's shops, local processing units, supermarkets and hypermarkets during summer to early winter seasons in the capital city of Iran, Tehran.

2. Materials and methods

2.1. Type of samples and collection

A cross-sectional study was conducted from August 2019 to December 2019 in Tehran, the capital of Iran. Iran has many different types of climates such as subtropical dry to extremely dry zone in the eastern half and some central areas, wet to extremely wet zone in the southern coastal plains of the Caspian Sea, relatively wet zone in some areas in the west, and semi-dry zones over the rest of the country (Alizadeh-Choobari and Najafi, 2018; Vaghefi et al., 2019). However, 94.8% of the country is dominated by an arid and semi-arid climate with low atmospheric precipitation and high evaporation–transpiration. The average precipitation in Iran is approximately 62.1–344.8 mm yearly (Khalili et al., 2016). The weather condition during our data collection period was hot and dry during August and September, and mild with some light rain in December. During this period, different types of vegetables were readily available on the farms, vegetable markets, greengrocer shops, local processing units, supermarkets and hypermarkets. A total of 366 samples of raw vegetables including leek, watercress, basil, savory, parsley and radish were randomly collected from 21 districts of Tehran; on each occasion 5 to 35 samples were taken. The samples obtained fall into two main categories including FL (untreated, unwashed and unpacked samples) and RTE (washed, cut and packaged samples) vegetables. Samples (FL vegetables $n = 274$; RTE vegetables $n = 92$) were kept in sterile bags, transported immediately to the laboratory and analyzed for isolation and identification of pathogenic bacteria following standard microbiological methods.

2.2. Sample preparation

Twenty-five grams of each sample was weighed and chopped thoroughly using a sterile sharp kitchen knife, homogenized with 225 ml buffer saline phosphate (PBS) with a stomacher (Seward Co. Ltd., London, UK) for 3–5 min at 230 rotations per minute. The homogenized samples were then filtered into 50 ml tubes and centrifuged at 4000 rpm (rcf = 3220×*g*). After centrifugation, the supernatant was discarded and the pellet was dissolved in sterile PBS (1 ml for each enrichment media). Finally, one ml of homogenized samples was inoculated onto 10 ml of specific enrichment broth media and incubated according to the conditions indicated in Table S1 for each specific bacterium. The remaining homogenized samples were stored at −20 °C until used in molecular assay. A workflow summary of the FBP detection in vegetables is illustrated in Fig. 1.

2.3. Bacterial isolation and identification

Seventeen FBPs including *Clostridium perfringens*, *Bacillus cereus*, *L. monocytogenes*, *S. aureus*, *Campylobacter coli*, *Campylobacter jejuni*, Shiga toxin-producing *E. coli*/enterohemorrhagic *E. coli* (STEC/EHEC), *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. *enterica*, *Salmonella enterica* subsp. *enterica* serovar Enteritidis, *Salmonella enterica* subsp. *enterica serovar* Tiphymurium, *Shigella flexneri, Shigella boydii*, *Shigella sonnei*, *Yersinia enterocolitica*, *V. cholerae* were investigated by using conventional culture method and real-time PCR. Identification of *Clostridioides difficile*, *Aeromonas hydrophila* and *Helicobacter pylori* was done only using real-time PCR.

For detection of FBPs, all bacterial isolation and identification processes were according to the recommendations described by the Institute of Standards and Industrial Research of Iran (ISIRI: http://isiri.gov.ir/portal/home/) with some modifications. Breifly, a hundred microliter of each enrichment broth was streaked onto a plate of the appropriate growth medium. Cultured plates were incubated under aerobic, anaerobic, and microaerophilic conditions for 24–72 h. Detection of aerobic bacteria was done by using incubation of cultured plates at 37 °C for 24–48 h. For isolation of anaerobic bacteria, cultured plates were

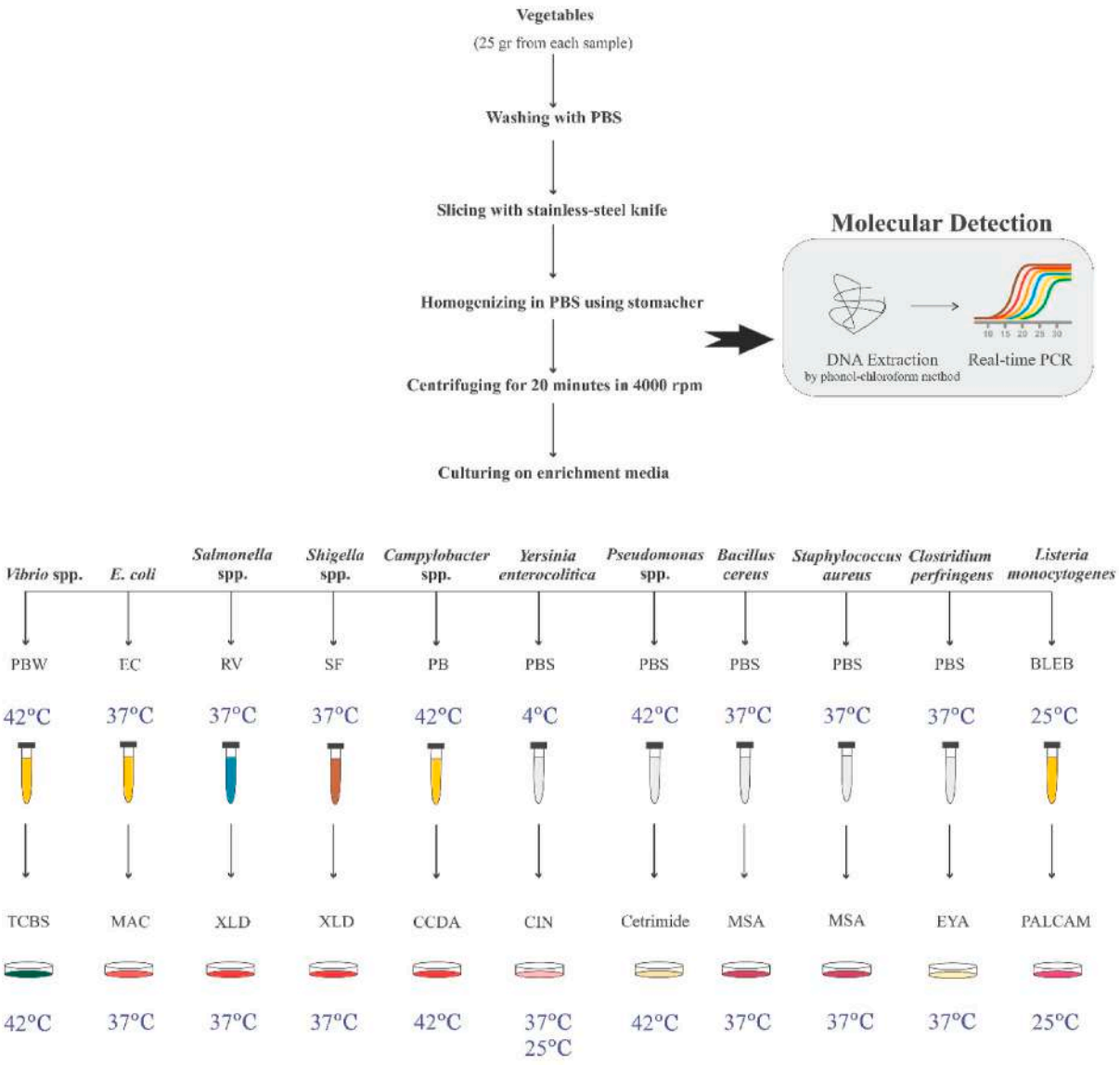

Fig. 1. Workflow of the foodborne pathogen detection in vegetables. Each step of the procedure, sample enrichment, and culture conditions are schematically illustrated from top to bottom.

incubated under anaerobic atmosphere (85% N_2, 10% CO_2 and 5% H_2) generated by Anoxomat® Gas Exchange System (Mart Microbiology BV, Holland) at 37 °C for 48–72 h. For detection of *Campylobacter* spp., inoculated plates were incubated at 37 °C in a CO_2 incubator (Innova CO-170; New Brunswick Scientific, USA) under microaerophilic conditions containing approximately 5% O_2, 10% CO_2, and 85% N_2 for 24–48 h. The suspected colonies were subjected to identification by colony morphology, Gram staining and standard biochemical tests. Bacterial strains and culture media used in this study are summarized in Table S1 and Fig. 1.

2.4. DNA preparation

Genomic DNA was extracted from the homogenized samples by classical cetyltrimethylammonium bromide (CTAB)/phenol chloroform method as described by Vu-Thien et al. (2007). Briefly, the homogenized samples were centrifuged at 4000 rpm for 10 min at 25 °C and the pellets were lysed into a solution containing 10 mM Tris HCl (pH 8), 10 mM EDTA, 10 mM NaCl, and 0.5% sodium dodecyl sulfate (SDS) and incubated overnight at 37 °C with 100 μg/ml proteinase K. One hundred microliters of 5 M NaCl was added to 0.6 ml lysate (final concentration, 0.7 M) and homogenized, followed by the addition of 40 μl of 10% CTAB in 0.7 M NaCl. After 10 min at 65 °C, the CTAB precipitate was extracted with 1 volume of chloroform, and the supernatant was transferred into a fresh tube. The DNA was purified by three successive extractions with phenol (pH 7.5), phenol-chloroform (1/1), and chloroform. The nucleic acids were precipitated with 2 M NaCl and 2 vol of ethanol. Extracted DNA samples were suspended into TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.5) and used as a template for amplification with specific primers. The quality of DNA extracts was determined spectrophotometrically by using NanoDrop spectrophotometer (ND-1000, Thermo Scientific, USA) by the A260/280 ratio and visualization on 2% agarose gel electrophoresis.

2.5. Real-time PCR assay

Genomic DNA extracted from the homogenized samples was employed as a template for molecular detection of FBPs by using a SYBR Green based real-time PCR assay. Each PCR reaction was performed in a final volume 20 μl comprising 10 μl of RealQ Plus 2x Master Mix Green (Ampliqon, Odense, Denmark), 0.4 μL of 10 pmol of forward and reverse primers, and 100 ng of DNA template. The reaction parameters for

amplification were 95 °C for 10 min, followed by 40 cycles at 95 °C for 20s, 30s of annealing at optimal temperature for each primer pair as indicated in Table 1, and 72 °C for 20s. All PCR amplifications were carried out in a Rotor-Gene® Q (Qiagen, Hilden, Germany) real-time PCR system. To prevent formation of primer dimers and non-specific products during amplification, melt curve analysis with increasing temperature from 60 °C to 95 °C (at regular increment of 0.5 °C for 5s) was performed. The specific oligonucleotide sequences used for detection of the FBPs by real-time PCR assay are presented in Table 1.

2.6. Statistical analysis

The IBM SPSS Statistics for Windows version 21.0 (IBM, Armonk, NY, USA) was used for testing differences between groups by using the Chi-squared test. A two-sided *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. District distribution and origin of vegetable samples

The majority of FL vegetables were collected from district 1 ($n = 32$) followed by district 20 ($n = 30$), district 4 ($n = 24$), district 5 ($n = 20$), and districts 2 and 3 ($n = 18$ each). The remaining 132 FL samples were collected from other districts accounting for less than 15 samples each. Additionally, most of the RTE samples were obtained from district 5 ($n = 25$), district 6 ($n = 11$), district 2 ($n = 9$), district 4 ($n = 6$), and districts 11 and 16 (each district, $n = 5$). The remaining 31 RTE samples were collected from other districts accounted for less than 5 samples each, with the exception of districts 13, 15, and 18 in which RTE vegetables were not available during the sampling period.

The vegetable samples originated from different cities and regions in Iran and were sold in Tehran. The most common sources of vegetables were from Shahriar ($n = 146$, 39.9%), wholesale markets in south of Tehran ($n = 37$, 10.1%), the farms around Tehran ($n = 16$, 4.4%), Alborz province ($n = 13$, 3.5%), Arak ($n = 12$, 3.3%), Yaftabad, a locality in

Table 1
List of oligonucleotide sequences used for detection of the foodborne pathogens by real-time PCR assays.

Bacteria	Target gene	Primers	Sequencing (5′–3′)	Annealing (°C)	References
Clostridioides difficile	NAD-specific glutamate dehydrogenase	Cdiff1fw	CCTAATTTAGCAGCAGCTTC	55	Du et al. (2014)
		Cdiff1rv	CTTGGATGGTTGATGAGTAC		
Clostridium perfringens	16S ribosomal RNA gene	16S rRNAfw	AAAGATGGCTCATTCAAC	58	Wu et al. (2009)
		16S rRNArv	TACCGTCATTATCTTCCCCAAA		
	Cpe gene	Cpefw	TTCAGTTGGATTTACTTCTG	55	Azimirad et al. (2019)
		Cperv	TGTCCAGTAGCTGTAATTGT		
Bacillus cereus	Ces gene	Bcer2fw	ACGCCGAAAGTGATTATACC	53	Wehrle et al. (2010)
		Bcer2rv	ATAAAACCACTGAGATAGTG		
Listeria monocytogenes	Cell wall hydrolases A	Lmon1fw	GTGTTGGTGCAACAGGAGTG	55	Bai et al. (2010)
		Lmon1rv	TAGTGGCGCTGGTGTTGATA		
Staphylococcus aureus	Membrane protein	Stau1fw	CACGACTAAATAAACGCTCA	55	Bai et al. (2010)
		Stau1rv	TCTCGTATGACCAGCTTCGG		
Campylobacter coli	CadF gene	Ccol1fw	TGTGAGACTACAGGAGCTGG	53	Ferrario et al. (2017)
		Ccol1rv	TTCCATGATGCAGATCATAG		
Campylobacter jejuni	HipO gene	Cjej2fw	AAGTTATTGGAAGAGGTGGT	53	Ferrario et al. (2017)
		Cjej2rv	TTAATCGTTGCAATATCTGG		
Shiga toxin-producing *E. coli*/enterohemorrhagic *E. coli* (STEC/EHEC)	Shiga toxin 1 subunit A (*stx1A*)	stx1Afw	CCATTCTGGCAACTCGCG	50	Carey et al. (2009)
		stx1Arv	GGCAAGAGCGATGTTACGGT		
		stx2Afw	TTGCTGTGGATATACGAGGGC		
	Shiga toxin 2 subunit A (*stx2A*)	stx2Arv	TCCGTTGTCATGGAAACCG		
Helicobacter pylori	Urease subunit alpha	Hpyl1fw	AACCGGATGATGTGATGGAT	53	Ferrario et al. (2017)
		Hpyl1ev	CCTTCGTTGATAGTGATGTC		
Pseudomonas aeruginosa	Enterotoxin	Paer1fw	CGATGACTGATGACCGTGGG	53	Ferrario et al. (2017)
		Paer1rv	TGTTGTGCCTGCTCGACCCG		
Salmonella enterica subsp. *enterica*	Hypothetical protein	See1fw	CGAGCTTGATGACAAACCTG	56	Ferrario et al. (2017)
		See1rv	GCTTCGCTTTTCCAACTGCC		
Salmonella enterica subsp. *enterica* serovar Enteritidis	Pilus formation protein (SafA)	Sent1fw	GGTTGCTAACACGACACTG	53	Ferrario et al. (2017)
		Sent1rv	TGGGGCATTGGTATCAAAG		
	Fimbrial biosynthesis protein	Sent2fw	GCCGTACACGAGCTTATAGA	56	Ferrario et al. (2017)
		Sent2rv	TGACTCTCTGTAGCTCGACC		
Salmonella enterica subsp. *enterica* serovar Tiphymurium	Putative cytoplasmic protein	Stiph1fw	CTGAACGTGGGTTATTTGAC	56	Maruthai et al. (2015)
		Stiph1rv	AGCGGCCAGGCGTTACCCAT		
	Sigma F factor of RNA polymerase	Stiph2fw	CATTACACCTTCAGCGGTAT	56	Ferrario et al. (2017)
		Stiph2rv	TGGTAAGAGAGCCTTATAGG		
Shigella flexneri	Hypothetical protein	Sflex1fw	TCTTCCTCATATCGAGTCTC	56	Ferrario et al. (2017)
		Sflex1rv	TGGTGCTTGTTGAGCAACTC		
Shigella boydii	Hypothetical protein	Sboy1fw	TGATGTCACTCTTTGCGAG	53	Ranjbar et al. (2014)
		Sboy1rv	GTAAAGGATAACTACTTCAC		
Shigella sonnei	RshC gene	Sson1fw	TCGAACTTCGATGCCAATCC	53	This study
		Sson1rv	CGGCAGACAGACGGATGCCG		
	BsuBI-PstI family restriction endonuclease	Sson2fw	CTTGAAGGAGATTCGCTGCT	55	Ranjba et al. (2014)
		Sson2rv	ACTTCGATGACGGCCTTAGC		
Yersinia enterocolitica	Attachment invasion locus	Yent2fw	ACTCGATGATAACTGGGGAG	55	Olsen et al. (1995)
		Yent2rv	CCCCCAGTAATCCATAAAGG		
Aeromonas hydrophila	Haemolysin	Ahyd1fw	TGGCCTTCTACCTCAACGTC	55	Singh et al. (2007)
		Ahyd1rv	ATCCGCACTATCTTGGCATC		
Vibrio cholerae	OprF membrane domain protein	Vch1fw	TTCCGTCCATATGTTGGTG	55	Ferrario et al. (2017)
		Vch1rv	AAGCGTTGAGGAACCAGCTA		

Tehran ($n = 9$, 2.5%) and cities of Shahr-e-Rey, Eslamshahr, Varamin, Isfahan (each city, $n = 6$, 1.6%) and the remaining from cities of Sari ($n = 5$, 1.4%), Hamedan ($n = 3$, 0.8%), Tabriz ($n = 2$, 0.5%), Dezful ($n = 2$, 0.5%), Safadasht ($n = 2$, 0.5%), Kashan ($n = 1$, 0.3%) and Khavaran, a neighborhood of Tehran ($n = 1$, 0.3%).

3.2. Occurrence of FBPs by conventional microbiological culturing methods

The results obtained from conventional microbiological culture methods are summarized in Table 2 and Fig. 2. Of the 11 pathogens searched by microbiological methods out of the twenty included in the study, the most prevalent was *S. aureus*, 134 positive samples out of 366 (36.6%), followed by *E. coli* (85/366, 23.2%) and *C. perfringens* (66/366, 18%); of these 116 (42.3%) *S. aureus*, 57 (20.8%) *E. coli* and 58 (21.2%) *C. perfringens* were detected in FL samples, and 18 (19.6%) *S. aureus*, 28 (30.4%) *E. coli* and 8 (8. 7%) *C. perfringens* bacteria were found in RTE vegetables. B. cereus was detected in 15 (5.5%) FL and 10 (10.9%) RTE samples. *L. monocytogenes* was identified in 15 (5.5%) FL and 4 (4.3%) RTE samples. Eighteen (4.1) samples were positive for *Salmonella* spp.; of these, three (3.3%) were detected in RTE and 15 (5.5%) in FL samples. Regarding *Shigella* spp., 15 (5.5%) isolates were detected in FL ($n = 13$, 4.7%) and RTE ($n = 2$, 2.2%) samples. *V. cholerae* was not detected in any of the samples tested and *Campylobacter* spp. and *Yersinia enterocolitica* were found in only 3.5% and 0.8% of the samples, respectively. The proportion of FL and RTE positive samples obtained by conventional microbiological methods was significantly different ($P < 0.05$) for *C. perfringens*, *Campylobacter* spp. and *S. aureus*.

3.3. Microbiological results obtained for FL and RTE vegetables by using real-time PCR assays

The results obtained from real-time PCR assays are also shown in Table 2 and Fig. 3. The presence of *C. difficile* and *H. pylori* was confirmed in 105/366 (28.7%) and 42/366 (11.5%) of all samples, respectively. All *C. perfringens* isolates were confirmed by real-time PCR using 16S ribosomal RNA gene. *L. monocytogenes* was detected in 28/366 (7.6%) of all samples, and more frequently in FL than in RTE vegetables. All *S. aureus* and *Y. enterocolitica* isolates were confirmed by real-time PCR assays. *A. hydrophila* was detetctd as the most common (39.3%; 110 FL and 34 RTE samples) bacterial agent among all samples by real-time PCR. Among the *Salmonella* spp., 19 (5.2%) isolates were identified as *S. enterica* subsp. *enterica*, one was *S.* Enteritidis and two were *S.* Tiphymurium by real-time PCR. All the 15 (4.1%) *Shigella* strains detected by cultural methods were confirmed as *S. flexneri*. Of the 85 *E. coli* isolates and 6 (1.6%) STEC/EHEC. Fifty-two samples (14.2%; 40 FL and 12 RTE samples), were positive for *C. coli* (CadF gene) and 24 for *C. jejuni* (HipO gene). The proportion of positive samples in FL and RTE vegetables was significantly different ($P < 0.05$) for *C. perfringens*, *S. aureus, H. pylori* and STEC/EHEC, the last one was found more frequently in RTE than in FL samples. We observed a noticeable reduction in the proportion of *Campylobacter* positive samples using conventional microbiological methods compared to the real-time PCR method, with overall 3.5% of positive samples with microbiological method and about 20.7% of positive with real-time PCR assay (Table 2). This notable difference was not observed for the other pathogens examined in this study.

4. Discussion

Conventional microbiological culture-based methods are still considered as the gold standard for microbiological analysis of food-borne outbreaks due to their reliability, efficiency, sensitivity, and their ability to determine viable bacteria present in the food samples (Ferone et al., 2020; Foddai and Grant, 2020). However, these procedures are laborious and time-consuming and incapable of simultaneously

Table 2
Microbiological results obtained for fresh leafy (FL) and ready-to-eat (RTE) vegetables ($n = 366$) by using reference culturing and real-time PCR assays.

	Culture			Real-time PCR		
Bacteria	FL ($n = 274$)	RTE ($n = 92$)	Total (%)	FL ($n = 274$)	RTE ($n = 92$)	Total (%)
Clostridioides difficile	NC	NC	NC	–	–	–
NAD-specific glutamate dehydrogenase	–	–	–	74 (27)	31 (33.7)	105 (28.7)
Clostridium perfringens	58 (21.2)	8 (8.7)	66 (18)	–	–	–
16S ribosomal RNA gene	–	–	–	58 (21.2)	8 (8.7)	66 (18)
Cpe gene	–	–	–	0	0	0
Bacillus cereus	15 (5.5)	10 (10.9)	25 (6.8)	–	–	–
Ces gene	–	–	–	0	0	0
Listeria monocytogenes	15 (5.5)	4 (4.3)	19 (5.2)	–	–	–
Cell wall hydrolases A	–	–	–	21 (7.7)	7 (7.6)	28 (7.6)
Staphylococcus aureus	116 (42.3)	18 (19.6)	134 (36.6)	–	–	–
Membrane protein	–	–	–	116 (42.3)	18 (19.6)	134 (36.6)
Vibrio cholerae	0	0	0	–	–	–
OprF membrane domain protein	–	–	–	0	0	0
Aeromonas hydrophila	NC	NC	NC	–	–	–
Haemolysin	–	–	–	110 (40.1)	34 (36.9)	144 (39.3)
Yersinia enterocolitica	3 (1.1)	0	3 (0.8)	–	–	–
Attachment invasion locus	–	–	–	3 (1.1)	0	3 (0.8)
Helicobacter pylori	NC	NC	NC	–	–	–
Urease subunit alpha	–	–	–	39 (14.2)	3 (3.3)	42 (11.5)
Pseudomonas aeruginosa	27 (9.8)	4 (4.3)	31 (8.5%)	–	–	–
Enterotoxin	–	–	–	0	0	0
Campylobacter spp.	13 (4.7)	0	13 (3.5)	–	–	–
Campylobacter coli (CadF gene)	–	–	–	40 (14.6)	12 (13)	52 (14.2)
Campylobacter jejuni (HipO gene)	–	–	–	18 (6.6)	6 (6.5)	24 (6.5)
Salmonella spp.[a]	15 (5.5)	3 (3.3)	18 (4.9)	–	–	–
Salmonella enterica subsp. *enterica*	–	–	–	15 (5.5)	4 (4.3)	19 (5.2)
Salmonella enterica subsp. *enterica* serovar Enteritidis	–	–	–	0	1 (1.1)	1 (0.3)
Salmonella enterica subsp. *enterica* serovar Tiphymurium	–	–	–	0	2 (2.2)	2 (0.5)
E. coli	57 (20.8)	28 (30.4)	85 (23.2)	–	–	–
STEC/EHEC				1 (0.4)	5 (5.4)	6 (1.6)
Shigella spp.	13 (4.7)	2 (2.2)	15 (4.1)	–	–	–
Shigella flexneri (hypothetical protein)	13 (4.7)	2 (2.2)	15 (4.1)	13 (4.7)	2 (2.2)	15 (4.1)
Shigella boydii (hypothetical protein)	0	0	0	0	0	0
Shigella sonnei (RshC and BsuBI-PstI genes)	0	0	0	0	0	0

STEC/EHEC: Shiga toxin-producing *E.* coli/enterohemorrhagic *E. coli.* NC: Not cultivated.

[a] *Salmonella enterica* subsp. *enterica* (hypothetical protein), *Salmonella enterica* subsp. *enterica* serovar Enteritidis (pilus formation protein and fimbrial biosynthesis protein), and *Salmonella enterica* subsp. *enterica* serovar Tiphymurium (putative cytoplasmic protein and sigma F factor of RNA polymerase).

detecting several different FBPs, and results are only observed after several days (Ferrario et al., 2017; Liu et al., 2019). Moreover, traditional approach for microbiological identification which is largely based on cultivation proceedings and phenotypic, morphological and biochemical features may result in frequent false positives especially when exploring closely related microbial species (Abayasekara et al., 2017; Franco-Duarte et al., 2019). On the other hand, DNA-based diagnostic methods typically offer a faster, more specific and sensitive alternative to convetional and culture-based techniques for detection of FBPs (Li et al., 2020). They also enable detection of non-cultivable bacteria and sub-dominant populations, even in the absence of a selective enrichment medium (Postollec et al., 2011). Taken together, in this study we applied both culturing and real-time PCR methods to investigate the presence of FBPs in FL and RTE vegetables in Tehran.

The results of the presented study showed the level of contamination of FL and RTE vegetables in Tehran, Iran, by important bacterial FBPs and confirmed the higher occurrence of bacterial contamination in FL samples compared to RTE vegetables. We found higher proportion of contaminated samples in FL than in RTE vegetables for *C. perfringens*, *S. aureus*, *Campylobacter* spp. and *H. pylori* but not for *E. coli*, in particular for the STEC/EHEC. Since RTE but not FL are washed, this is probably due to the effect of the washing, that although did not completely eliminate the microbial contamination, reduced the final microbial load and the pathogens in RTE vegetables. On the other hand, the contamination of RTE with EHEC is a cause of concern, EHEC bacteria as subgroup of STEC are associated with severe clinical illness and outbreaks (Karmali et al., 2003; Bugarel et al., 2010). Vegetables can be contaminated by EHEC at any step of the production chain, and studies conducted on *E. coli* O157:H7 showed its ability to internalize plant tissue and gain protection from environmental influences and from washing and sanitizing processes (Solomon et al., 2002; Luna-Guevara et al., 2019). Bacterial invasion can occur in intact and damaged leaves, but damaged tissues offer more favourable conditions for bacterial survival and/or multiplication (Luna-Guevara et al., 2019). Thus, it is possible that the processing of RTE (washing, cutting and packaging) and consequent tissue damage should have facilitated the persistence of STEC/EHEC and the higher contamination rate in RTE than in FL.

Among the 11 FBPs included in the survey that were searched with microbiological methods, 10 were isolated from the FL and 8 from the

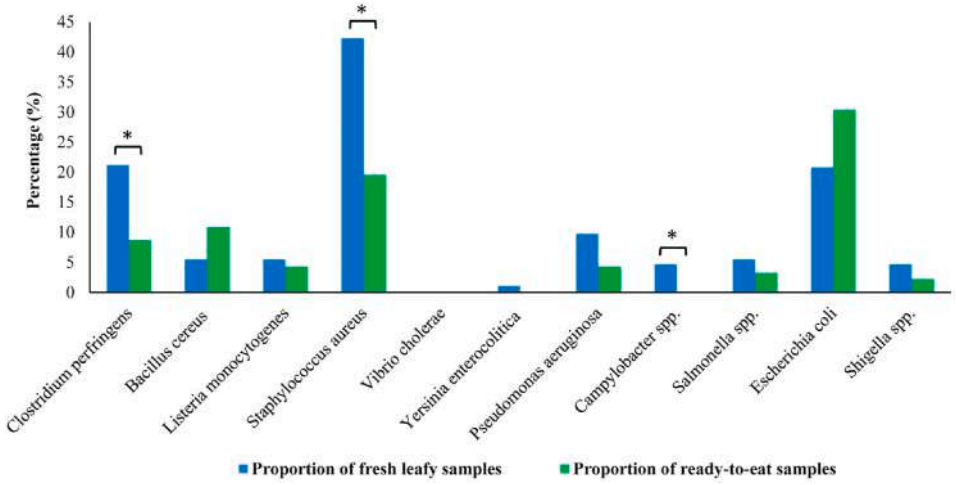

Fig. 2. Proportion of fresh leafy (FL) and ready-to-eat (RTE) samples positive for the eleven pathogens searched by conventional microbiological methods. The (*) means for that pathogen the proportion of FL and RTE positive samples was statistically ($P < 0.05$) different.

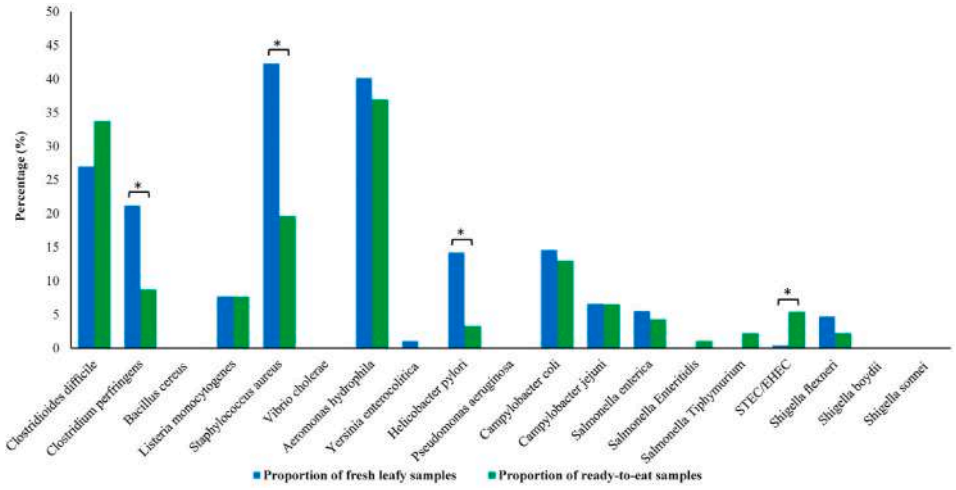

Fig. 3. Proportion of fresh leafy (FL) and ready-to-eat (RTE) samples positive for the twenty pathogens searched by real-time PCR. The (*) means for that pathogen the proportion of FL and RTE positive samples was statistically ($P < 0.05$) different.

RTE samples, confirming the higher level of hygiene of RTE compared to FL samples. *V. cholerae* was not detected in any of the samples processed, neither by cultural nor by molecular methods.

We also found some differences in the proportion of positive samples according to the method used, in particular for *Campylobacter* spp., that was more frequently detected by real-time PCR than cultural methods, as observed also by Losio et al. (2015). This is probably related to the difficulty to detect *Campylobacter* by culture methods and the higher sensitivity of molecular methods that, on the other hand, cannot distinguish between infective and non-infective bacteria.

S. aureus was the predominant bacterial contaminant in our study, detected in both FL and RTE samples. *S. aureus* strains that produce staphylococcal enterotoxins (SEs) are common causes of reported staphylococcal food poisoning. *S. aureus* is usually detected in RTE foods (Elizaquivel and Aznar, 2008; Chajęcka-Wierzchowska et al., 2014). We did not check our isolates for SEs production, but a study in Iran conducted on milk samples found around 13% of the isolates positive for SEs (Saadat et al., 2014).

In our study we found a high proportion of samples contaminated by sporogenous bacteria, in particular *C. difficile*, *C. perfringens* and *B. cereus*. *C. perfringens* and *B. cereus* are considered as a concern when food is handled in a manner that allow germination of spores and growth of vegetative cells with production of toxins (Gómez-Govea et al., 2012). The detection of *C. difficile* only by real-time PCR did not allow to understand if viable bacterial cells and not only nucleic acids were present in the positive samples. However, FL vegetables are identified as high risk food for transmission of *C. difficile* to humans (Rodriguez-Palacios et al., 2020).

We detected high rates of samples positive for *A. hydrophila* by real-time PCR in both FL and RTE vegetables. Aeromonas is an environmental microorganism distributed worldwide, and mesophilic motile aeromonads are present in fresh water, sewage and brackish water (Wei et al., 2015), and also in chlorinated and unchlorinated drinking water (Janda and Abbott, 2010; Abraham and Abraham, 2011). In the last decades *Aeromonas* spp. have emerged as an important human pathogen and vegetables contaminated with poorly sanitized or polluted water have been implicated in a foodborne outbreak in China (Zhang et al., 2012). The presence of *Salmonella* spp., *Campylobacter* spp., *Shigella* spp. and *Y. enterocolitica* in vegetables has frequently reported in association with human infections and outbreaks (Taban and Halkman, 2011; Mohammadpour et al., 2018).

H. pylori was found by real-time PCR in both FL and RTE samples. This highly adapted human gastric pathogen is linked to some of the most common chronic clinical disorders of the upper gastrointestinal tract in humans (Farzi et al., 2018; Yadegar et al., 2019). It is also recognized by IARC as class I carcinogens (Takahashi-Kanemitsu et al., 2020). *H. pylori* was detected in water, vegetables and foods of animal origin pointing out the role of the food as a source of human infection (Quaglia and Dambrosio, 2018). In Iran, the prevealnce rate of H. pylori infection varries in different parts of the country and nearly 50–69% of adult population are infected with this bacterial pathogen (Hooi et al., 2017; Farzi et al., 2018).

Several food surveys throughout the world reported the detection of *L. monocytogenes* in raw RTE vegetables sold at market retails. In this study *L. monocytogenes* was detected in FL and RTE in rates comparable with a survey carried out in China (Chen et al., 2019), but lower than the prevalence found in other parts of the world (Cordano and Jacquet, 2009; Ponniah et al., 2010; Ajayeoba et al., 2015). Accordingly, the microbiological safety of RTE vegetables seems to be a challenging issue because, on the basis of the theory that they have been already washed, people consume them dierectly and without prior preparation. Thus, inadequate practices during the preparation of RTE vegetables may increase the risk of contamiantion and cause foodborne disease outbreaks (Ahmed et al., 2017; Makinde et al., 2020).

Our study presents some limitations, in particular the representativeness of the sampling, that included Tehran city only, and the time period, that was less than one year, not allowing a more detailed representation of the variability according to seasonality of the occurrence of the different bacteria in the collected samples. Our investigation also lacks the monitoring of viral FBPs, however recently developed international standard methods have been released for the quantification of norovirus and HAV (https://www.iso.org/standard/65681.html; https://www.iso.org/standard/74263.html), and some research is invested into how to determine viral infectivity (Leifels et al., 2021). Thus, this is an issue for future research to explore detection of viral FBPs in LF and RTE vegetables consumed in our country.

5. Conclusions

Taken altogether our findings indicated a contamination of FL and RTE vegetables in Iran with a range of well-known and emerging FBPs. The proportion of positivity and the distribution of bacterial species indicate different contamination sources for the vegetables, including environment, direct or indirect animal and human fecal contamination, and overall a lack of effective decontamination steps during the production chain. Additional information about the quality of the water used for irrigation and washing, the hygiene measures implemented during the processing, storage and marketing of the vegetables are needed to better identify the critical points and define the proper measures. A schematic representation of potential sources and contamination routes of microbial pathogens to vegetables during field cultivation and farm-to-fork chain is presented in Fig. S1. Moreover, the importance to follow standard hygiene measures during food preparation, including thorough washing and disinfection of vegetables before consumption must be conveyed to consumers.

Author contributions

MA, BN, HA, SNP, SMVB, FA, FG, and PJ performed the microbiological examinations. BN, HA, SNP, SMVB, FA, FG, and PJ collected the vegetable samples. MA, BN, AY and LB reviewed the literature and wrote the manuscript. AY and LB, HAA and MRZ critically revised the final version of the manuscrip. We also confirm that all authors have read and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no competing interests or relationships that could influence the results reported in this study.

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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.2021.113824.

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Particle and metal exposure in Parisian subway: Relationship between exposure biomarkers in air, exhaled breath condensate, and urine

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ABSTRACT

Subway particulate toxicity results from *in vitro* and *in vivo* studies diverge and call for applied human research on outcomes from chronic exposures and potential exposure biomarkers. We aimed to (1) quantify airborne particulate matter (PM) concentrations (mass and number) and metal concentrations in exhaled breath condensate (EBC), urine, and PM; (2) investigate their associations (EBC vs. PM vs. urine); and (3) assess the relevance of EBC in biomonitoring. Nine subway workers in three jobs: station agents, locomotive operators and security guards were monitored during their 6-h shifts over two consecutive weeks. Six-hour weighed average mass concentrations expressed as PM10, PM2.5 and their metal concentrations were determined. Urine and EBC samples were collected pre- and post-shift. Ultrafine particle (UFP) number concentrations were quantified in PM and EBC samples. Metal concentrations in urine and EBC were standardized by creatinine and EBC volume, respectively, and log-transformed. Associations were investigated using Pearson correlation and linear mixed regression models, with participant's ID as random effect. PM concentrations were below occupational exposure limits (OEL) and varied significantly between jobs. Locomotive operators had the highest exposure (189 and 137 $\mu g/m^3$ for PM10 and PM2.5, respectively), while station agents had the highest UFP exposure (1.97×10^4 particles/cm^3). Five metals (Al, Fe, Zn, Cu, and Mn) in PM2.5 and three (Al, Fe, and Zn) in PM10 were above the limit of quantification (LOQ). Fe, Cu, Al and Zn were the most abundant by mass fraction in PM. In EBC, the metal concentrations in decreasing order were: Zn > Cu > Ni > Ba > Mn. Security guards had the highest EBC metal concentrations, and in particular Zn and Cu. Urinary metal concentrations in decreasing order were: Si > Zn > Mo > Ti > Cu > Ba ≈ Ni > Co. All urinary metal concentrations from the subway workers were similar to concentrations found in the general population. A statistically significant relationship was found for ultrafine particle number concentrations in PM and in EBC. Zn and Cu concentrations in post-shift EBC were associated with Zn and Cu concentrations in PM10 and with post-shift urinary Zn and Cu concentrations. Therefore, EBC appears a relevant matrix for assessing exposure to UFP in human biomonitoring when inhalation is a primary route of exposure. We found different temporal variation patterns between particle and metal exposures in three matrices (PM, urine, EBC) quantified daily over two full weeks in subway workers. These patterns might be related to metal oxidation, particulates' solubility and size as well as their lung absorption capabilities, which need to be further explored in toxicological research. Further research should also focus on understanding possible influences of low chronic exposures to subway particulates on health in larger cohorts.

1. Introduction

Subways (also called metros, undergrounds or underground railways) are the most commonly used mode of public transportation in large cities (Wen et al., 2020). Subways are low-carbon transport modes and crucial in meeting climate goals. Public authorities worldwide have adopted clean air policies since 2000 and interventions on public transport systems were shown effective (Burns et al., 2020) in reducing particulate matter (PM) emissions (EEA., 2020). The toxicity of suspended particles is mainly due to aerosolized particles with a diameter of less than 10 μm. PM concentrations with a median diameter of less

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Abbreviations

BMI	Body mass index
EBC	exhaled breath condensate
GV	indoor air quality guide values
ICP-MS	inductively coupled plasma mass spectroscopy
LDSA	lung deposited specific area
LEM	Laboratory of Essays and Measurements
LOD	limit of detection
LOQ	limit of quantification
NTA	nanotracking analysis
OEL	occupational exposure limits
PBZ	personal breathing zone
PM	particulate matter
PNOS	particles not otherwise specified
ROS	reactive oxygen species
RTV	reference toxicological value
SD	Standard deviation
WHO	World Health Organization

than 10 μm are reported as PM10 and those less than 2.5 μm as PM2.5. Many countries have reported PM concentrations in subway air exceeding the guidelines for indoor air quality from the World Health Organization (WHO) currently set at 50 and 25 $\mu g/m^3$ for PM10 and PM2.5, respectively, for 24 h exposure (WHO, 2005). Poor subway air quality might present a potential health risk for regular subway users and in particular, for workers (Smith et al., 2020; Loxham et al., 2013). Environmental exposure assessments and guidelines for interpreting PM2.5 and PM10 concentrations are used for subway users, while occupational exposure assessments and regulations use total inhalable and respirable dust with median particle diameter of less than 100 μm and 4 μm, respectively. Inhalable and respirable dust concentrations are regulated with occupational exposure limits (OELs). The OEL set for dust exposures among subway workers is 'particles not otherwise specified' (PNOS).

Environmental exposure studies consistently document a very specific physical-chemical composition and size distribution of subway PM. Subway PM is highly ferruginous, with up to 67% iron oxide in PM2.5 (Seaton et al., 2005) and up to 50% in PM10 (Park et al., 2012). Subway PM also contains trace metals (Mg, Al, Si, Ti, V, Cr, Mn, Fe, Ni, Cu, Zn, Ba, and Pb), some of which have known adverse health effects. Subway PM toxicity results from *in vivo* studies are inconclusive and diverge from those of *in vitro* studies. The latter show that subway PM generates more reactive oxygen species (ROS) and oxidative stress related outcomes compared to other PM (Loxham and Nieuwenhuijsen, 2019), although a direct evidence for the clinical significance of ROS generation *in vivo* is limited (Loxham and Nieuwenhuijsen, 2019). The controversy between *in vitro* and *in vivo* studies may be due to disparities between *in vivo* exposures and *in vitro* models, and differences in exposure doses, as well as lack of statistical power in *in vivo* studies of chronic exposures. Future research recommendations are to focus on outcomes from chronic *in vivo* exposures and understanding mechanisms and potential biomarkers of exposure (Wen et al., 2020; Loxham and Nieuwenhuijsen, 2019).

Following this recommendation, we launched a Franco-Swiss epidemiological research project, called "ROBoCoP" for the Respiratory disease Occupational Biomonitoring Collaborative Project. ROBoCoP aimed at assessing occupational exposures to airborne PM, exposure biomarkers related to PM exposures, and early respiratory effects among subway workers employed by the Parisian urban transport company (RATP) (Guseva Canu et al., 2021).

In this study, we present 6 h-weighed particle mass concentration assessed over two work-weeks in subway workers using both environmental and occupational exposure assessment approaches. Moreover, we determined metal concentrations in three matrices: exhaled breath condensate (EBC), urine, and PM (PM2.5 and PM10). We also assessed the particle number concentrations in EBC and PM in air with direct-reading instruments. Our aims were to (1) quantify particulate matter (PM) concentrations (mass and number) and metal concentrations in exhaled breath condensate (EBC), urine, and PM; (2) investigate their associations (urine vs. PM vs. EBC); and (3) assess the relevance of EBC in biomonitoring.

2. Material and methods

2.1. Study design, setting and participants

This six-week occupational pilot-study was conducted at the Parisian urban transport company (RATP) in France according to a registered research protocol (Guseva Canu et al., 2021). Collected samples were analyzed at Unisanté in Switzerland and RATP (LEM laboratories). Workers in three different jobs were included: subway station agents, locomotive operators, and security guards. Station agents oversee passenger information and ticket sale. They operate the ticket counters and have a mobile activity checking ticket distributors in the stations' concourses and controlling purchased tickets among travelers. Each station agent work on one assigned subway line during a work shift. Locomotive operators run the subway trains and spend the majority of their work shift inside the train cabin physically separate from the passenger rail cars. Security guards patrol stations on demand, constantly moving from one to another across all subway lines. We selected subway line 7 for our study, as it is underground, has no mechanical ventilation and therefore represents a worst-case scenario in terms of PM exposures. The convenience sample approach included nine (three per job or professional type) non-smoking subway workers of both sexes. We collected samples by job type: two weeks per type of subway professionals from October 7th to November 15th' 2019. Moreover, all participants filled in a standardized epidemiological questionnaire describing factors that may influence exposures and biological sample analysis (Guseva Canu et al., 2021).

2.2. Air sample collection and analyses

RATP safety regulations do not allow any RATP professionals to wear any equipment other than those used for their regular work. Consequently, airborne PM were collected with appropriate equipment but carried by two or three RATP LEM technicians job-shadowing the RATP worker for the entire shifts. According to our protocol (Guseva Canu et al., 2021), the RATP LEM technicians would don the air sampling equipment in the personal breathing zone (PBZ); however, this was not physically possible in the cramped space for station agents and locomotive operators. Therefore, the RATP LEM technicians carried all air sampling equipment and instruments in a backpack (security guards) or placed this backpack close to the sitting workers.

The sampling train for measuring PM2.5 and PM10 was equipped with a filter (PTFE Membrane Filters (37 mm), Sigma-Aldrich, France) in a cassette holder (Personal Impactor H-PEM, BGI, USA) connected to a cyclone and attached with flexible tubing to a pump (GilAir Plus, Sensidyne, Germany) operating at 4L/min. Inhalable (PM100) and respirable (PM4) dust were collected actively (pump rate at 10 L/min) on foam using individual dust samplers CIP 10-I and CIP 10-R, respectively (Tecora, France). A particle counter ("DISCmini", (Testo, Mönchaltorf, Suisse) measured particles from 10 to 300 nm, particle number concentration ($\#/cm^3$), and lung deposited specific area (LDSA) (recorded every 10 s). Both particle size and number concentrations are exposure metrics related to adverse health outcomes of ultrafine particles (UFP) (Schraufnagel, 2020). A second sampling train included the Mini-Particle Sampler, MPS® (INERIS, France) equipped with a transmission electronic microscopy (TEM) grid (Q310AR-14; Quantifoil R 1/4, 300 Mesh, Gold, Quantifoil Micro Tools GmbH, Germany) for

microscopy analyses (R'mili et al., 2013).

Random grid surfaces were analyzed with a TEM (CM100 Biotwin, at 80 kV, Philips) and a scanning electronic microscopy (SEM) (Phenom ProX, at 15 kV, Thermo Scientific) coupled with Energy-Dispersive X-ray detector (EDX). Particle morphology, size and chemical composition were determined. Quantification of mass concentration was determined using standard gravimetric analysis for total inhalable and respirable dust and for PM2.5 and PM10. PM were also analyzed for 11 elements (Al, As, Ba, Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) using two acidic digestion steps (95°C; HCl 30% for 25 min then HNO_3 65% for 15 min) and inductively coupled plasma mass spectroscopy (ICP-MS). The measured concentrations were integrated over sampling time, which was equal to the 6-h work-shift duration. All laboratories were accredited for regulatory analysis and used certified methods.

2.3. Exhaled breath condensate sampling and analyses

The pre- and post-shift EBC samples were collected daily over two consecutive weeks, using a portable collection device (Turbo-DECCS, Medivac, Parma, Italy) set at −10 °C. The recommendations of the American Thoracic Society (ATS) and the European Respiratory Society Task Force (ERSTF) (Horváth et al., 2005, 2017) were strictly applied. None of the participants declared drinking coffee an hour before EBC collection. A volume of 2–3 mL of EBC was collected from each participant (20 min). EBC samples were aliquoted and conserved at −80 °C until analysis. Biological sample collection, aliquoting and storage were operated in a closed clean room equipped for study purposes.

EBC metal concentrations were quantified by ICP-MS (ICap TQ, Thermo Scientific) at the Unisanté laboratory. The calibration curve was prepared by diluting a multi-element certified stock solution (Plasma Cal, SCP Science, France) with water to 0–50 µg/L range. The HNO_3 (40 µl, Plasma Plus pure, 67–70%, SCP Science, France) containing the internal standards Y, Rh, and Ir (100 µg/L) was added to 400 µl of calibration or EBC sample. The mixture was directly introduced in the plasma by aspiration. The QTegra vers 2.10 software was used for the signal acquisition and treatment was done using. All metal concentrations were standardized per EBC volume and expressed in µg/L. The observed limit of quantifications (LOQs) was 10.0 µg/L for Ba, Co, Cr, Cu, Mn, Mo, Ni, Pb, Sb, V, 1 µg/L for Fe, Ti and Zn, 5.0 µg/L for Al, and 20.0 µg/L for Si.

The number of sub-micron particles in the EBC samples was quantified using the nanotracking analysis (NTA), which also determines the hydrodynamic size distribution with a diameter of approximately 40–1000 nm (nm) in liquid suspension (Sauvain et al., 2017). About 400 µl of EBC sample was introduced into the cell of a NTA instrument (LM10, Malvern Pananalytical, Malvern, UK), which adds a laser beam to excite the particles in Brownian motion and track their movements with a camera. A total of 5 videos of 60 s was recorded and analyzed using the NTA software (version 3.1) (Sauvain et al., 2017).

2.4. Urine sampling and analyses

The pre- and post-shift urine samples were collected daily over two weeks. Urinary metal concentrations were quantified at Unisanté laboratory using ICP-MS (ICap TQ, Thermo Scientific, Switzerland). Element LOQs were as follows: 12.50 µg/L for Al, 0.39 µg/L for Ba, 0.20 µg/L for Co, 2.80 µg/L for Cu, 0.33 µg/L for Cr, 25.00 µg/L for Fe, 0.08 µg/L for Mn, 4.80 µg/L for Mo, 0.26 µg/L for Ni, 1.42 µg/L for Pb, 0.28 µg/L for Sb, 400.00 µg/L for Si, 20.00 µg/L for Ti, 0.31 µg/L for V and 24.80 µg/L for Zn.

Urinary concentrations were standardized per gram of creatinine to remove the influence of urine dilution on exposure biomarkers measured in spot samples. Urinary creatinine concentrations were measured with the Jaffe method (Jaffe, 1986). Only urine samples with creatinine concentrations in the normal range (0.5–3 g/L) were included in the analysis.

2.5. Data management and statistical analysis

2.5.1. Modelling of particle and metal concentrations

We first examined graphically the distribution of continuous quantitative variables corresponding to the measured particle or metal concentrations and log-transformed the variables that were log-distributed. For some elements, a proportion of the measurements fell below the LOQ or in the interval between limit of detection (LOD) and LOQ. In that case, we used the multilevel mixed-effects interval regression models, where the dependent variable, can be left- or interval-censored and recorded using two variables corresponding to its lower and upper values (Gelman and Hill, 2006). Among independent variables we considered Job and Day of the week as fixed effect variables and Participant's ID as random effect variable to account for intra-cluster correlation when modelling the airborne PM and metal concentrations. The inter-subject variance was compared with intra-subject variance (corresponding to the residual variance in each model), and reported as intra-class-correlation (i.e., ratio inter-subject variance/total variance). When modelling particle (EBC) and metal concentrations (EBC and urine), the sampling time (pre-or post-working shift) alone and in interaction with Job were considered as additional fixed effect variables. For each concentration, we then predicted the marginal mean concentration (dependent on the covariate pattern) with associated 95%-confidence intervals ($CI_{95\%}$), in original scale. For metals where a large proportion of measurements fell below the LOQ, the mixed effects modelling may result in biased estimates of the fixed effects and variability (Morton et al., 2014). Therefore, we limited the analysis to the metals with less than 50% of measurements below the LOQ to minimize the bias arising from censored data.

2.5.2. Analysis of the relationship between different exposure metrics

We first explored the relationships between different exposure matrices for every metal and PM sizes (PM10 and PM2.5). We considered three types of variables for EBC and urinary exposure biomarkers: pre- and post-shift concentrations and the ratio post-shift to pre-shift concentration (unit-less), expressing the change in exposure biomarker concentrations over the work-shift. Moreover, we explored the values measured 24 h and 48 h before, notified as lag 1 and lag 2, respectively to assess temporal variations in the biomarkers. This exploratory analysis was based on the pairwise Pearson correlation coefficients. We performed no adjustment for multiple testing since we wanted to identify possible relationships rather than testing an initial set of hypotheses (Rothman, 1990; Bender and Lange, 2001). We analyzed further exposure matrices with significant correlation using multilevel mixed-effects models, adjusted for participant's age, sex, and micronutrient/vitamin oral supplementation. All analyses were performed with STATA statistical software, version 16 (STATA, College Station, TX, USA).

3. Results and discussion

3.1. Description of study sample

Nine subway professionals participated to our study, and are described in Table 1.

All participants completed the questionnaires and provided all required biological samples (100% participation). Creatinine concentration was within normal range in 86% (N = 144) of urine samples.

3.2. PM and metal concentrations in air

3.2.1. Particle concentrations

We present the 6-h weighed average PM concentrations in Table 2. PM concentrations irrespective of particle size were lowest among station agents and highest among locomotive operators (Table 2, Fig. 1). The ticket booth equipped with a general ventilation probably contributed to the low airborne PM concentrations for the station agents.

Table 1
Description of the study sample.

Characteristics	Station agents	Locomotive operators	Security guards
Number of participants (n (%))	3 (100%)	3 (100%)	3 (100%)
Sex	Women	Men	Men
Age (in years, Mean ± SD)	42.00 ± 10.10	49.86 ± 12.32	49.83 ± 6.35
Length of employment (years, Mean ± SD)	10.66 ± 12.42	15.00 ± 2.64	18.33 ± 1.15
General health score (on 8-point scale)	2.00 ± 0.00	3.33 ± 0.58	1.33 ± 0.58
BMI (Mean ± SD)	23.70 ± 1.94	27.68 ± 2.17	25.25 ± 0.55
Use of vitamins/ supplementation (n (%))	0 (0%)	1 (33%)	0 (0%)
Home to work commuting (min., Mean ± SD)	58.33 ± 15.27	60.00 ± 47.69	64.16 ± 31.05
Use of motor vehicle for commuting (n (%))	0 (0%)	1 (33%)	1 (33%)
Use of bicycle for commuting (n (%))	0 (0%)	0 (0%)	0 (0%)
Commuting by foot (n (%))	1 (33%)	1 (33%)	1 (33%)
Follow-up period	7–18.10.2019	21–31.10.2019	4–15.11.2019

Station agents were exposed to the highest number concentrations of UFP, but converted to LDSA metrics, locomotive operators had the highest exposure (44.79 $\mu m^2/cm^3$, $CI_{95\%}$ = 39.36–50.21) closely followed by station agents (37.79 $\mu m^2/cm^3$, $CI_{95\%}$ = 28.99–46.59). Both metrics exhibited statistically significant time-dependence, varying not only from day to day, but also over a much shorter time span (Petremand et al., 2021).

PM10 exhibited statistically significant daily variation. In the Stockholm subway, the PM10 concentrations were correlated with hourly train frequencies and the number of rail cars (Tu and Olofsson, 2021). We did not find any particular pattern for these variables in our study (Table 2). Our PM10 concentrations are in line with PM10 values in underground stations reported in the scientific literature (Tu and Olofsson, 2021).

The PNOS concentrations were below current French OELs; 4 mg/m^3 and 0.9 mg/m^3 for inhalable and respirable particles, respectively (Guillou et al., 2020). The OEL set for PNOS are used in the instances where the metals do not have their own OEL such as for titanium dioxide, which has been classified suspected carcinogen (Guseva Canu et al., 2020). Workers exposed to titanium dioxide may therefore have an increased risk of cancer, which goes undetected. Metals with specific effects deserve specific exposure assessments.

3.2.2. PM2.5 and PM10 metal concentrations

Metal concentrations in PM10 and PM2.5 are presented in Table 2. Fifty percent of the values for six (As, Ba, Cd, Cr, Ni, Pb) of the 11 metals quantified in PM10 were below LOQ. PM10 contained up to 40% Fe and 20% Al, and less than 2% Cu, Zn and Mn (results not shown) and varied significantly across jobs (Table 1). The locomotive operators had the highest exposure to Fe, Zn, and Mn, while the security guards had the highest exposures to Al, which was twice that of the other jobs for PM10 and almost twice for PM2.5 (Table 1). PM10 Cu concentrations were the same for locomotive operators and security guards, and three times greater than for station agents.

Table 2
PM and metal concentrations in the personal air samples of Paris subway workers.

Parameter measured	Fixed effects (p-value)		Intra-class correlation	Geometric Mean [95% Confidence Interval]*											
	Day of the week	Job		Station agents				Locomotive operators				Security guards			
Ultrafine particles <300 nm (10^4#/cm3)	<0.001	<0.001	0.49	1.97	[1.48	;	2.46]	1.59	[1.39	;	1.80]	0.95	[0.82	;	1.08]
Ultrafine particles <300 nm (µm2/cm3)	< **0.001**	< **0.001**	0.49	37.79	[28.99	;	46.59]	44.79	[39.36	;	50.21]	25.45	[22.19	;	28.71]
Respirable particles (mg/m3)	0.69	**0.05**	0.49	0.07	[0.06	;	0.08]	0.10	[0.07	;	0.12]	0.09	[0.07	;	0.11]
Inhalable particles (mg/m3)	0.43	< **0.001**	0.48	0.06	[0.05	;	0.08]	0.20	[0.15	;	0.26]	0.14	[0.10	;	0.18]
PM2.5 (µg/m3)	0.73	< **0.001**	0.44	44.49	[25.47	;	63.52]	136.83	[70.04	;	203.62]	47.79	[22.22	;	73.37]
PM10 (µg/m3)	**0.03**	< **0.001**	0.46	54.20	[36.37	;	72.03]	188.50	[101.93	;	275.07]	79.71	[43.95	;	115.46]
Al in PM2.5 (µg/m3)	0.61	0.28	0.44	5.76	[3.77	;	7.76]	4.24	[2.08	;	6.41]	7.74	[3.73	;	11.75]
Fe in PM2.5 (µg/m3)	0.49	< **0.001**	0.47	1.48	[0.98	;	1.97]	15.65	[9.03	;	22.26]	5.11	[2.60	;	7.63]
Zn in PM2.5 (µg/m3)	0.38	< **0.001**	0.49	0.40	[0.34	;	0.46]	0.64	[0.50	;	0.78]	0.40	[0.31	;	0.49]
Al in PM10 (µg/m3)	0.66	**0.01**	0.43	5.20	[3.38	;	7.02]	6.61	[2.97	;	10.26]	13.22	[6.27	;	20.17]
Fe in PM10 (µg/m3)	0.93	< **0.001**	0.47	2.49	[1.23	;	3.75]	48.11	[21.72	;	74.50]	12.32	[3.40	;	21.25]
Zn in PM10 (µg/m3)	0.61	< **0.001**	0.48	0.61	[0.49	;	0.73]	1.08	[0.76	;	1.39]	0.42	[0.30	;	0.54]
Cu in PM10 (µg/m3)	0.51	< **0.001**	0.46	0.05	[0.03	;	0.07]	0.15	[0.08	;	0.21]	0.15	[0.08	;	0.21]
Mn in PM10 (µg/m3)	0.82	< **0.001**	0.48	0.06	[0.05	;	0.08]	0.45	[0.32	;	0.59]	0.13	[0.09	;	0.17]

Statistically significant results are shown in bold.
* Marginal mean concentration predicted by the model.

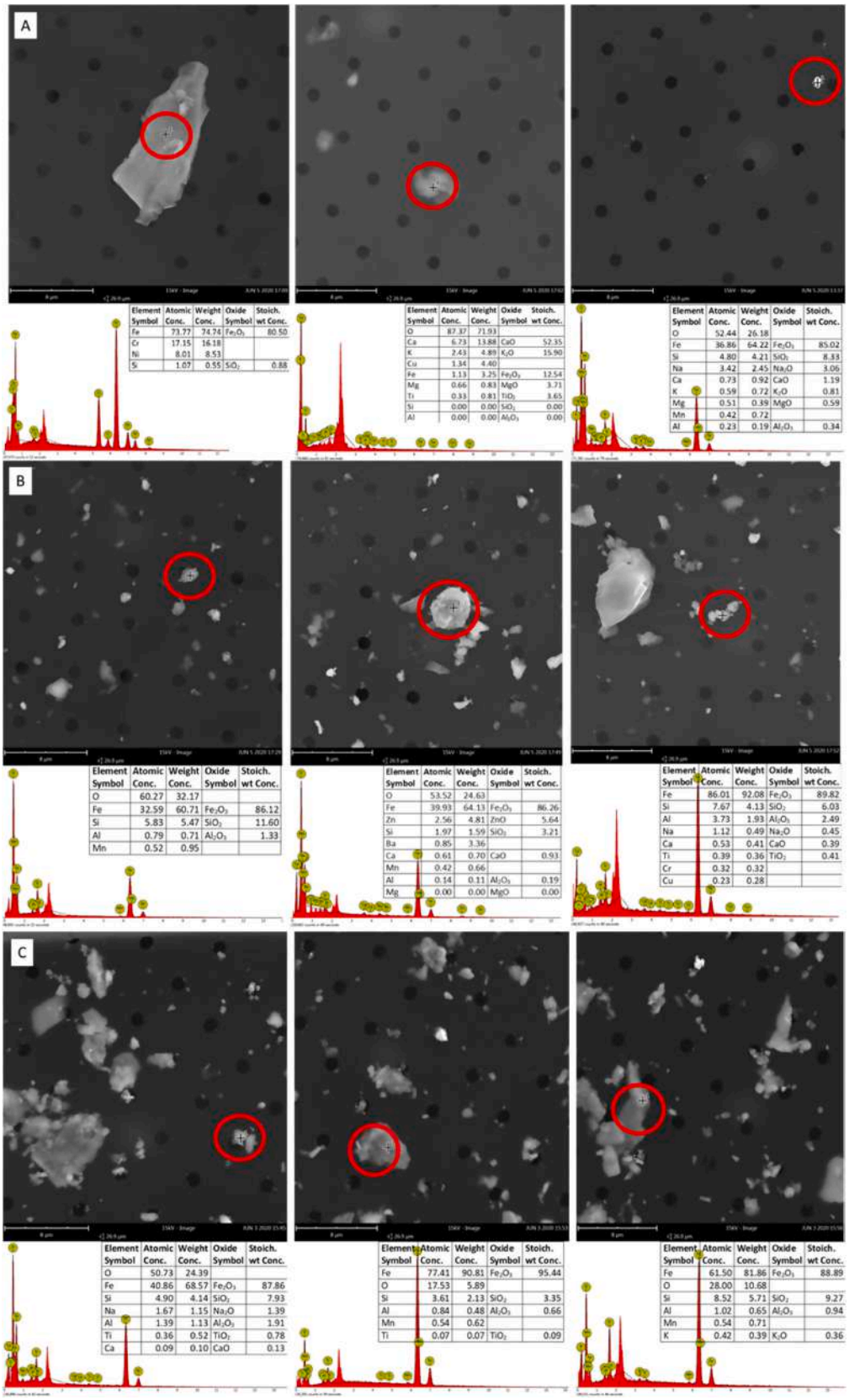

Fig. 1. SEM of an aerosol sample from A) a station agent at the ticket sale counter, station *Porte de la Vilette*, line 7; B) a locomotive operator in the subway cabin when driving between *Chatelet* and *Pont-Marie* stations, line 7; and C) a security guard on the subway platform at the *République* station - Direction *Créteil*, line 8. Red circles indicate particles analyzed with EDX for elemental composition (emission spectrum indicated under the picture). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Our metal concentrations are in line with previously published subway studies and have multiple origins. For instance, CuO emissions usually arise from short circuits incurred when a catenary wire is attached to a pantograph to provide discharge or from draft lines abrasion (Li et al., 2018). Fe, Mn, and Cr contents are mainly generated by sparks from the brakes, wheels, rails, and electric cabin tracks (Johansson and Johansson, 2003). Emissions of trace elements Ba, Zn, Sb and Cu are attributed to brake abrasion, although their concentration depends on brake type. The Ba and Zn concentrations are higher in frontal brake pads whereas Sb and Cu are higher in lateral brake pads (Moreno et al., 2017). The origin of Al and Si is to soil materials and construction material deterioration. The interaction between both pantographs and catenaries supplying electricity and by the mechanical wear–tear and friction processes due to brake–wheel–rail contact generate Fe, K, Mn, Ba, Ca, Zn, Cr, Ni, Cu and Si, along with resuspension caused by the piston effect (Cartenì et al., 2020).

Some elements can enter an underground subway station through vents and as dust generated by the cabins or transported on the clothes and shoes of passengers. Such elements can also access the passenger cabins as air is exchanged when passengers enter and exit the subway (Park et al., 2012). Microscopy analysis revealed that the particles' size, shape, and composition differed by job (Fig. 1), and visualized with the TEM images (Fig. 2). The shape and size of subway particles are useful in understanding sources. Abrasions of train vehicles, wheels and rail are sources of flake particles (Fig. 1B and C), while thermal processes due to the heating created by mechanical braking and wheel-rail contact generate spherical and semi-spherical particles (Loxham et al., 2013). UFP are generated by high temperature friction phenomena followed by vaporization or by electric arcs on the power supply system (Loxham et al., 2013). The EDX analysis of sub-micron particles (Fig. 1A, right picture) showed that they contain mostly iron. EDX analysis uses Fe_2O_3 as a standard (Fig. 1) and does not allow specification of iron species. We were therefore not able to distinguish Fe_2O_3 (hematite) from Fe_3O_4 (magnetite). Several studies have reported that Fe usually exists as Fe_3O_4 in the subway environments and as Fe_2O_3 in atmospheric PM (Karlsson et al., 2005; Eom et al., 2013; Moreno et al., 2015; Loxham et al., 2020), while others have reported the opposite (Smith et al., 2020; Querol et al., 2012). Iron oxides influence the ROS-related outcomes and thus may have an influence on respiratory diseases (Loxham et al., 2020). We believe that iron specification in PM from the Parisian subway deserves further attention.

3.3. PM and metal concentrations measured in EBC

3.3.1. Particle number concentrations and sizes

Particle number concentrations in EBCs were dependent on sampling time point (higher in pre-shift EBC than in post-shift) and on the day of the week (Table 3). Job did not have an effect per se on particle number concentrations, but acted as an effect modifier in interaction with the sampling time. This has been observed previously (Fireman et al., 2017).

The median particle size was independent of job, sampling time, or day of the week, though the latter was of borderline significance (Table 3). Locomotive operators had the highest concentration of particles in EBC compared to other subway professionals. NTA is a recent tool; we could therefore not find NTA data to compare our findings with others. Sauvain et al. reported EBC particle number concentrations in Brazilian workers processing crystal and quartz ($20.1 \pm 4.6\ 10^7$ #/ml) or soapstone ($4.2 \pm 1.9\ 10^7$#/ml) and compared these to university administration workers ($2.8 \pm 1.6\ 10^7$ #/ml). The EBC particle concentrations found in our study were an order of magnitude lower than the Brazilian administration workers. Furthermore, Sauvain et al. found that the particle size distributions in the EBC were similar for all three groups (Sauvain et al., 2017). EBC particles smaller than 100 nm can be both of endogenous (e.g., lipidic bilayered vesicles or exosomes) and exogenous sources (Sauvain et al., 2017). Therefore, analysis of the EBC elemental content might help to understand particle nature and origin.

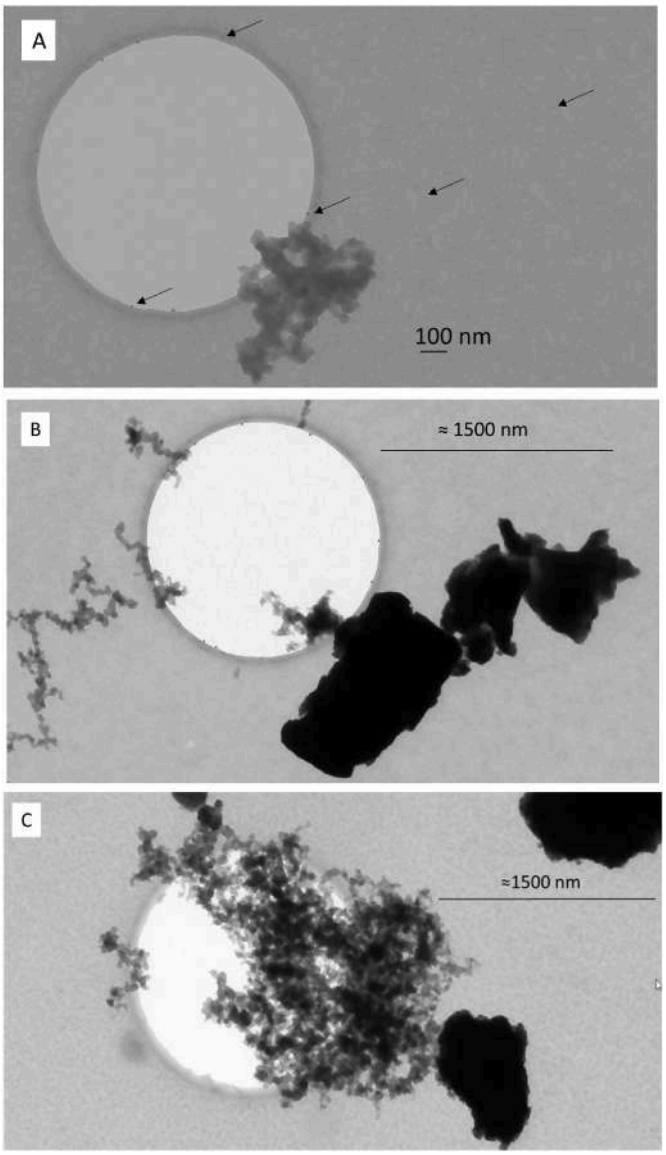

Fig. 2. TEM of an aerosol sample from A) a station agent in the ticket booth, station *Porte de la Villette*, line 7; B) a locomotive operator in the subway cabin when driving between *Chatelet* and *Pont-Marie* stations, line 7; and C) a security guard on the subway platform at the *Gare de Lyon* station - Direction *Boissy*, RER A. Arrows indicate non agglomerated nano-sized particles (median diameter 14–17 nm).

3.3.2. Metals

Among 15 metals quantified in EBC, six metal concentrations (Al, Fe, Mo, Si, Ti, and V) had more than half of the measurements below LOQ. Consequently, the statistical analyses were restricted to the other metals (Table 3). It is worth noticing that Fe and Al concentrations were below LOQ. This was unexpected given their high concentrations in subway PM. There is no data about respiratory clearance mechanisms of these metals in humans. However, our finding is consistent with the published literature on metals in EBC. Hulo et al. found that only 24% of Al concentrations in EBC in unexposed controls (67% smokers) were >LOQ (0.1 μg/L) (Hulo et al., 2016). The number of values for Al in EBC below LOQ in our study could be due to the 5-fold higher LOQ compared to Hulo et al. Other studies have also quantified metals in EBC but LOQs nor the numbers of values > LOQ were reported (Marie-Desvergne et al., 2016; Hulo et al., 2014; Ghio et al., 2018a).

The very low Fe levels in EBC can indicate a strong sequestration of this metal, as it is an essential element for life. Guio et al. suggested that

Table 3
Particle and metal concentration in the exhaled breath condensate (EBC) of Paris subway workers.

Parameter measured	Fixed effects (p-value)				Intra-class correlation	EBC sample	Geometric Mean [95% Confidence Interval]								
	Job	Shift	Day of the week	Job*Shift interaction			Station agents			Locomotive operators			Security guards		
Particles number concentration (10^6 #/mL)	0.29	**0.01**	**0.03**	**0.03**	0.38	pre-shift	31.34	[20.02;	42.66]	45.78	[29.5;	62.02]	39.85	[25.8;	53.9]
						post-shift	22.77	[14.4;	31.13]	33.25	[21.3;	45.23]	28.95	[18.5;	39.38]
Median particle diameter (nm)	0.69	0.38	**0.05**	0.55	<0.001	post-shift	138.25	[122;	154.5]	146.4	[130;	162.8]	150.3	[134;	166.6]
Metal concentration (μg/L)															
Cr	0.11	0.14	0.78	**0.01**	0.34	pre-shift	0.02	[0.01;	0.03]	0.04	[0.02;	0.05]	0.04	[0.03;	0.06]
						post-shift	0.03	[0.02;	0.04]	0.04	[0.03;	0.05]	0.04	[0.03;	0.06]
Mn	0.58	0.40	0.16	0.14	0.40	pre-shift	0.12	[0.08;	0.17]	0.12	[0.08;	0.17]	0.22	[0.15;	0.30]
						post-shift	0.14	[0.09;	0.19]	0.14	[0.10;	0.19]	0.18	[0.12;	0.24]
Co	0.99	0.72	0.70	0.94	0.39	pre-shift	0.04	[0.03;	0.04]	0.04	[0.03;	0.04]	0.03	[0.03;	0.04]
						post-shift	0.04	[0.03;	0.04]	0.04	[0.03;	0.04]	0.04	[0.03;	0.04]
Ni	0.35	**0.01**	0.11	**0.02**	0.34	pre-shift	0.41	[0.26;	0.57]	0.20	[0.14;	0.27]	0.31	[0.21;	0.41]
						post-shift	0.23	[0.15;	0.30]	0.30	[0.20;	0.39]	0.22	[0.15;	0.30]
Cu	0.34	0.24	0.31	**0.01**	0.36	pre-shift	0.98	[0.71;	1.25]	0.75	[0.57;	0.93]	1.38	[1.06;	1.70]
						post-shift	0.79	[0.60;	0.99]	1.01	[0.77;	1.25]	0.96	[0.71;	1.20]
Zn	0.28	0.36	0.97	**0.05**	0.38	pre-shift	8.65	[6.00;	11.30]	6.69	[4.95;	8.44]	11.68	[8.65;	14.72]
						post-shift	10.25	[7.47;	13.03]	7.58	[5.60;	9.56]	8.42	[6.08;	10.75]
Sb	0.87	0.85	0.87	0.75	0.34	pre-shift	0.02	[0.01;	0.03]	0.02	[0.01;	0.02]	0.03	[0.02;	0.03]
						post-shift	0.02	[0.01;	0.03]	0.02	[0.02;	0.03]	0.02	[0.02;	0.03]
Ba	0.08	1.00	0.17	**0.04**	0.30	pre-shift	0.21	[0.13;	0.29]	0.23	[0.15;	0.30]	0.37	[0.25;	0.48]
						post-shift	0.21	[0.14;	0.28]	0.20	[0.13;	0.26]	0.33	[0.21;	0.45]
Pb	**0.02**	0.34	**0.02**	**0.03**	0.32	pre-shift	0.06	[0.04;	0.08]	0.03	[0.02;	0.04]	0.05	[0.03;	0.06]
						post-shift	0.04	[0.03;	0.06]	0.06	[0.04;	0.07]	0.03	[0.02;	0.04]

Statistically significant results are shown in bold.

PM exposure induces changes in iron homeostasis Fe through the Fe complexion/chelation or displacement from pivotal sites in the cell, resulting in cellular Fe sequestration (Ghio et al., 2020).

Zn concentrations in EBCs were independent of sex, age, and smoking status and interestingly, 34-fold greater than the Fe concentrations (Ghio et al., 2018a). Similarly, we observed no effect of sex or job in our study on Zn concentrations in EBC. Our values were all below the values of Zn concentration in EBC reported in recent literature reviews (Ghio et al., 2018b; Corradi et al., 2009).

Cu concentrations in EBC were highest in security guards (Table 3) and greater than the median values reported for healthy adults (Corradi et al., 2009; Mutti et al., 2006)), but within the 25th and 75th percentile interval (0.30–1.80 μg/L) (Corradi et al., 2009). Ni concentrations in EBC were highest in station agents, but were affected by the sampling time as well as an interaction term between job and sampling time. Our results are in line with Ni concentrations in EBC reported for healthy adults (Ghio et al., 2018b). Ba concentrations in EBC were highest in security guards and remained stable across weekdays and work-shifts, which is consistent with its long clearance half-lives in experimental animals (EPA, 1998). Mn concentrations in EBC were also highest in security guards, and higher in pre-shift compared to post-shift EBC. However, the values measured are in line with values reported for healthy adults (Ghio et al., 2018b). All Cr concentrations in EBC were greater than the 75th percentile in healthy non-smoking adults (Corradi et al., 2009). Pb concentrations in EBC varied by job and weekday, but were in line with other studies (Corradi et al., 2009; Mutti et al., 2006). We found no effect of gender, job, weekday or shift for Co and Sb concentrations in EBC, and no literature values for comparison. The inter-class correlation was rather similar for all detected metals, ranging between 30 and 40% (Table 3).

3.4. Metal concentrations in urine

Among the 15 metals quantified in urine, seven metal concentrations (Al, Cr, Fe, Mn, Pb, Sb, and V) had more than half of the measurements below the LOQ. The statistical analyses was thus restricted to the eight metals above the LOQ (Table 4). The average creatinine-adjusted metal concentrations in urine were in decreasing order: Si > Zn ≫ Mo > Ti >

Cu > Ba ≈ Ni > Co, regardless of urine sampling time. As for EBC, iron I not found consistently in urine, suggestive of an effective sequestration in the body and a low excretion rate. Cu, Mo, Ba, and Si concentrations were greater post-shift compared to pre-shift. Zn was the only metal that had greater pre-shift than post-shift urine concentrations for all workers. For other metals (Ti, Ni, Co), the pre- and post-shift variations were limited to one or two jobs (Table 4). The highest inter-subject variability was observed for Zn and Co concentrations in urine (about 60% of total variance), and the lowest for Si and Mo (Table 4).

We relied on general population values from biomonitoring surveys when occupational biological limit values for urinary metal concentrations did not exist. The American Conference for Industrial Hygienists (ACGIH) has developed a biological exposure index for Co (30 μg/L) and several for Ni; metal (45 μg/L), Ni soluble salt (40 μg/L) and Ni insoluble salt (10 μg/L) (Hopf and Fustinoni, 2021). Our workers had urinary Co and Ni concentrations well below these as well as the 95th percentile reported for 40-59-year old adults in the French National Nutrition and Health Survey (FNNHS) (Fréry et al., 2017). The urinary Co concentrations in station agents and the urinary Ni concentrations in security agents appeared slightly above the central estimates of the creatinine-corrected concentration reported in FNNHS (Fréry et al., 2017). It is worth mentioning that the two- and tree-fold higher urinary Co concentrations we observed for station agents compared to the other professionals (Table 4), are likely a sex effect rather than a difference in occupational metal exposures. This difference is likely due to higher prevalence of iron deficiencies in females (Meltzer et al., 2010). Mo concentrations in urine from our workers were well below the 90th percentile reported for healthy non-smoking Swedish adults (Barregard et al., 2021). This was also the case for Cu concentrations in urine for males in our study, while female station workers' values were slightly higher than the Swedish values (Barregard et al., 2021), but lower than the Belgian (Hoet et al., 2013) and UK values (Morton et al., 2014). Urinary Cu concentrations were significantly higher in females than in males, which is consistent with our findings. This difference between the sexes can be due to the use of oral contraceptives (ATSDR, 2004). Moreover, the day-to-day variation in urinary Cu concentrations was low in comparison to the other metals (after adjusting for sex) (Morton et al., 2014).

In contrast to Cu and Co, urinary Zn concentrations were higher in males than in females. They were particularly elevated among locomotive operators being above the general population values reported for Swedish, Belgian, and UK adults (Morton et al., 2014; Barregard et al., 2021; Hoet et al., 2013) but below the biomonitoring equivalents for Zn concentrations in urine (Poddalgoda et al., 2019). Urinary Ba concentrations were highest in station agents followed by locomotive operators, who had twice the value compared to the security guards (Table 4). All values were below urinary Ba concentrations for the general population in Britain, Belgium, and France (Morton et al., 2014; Hoet et al., 2013; CDC, 2021; Goullé et al., 2005). Urinary Ti concentrations measured in our subway workers were all below British healthy non-smoking adults (Morton et al., 2014).

Si was the most abundant metal measured in the subway workers' urine (Table 4) with post-shift higher than pre-shift concentrations in station agents and security guards suggesting an occupational origin of this exposure. The urinary Si concentrations in security guards and particularly, in station agents were notably higher than those reported for Swedish healthy adults (Magnusson et al., 2020). Fe, Mn, Cu, Zn, and Mo are considered essential elements in human nutrition. As such, their concentration in the body is strictly regulated, thus diet and environmental factors have less effects on their concentrations (Morton et al., 2014). The excretion of these essential elements is essentially via bile (in feces); therefore, urine is not the most relevant biological matrix for biomonitoring. Notwithstanding, for Zn, the recent evidence suggests that in a health-risk context, urinary Zn is more reliable biomarker of exposure than blood due to homeostasis in blood (Poddalgoda et al., 2019).

Table 4
Metal concentration in urine (μg/g creatinine) of Paris subway workers.

Metal	Fixed effects (p-value)				Intra- class Cor.	Urine sampling	Mean [95% Confidence Interval]							
							Station agents			Locomotive operators			Security guards	
	Job	Shift	Day of the week	Job*Shift interaction										
Co	0.14	**0.01**	0.51	**< 0.001**	0.60	pre-shift	0.64	[0.09;	1.20]	0.24	[0.03;	0.44]	0.11	[0.01;	0.21]
						post-shift	0.49	[0.06;	0.92]	0.24	[0.03;	0.46]	0.14	[0.02;	0.27]
Ni	0.28	**0.05**	0.15	**0.01**	0.52	pre-shift	1.16	[0.54;	1.78]	0.74	[0.34;	1.13]	0.92	[0.43;	1.42]
						post-shift	0.92	[0.42;	1.42]	0.69	[0.31;	1.06]	1.28	[0.60;	1.97]
Cu	**< 0.001**	0.88	0.90	**< 0.001**	0.50	pre-shift	6.91	[5.47;	8.36]	4.27	[3.37;	5.17]	3.97	[3.14;	4.81]
						post-shift	6.97	[5.47;	8.47]	4.91	[3.86;	5.95]	3.92	[3.10;	4.74]
Zn	0.27	0.01	0.82	**< 0.001**	0.57	pre-shift	137.69	[54.25;	221.13]	261.54	[102.90;	420.18]	233.74	[91.97;	375.51]
						post-shift	114.20	[44.56;	183.83]	232.95	[91.43;	374.48]	161.36	[63.54;	259.18]
Mo	0.39	0.22	0.97	0.42	0.44	pre-shift	18.41	[12.08;	24.74]	16.76	[10.91;	22.61]	26.62	[17.34;	35.91]
						post-shift	22.27	[13.99;	30.55]	17.73	[11.40;	24.06]	25.06	[16.38;	33.73]
Ba	**< 0.001**	**< 0.001**	0.18	**< 0.001**	0.52	pre-shift	1.24	[0.47;	2.02]	0.91	[0.34;	1.49]	0.48	[0.18;	0.78]
						post-shift	1.93	[0.71;	3.16]	1.13	[0.42;	1.84]	0.35	[0.13;	0.57]
Ti	0.07	**< 0.001**	0.80	**0.01**	0.54	pre-shift	22.70	[4.91;	40.49]	14.40	[3.06;	25.75]	15.09	[3.18;	27.01]
						post-shift	36.69	[7.44;	65.95]	12.50	[2.54;	22.46]	11.20	[2.35;	20.06]
Si	**< 0.001**	**< 0.001**	0.07	**< 0.001**	0.45	pre-shift	6786.36	[5901.40;	7671.32]	4233.52	[3666.52;	4800.53]	5089.95	[4409.88;	5770.02]
						post-shift	8819.21	[7502.73;	10135.70]	3863.69	[3327.66;	4399.72]	6014.52	[5221.72;	6807.31]

Statistically significant results are shown in bold.

3.5. Relationship between different exposure metrics

The exploratory pairwise correlation analysis showed that UFP number concentrations in air and post-shift EBC were positively correlated and became significant when pre/post-shift ratio of the particle number concentration was used (Supplementary Material Table S1). Both post-shift Zn EBC and pre-post shift Zn ratio were negatively correlated with Zn concentrations in PM2.5 measured two days earlier. On the other hand, Zn concentrations in PM2.5 were positively correlated with urinary Zn concentrations measured the same day post-shift. The same was true for Cu concentrations in PM2.5. These results suggest a temporal effect, as Cu concentrations in PM2.5 the day before was positively correlated with the urinary Cu concentrations pre-shift. Cu concentrations in PM2.5 two days before was positively correlated with the urinary Cu concentrations 48 h later (post-shift). Post-shift urinary Cu concentrations was negatively correlated with pre-shift copper concentration in EBC. Negative correlations were observed between post-shift Zn concentrations in EBC and both, pre- and post-shift urinary Zn concentrations. These correlations were slightly higher with the work-shift change in Zn concentration in EBC. The same pattern of relationship was observed for Ni (Table S1). These findings, although exploratory by nature, suggest a complex interplay between different exposure metrics. The difference in temporal variation of measured concentrations depending on the biological matrix may reflect the mechanism of metal clearance after exposure trough inhalation, which remains poorly documented in humans, particularly for these metals.

In the multivariate analysis, we found a significantly positive relationship for Zn concentration between post-shift EBC and PM10, while this relationship was negative for Cu (Table 5). Moreover, the change in Zn concentrations in EBC over the work-shift was positively associated with the change in urinary Zn concentration, though the coefficient was of borderline statistical significance. The relationship between post-shift EBC and post-shift urinary Cu concentrations was positive and strong (Table 5). These results show an interdependence of metals in EBC and PM10 as well as EBC and urine, but not PM and urine.

3.6. Findings' biological relevance and implications

3.6.1. Metals in PM

Toxicological profiles for most metals, and particularly Zn and Cu, lack data on inhalation exposure and respiratory tract absorption (ATSDR, 2004; ATSDR, 2005) as well as short and long-term health effects. Environmental epidemiologists have identified inhalation of Cu, Fe, Ni, Si, K, V, and Zn in PM of particular health concern (Wolf et al., 2015; Chen et al., 2021). Zn, Si, Fe, Ni, V, and K in PM were associated with cardiovascular health effects (Yang et al., 2019), while Cu was associated with both cardiovascular and respiratory health effects (Rohr and Wyzga, 2012). For iron concentrations in PM, the evidence of adverse health effect is less conclusive than for Cu, Zn and Al (Rohr and Wyzga, 2012). The concentrations of these metals in PM2.5 and PM10 are rarely reported. Nevertheless, we recommend monitoring their concentrations in subway PM as long-term exposure to metals may trigger adverse health effects, especially for Ti, a suspected carcinogen, and V and K, which are associated with respiratory and cardiovascular morbidity (Chen et al., 2021; Yang et al., 2019). We did not detected V and K in these elements in EBC or urine. But a previous analysis of the RATP workers' mortality showed an excess of ischemic heart disease in males (Campagna et al., 2008). Unfortunately, our analytical methods were not able to quantify V and K concentrations in PM. Their airborne concentrations should be assessed in a future study.

3.6.2. Cu and Zn implication in oxidative stress mechanism

The fact that only Zn and Cu concentrations were >LOQ in all three types of samples, was surprising. Loxham et al. showed that exposing primary bronchial epithelial cells (a key site of PM deposition) for 6 h or 24 h to iron-rich ultrafine PM collected from a subway, yielded at both time-points an upregulation of metallothioneins with antioxidant activity (Loxham et al., 2020). The main function of these metallothioneins is the binding and homeostasis of Zn and Cu ions, but not Fe ions. Loxham et al. explained that *in vivo*, Fe(II) is unable to displace Zn(II) or Cu(I) from the metallothioneins binding sites. Therefore, it is possible that the metal sequestration by metallothioneins is relatively ineffective against direct toxicity from Fe-rich PM, and it may affect the homeostasis of other metals. Indeed, Zn-loaded metallothioneins appear less protective against iron-induced DNA strand breakage compared with Cu. Nevertheless, the presence of Fe can have a profound effect on metallothioneins (Cai et al., 1995). The metallothionein-Zn complex is able to reduce ferritin-bound Fe(III) to Fe(II), which results in release of redox active Fe(II) from complex with ferritin, and concomitantly oxidation of the metallothionein thiolate groups, resulting in release of Zn(II). This free Fe(II) is then able to participate in other ROS-generating reactions, thus increasing oxidative stress, while there may also be dysregulated Zn homeostasis (Krężel and Maret, 2017). It is unclear whether oxidative stress and/or the presence of ferritin–Fe(III) may have an impact on the sequestration of Cu by metallothioneins in the same way. If this were the case, and given the greater affinity of

Table 5
Results of the mixed multivariate models* of PBZ, urinary and EBC metal concentrations in Paris subway workers.

Dependent variable	Concentration in PM10 (μg/m3)				Concentration in PM2.5 (μg/m3)				EBC or Urine Concentration**			
	β	IC-inf		IC-sup	β	IC-inf		IC-sup	β	IC-inf		IC-sup
Urine pre-shift Zn (μg/g creatinine)	−0.06	−0.19	;	0.07	0.09	−0.09	;	0.26	−0.02	−0.12	;	0.08
Urine post-shift Zn (μg/g creatinine)	0.06	−0.19	;	0.31	−0.06	−0.43	;	0.31	0.03	−0.14	;	0.20
Urine post/pre-shift Zn ratio	0.17	−0.14	;	0.49	0.30	−0.12	;	0.72	0.06	−0.11	;	0.23
EBC pre-shift Zn (μg/L)	−0.21	−0.57	;	0.16	0.08	−0.41	;	0.56	−0.17	−0.91	;	0.58
EBC post-shift Zn (μg/L)	**0.52**	**0.04**	**;**	**1.01**	−0.72	−1.53	;	0.08	0.59	−0.15	;	1.34
EBC post/pre-shift Zn ratio	−0.21	−1.25	;	0.84	No convergence				***0.34***	***−0.94***		***1.61***
Urine pre-shift Cu (μg/g creatinine)	0.03	−0.08	;	0.15	−0.04	−0.50	;	0.42	0.00	−0.08	;	0.08
Urine post-shift Cu (μg/g creatinine)	0.12	−0.02	;	0.25	−0.02	−0.61	;	0.57	−0.02	−0.12	;	0.07
Urine post/pre-shift Cu ratio	0.08	−0.13	;	0.29	0.23	−0.50	;	0.95	0.07	−0.05	;	0.19
EBC pre-shift Cu (μg/L)	−0.31	−0.69	;	0.08	0.17	−1.02	;	1.36	0.08	−0.80	;	0.97
EBC post-shift Cu (μg/L)	**−0.67**	**−1.12**	**;**	**−0.21**	−0.12	−1.41	;	1.18	**0.81**	**0.03**	**;**	**1.58**
EBC post/pre-shift Cu ratio	No convergence				No convergence				0.12	−0.52		0.76

* All models are adjusted for age, sex, and vitamin or food supplement intake.

** β coefficient corresponds to EBC concentration (in μg/L) when urine concentration (in μg/g creatinine) is dependent variable and *vice versa*. Values in bold correspond to statistically significant estimates ($p < 0.05$); those in italic correspond to the estimates of borderline statistical significance.

metallothioneins for Cu compared to Zn, this may result in displacement of metallothionein-bound Zn, and thus further dysregulation of Zn metabolism (Loxham et al., 2020).

These results support our exploratory findings, calling for a further hypothesis-based investigation of subway PM Zn and Cu effect. Both EBC and urine concentrations of these metals should be better characterized. Currently, urinary Zn concentration is considered a reliable biomarker for oral zinc intake and not from inhalation (Poddalgoda et al., 2019). Zn concentrations in EBC might reflect daily airborne Zn exposure as we have shown here, but this is still debated (Monsé et al., 2021). Zn concentration in EBC and its ratio to EBC iron was shown varying between non-smokers, smokers and COPD patients, exhibiting the highest ratio in non-smokers (Ghio et al., 2018a). Regarding Cu, Mutti et al. reported that chronic oxidative stress may be associated with Cu depletion in EBC, the antioxidant responsive element, and that Cu levels in EBC may be of particular interest because of their positive correlation with lung function parameters in COPD patients (Mutti et al., 2006).

3.7. Relevance of biomonitoring

3.7.1. EBC as biological matrix

Our results suggest that EBC can be a proficient matrix to sample airborne exposures to UFP (particle number concentrations) and some metals (Zn and Cu). These metals in EBC were associated with their respective urine concentrations. Assessing the metal dose in the target tissue (lung) is an advantage as opposed to blood or urine metal concentrations. This study demonstrated that EBC as a biological matrix provides complementary data on internal particle and metal exposures, and some insights on their mechanism after inhalation of PM in the subway environment. Some researchers wish to extend its use (Corradi and Mutti, 2005), while others consider EBC analysis a "niche approach" in occupational health research (Maestrelli et al., 2020). The main EBC advantages are that it is safe, rapid, simple to perform, non-invasive compared to blood draws, and effort independent compared to spirometry. The potential for using EBC in biomonitoring is high, but depend on further characterization of human breath, as it contains upwards of 250 chemicals (Corradi and Mutti, 2005). We provide here nine metal concentrations in EBC, which adds to the almost non-existent EBC exposure data. Along with this, we have also shown that EBC can be used to determine exposures to UFP expressed as particle number concentrations. The main concerns with EBC use are the need for standardization of sampling and analytical methods (Maestrelli et al., 2020). The contamination from outdoor and indoor air and from devices and reagents used are additional issues that need to be considered carefully (Horváth et al., 2005; Hemmendinger et al., 2021). Thus, inter-lab comparisons are necessary in the near future.

3.7.2. Biomonitoring result communication

Communicating biomonitoring results to workers is a topic on its own. However, we would like to share some reflections from issues raised during our study. Under French regulations, the physicians are mandated to communicate results to the workers. Many physicians are uncomfortable with this, as the biomonitoring data are not readily interpretable. Biomonitoring studies provide metal concentrations in biological matrices (e.g., mg/g creatinine in urine), the exposure guidance values for toxicity, such as reference doses are reported as oral intake values (in mg mg/kg bw/day) rather than biological equivalents (available only for two of analyzed metals (Poddalgoda et al., 2019; Poddalgoda et al., 2017)). Only few biological limit values are available and when they are, many physicians have no training in how to interpret these as there is no direct clinical values (as opposed to cholesterol values), but are related to exposures (Fréry and El Yamani, 2020). Within this study, we discussed how the individual results should be interpreted and communicated to the study participants. Namely, we retrieved from the literature the metal concentrations in urine and EBC of general population and control groups of healthy unexposed workers, respectively, and when necessary harmonized units using appropriate unit conversion. For some biomarkers we also performed meta-analysis of available values (Graille et al., 2020a, 2020b; Hemmendinger et al., 2020; Shoman et al., 2020). Therefore, this study plea for a better standardization and generalization of exposure biomonitoring in occupational and environmental health research and practice.

3.8. Study limitations

The exploratory nature and therefore small sample size is likely limiting the statistical power, especially for the mixed multivariate models for EBC and urine relationships, and the results generalizability. Although the personal air sampling was performed as close as possible to the participants' personal breathing zone, the logistical constraints encountered, prevented us from satisfying the conditions required to consider the samples taken in the breathing zone.

4. Conclusion

Particle and metal exposure in Parisian subway was assessed using a triple approach: in PM, EBC and urine of subway professionals. PM2.5 and PM10 concentrations were in compliance with the French guidance values. Fe, Al, Zn, and Cu were the most abundant PM constituents; however, only Zn and Cu were consistently quantified in EBC and urine. The relationships between both metal concentrations in EBC and PM, and in EBC and urine, as well as a correlation between UFP exposures and particle number concentrations in EBC confirm the interest to use EBC as a collection matrix in exposure assessments, especially when inhalation is a primary route of exposure.

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Ethics approval and consent to participate

The study protocols were approved by the French Personal Protection Committees South-Est II (N°2019-A01652 55), Declaration of conformity to the French National Commission for Computing and Freedoms (CNIL) N° 2220108. Written consent to participate was obtained from all study participants.

Authors' contributions

CC, MH, AD, VJ, TBR, GS, SB, data collection; GS, MH, JJS, SB, lab analyses; IGC, PW: statistical analysis; IGC: drafted the manuscript; NH, CC, and SB critically reviewed it; All authors participated in the result interpretation, manuscript preparation and read and accepted its final version.

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2021.113837.

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Perfluorobutanoic acid (PFBA): No high-level accumulation in human lung and kidney tissue

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ABSTRACT

Perfluorobutanoic acid (PFBA) belongs to the complex group of synthetic perfluoroalkyl substances (PFAS) which have led to ubiquitous environmental contamination. While some of the long-chain compounds accumulate in the human body, the short-chain compound PFBA was found to have a relatively short half-life in blood of a few days, in agreement with relatively low PFBA serum/plasma levels of roughly 0.01 ng/ml in European studies. Surprisingly, very high median levels of PFBA of 807 and 263 ng/g tissue for human lung and kidney autopsy samples, respectively, were reported in a paper of Pérez et al. (2013). This would question the concept of PFAS blood analysis reflecting the body burden of these compounds.

To verify the results of high PFBA tissue accumulation in humans, we have analyzed PFBA in a set of 7 lung and 9 kidney samples from tumor patients with a different method of quantification, using high-resolution mass spectrometry with the accurate mass as analytical parameter.

The only human sample with a quantifiable amount of PFBA (peak area more than twice above the analytical background signals) contained approximately 0.17 ng/g lung tissue.

In the light of our results and considering the analytical problems with the short-chain compound PFBA exhibiting only one mass fragmentation, it appears to be likely that PFBA is not accumulating on a high level in human lung and kidney tissue. In general, the analysis of short-chain PFAS in complex matrices like food or tissue is very challenging with respect to instrumental quantification and possible sample contamination.

1. Introduction

Perfluoroalkyl substances (PFAS) are a complex group of synthetic chemicals composed of a fluorinated carbon backbone of varying length, primarily terminated by a carboxylate or a sulfonate as functional group. Due to their unique properties with water and oil repellency, the compounds have been used since decades for the production of many consumer products. From these everyday objects and other sources, PFAS have been released and have caused – due to their high persistence and mobility – ubiquitous environmental contamination and in part accumulation in the food chain. Consumption of food and drinking water is the main route of background exposure in humans. Internal exposure to PFAS in individuals, determined by an analysis of serum/plasma, revealed four compounds to typically represent more than 90% of detectable PFAS in adults in industrialized countries, namely perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluorohexane sulfonic acid (PFHxS) and perfluorononanoic acid (PFNA). Based on studies from 2007/2008 and onwards, median concentrations of PFOS, PFOA, PFHxS, and PFNA in the European adult population were found to be 7.7, 1.9, 0.67 and 0.61 ng/ml, respectively (EFSA 2020). This pattern results from the occurrence in food and drinking water on the one hand, and from accumulation due to half-lives in humans up to several years, resulting from missing metabolic degradation and low urinary excretion (EFSA 2020), on the other hand.

A broad spectrum of toxic effects of different PFAS was observed in experimental animals primarily at higher doses, and epidemiological studies conducted in recent years revealed associations between internal PFAS exposure and certain biological parameters even in humans exposed in the higher background range. Regarding the immune system, lower levels of vaccine antibodies associated with higher internal levels of PFAS were primarily observed in children. In its 2020 risk assessment, the European Food Safety Authority (EFSA) considered the findings of a decreased immune response as robust, since they were consistently observed in rodents (treated with PFOA/PFOS) and in humans.

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Therefore, EFSA (2020) has chosen the reduced production of vaccine antibodies in children as critical effect, using a study in one-year old children (Abraham et al., 2020) and kinetic modelling to derive a tolerable weekly intake (TWI) of 4.4 ng/kg body weight per week for the sum of PFOS, PFOA, PFHxS, and PFNA.

In contrast to these long-chain PFAS, short-chain PFAS typically have much shorter half-lives and are thought to have a low rate of accumulation in the human body. Perfluorobutanoic acid (PFBA) is one of these compounds with relevant external exposure via food in Europe, contributing to approximately 16% of the total PFAS exposure in adults (EFSA 2020), but with only low serum/plasma levels of roughly 0.01 ng/ml in the European adult population (EFSA 2020).[1] This obviously is due to its short serum elimination half-life of a few days reported for nine employers exposed at work: median 2.3 days, range 1.8–6.3 days (Chang et al., 2008). However, data on the distributions of PFBA in human tissues are not available, apart from those of Pérez et al. (2013) who analyzed autopsy tissues (liver, lung, kidney, brain and bone) of 20 subjects. They had been living in different areas of Tarragona County (Catalonia, Spain) at least for the last 10 years and died of different diseases in 2008. No specific PFAS exposure was described for these subjects. Very different PFAS accumulation patterns were reported for different human tissues, with highest PFBA accumulation in the lungs and kidneys showing median PFBA levels of 807 ng/g (range <0.01–4138) and 263 ng/g (range <0.01–4026), respectively. Plasma/serum samples were not available for comparison, but compared to PFBA plasma/serum levels typically found in adults from Europe (median 0.01 ng/ml, EFSA 2020), lung median levels would be higher by a factor of about 80,000. This would point to a very specific binding and a very long half-life of PFBA in lung and kidney, much longer than that in serum of a few days (Chang et al., 2008).

Such an extreme PFBA accumulation in the lung is theoretically conceivable in case of a very strong binding to specific tissue structures, but would raise a general question: Are PFAS plasma/serum levels reliable markers for the internal exposure, at least for PFBA? Considering the data of Pérez et al. (2013), obviously not. In case of other persistent organic pollutants, namely the lipophilic compounds like dioxins, it is known that they mainly distribute in the body according to the fat content of the tissues (e.g. DeVito et al., 1995). In case of PFAS, however, the compounds mainly bind to protein structures (EFSA 2020), and a tissue-specific accumulation seems possible, but not very plausible on such an extreme level of accumulation as calculated above.

A clarification of the question of PFBA accumulation especially in human lung tissue is of high relevance for two reasons. Firstly, a high accumulation of PFBA in certain organs is not expected to strongly correlate with its concentrations in blood on the individual level, and therefore, the relatively simple PFAS analysis of blood would lose its value to determine the individual internal exposure in epidemiological studies, at least in case of PFBA. Secondly, the findings of Pérez et al. (2013) have recently been discussed in the context of possible PFAS immunosuppression to provide a link between PFBA exposure and the severity of COVID-19 (Grandjean et al., 2020; Beans 2021; Catelan et al., 2021). To verify the results of Pérez et al. (2013), we therefore decided to analyze PFBA in human lung and kidney samples with a different method of quantification, using the accurate mass as analytical parameter.

[1] EFSA (2000) reported a PFBA median of 0.01 ng/ml (Table 16), but this is based on one study only, as EFSA only included studies where the median is above the limit of quantification (LOQ). In many other studies, the median of PFBA was found below the LOQ. Therefore, the value of 0.01 is relatively uncertain.

2. Material and methods

2.1. Human tissue samples

Anonymized tissue samples provided by Biopredic International (Rennes, France) were collected from tumor patients between 2011 and 2014 in France. Informed consent was given by the patients for the collection of surgical leftovers and their further use in scientific research (for details see: www.biopredic.com/rubrique-ethics). Immediately after surgery, the samples of non-neoplastic tissues from lung (n = 7, four males, age 72–80 years, and three females, age 64–72 years) and kidney (n = 9, four males, age 66–82 years, and five females, age 45–86 years) were stored at −80 °C.

2.2. Materials

HPLC-grade methanol, formic acid and sodium hydroxide pellets were obtained from Merck (Karlsruhe, Germany). HPLC-grade water was prepared using a Milli-Q Integral Water Purification System from Millipore Merck (Darmstadt, Germany). Ammonium acetate was from Fluka (Buchs, Switzerland). Solutions of perfluoro-n-butanoic acid (PFBA; 50 mg/L in methanol) and perfluoro-n-[$^{13}C_4$]butanoic acid ([$^{13}C_4$]PFBA; 1 mg/L in methanol) were purchased from Wellington Laboratories Inc. (Guelph, Canada).

2.3. Extraction

The sample preparation was based on an alkaline extraction with methanol (Sadia et al., 2020). Briefly, up to 9 g of the tissue samples were homogenized using a Tube Mill 100 control (IKA, Staufen, Germany). Portions of 0.5–1.3 g wet tissue were mixed with 100 µl [$^{13}C_4$] PFBA (10 µg/L in methanol) and 5 ml sodium hydroxide in methanol (100 mM). After shaking (30 min) and sonication (15 min), the samples were centrifuged for 10 min (3000×*g*). The extraction was repeated twice with 3 ml sodium hydroxide in methanol (100 mM). The combined supernatants were neutralized by addition of 30 µl formic acid. A volume of 1.2 ml was concentrated in a stream of nitrogen to approximately 200–400 µl, and the residuals were diluted with 4.5 ml 2 mM ammonium acetate buffer (pH 4). Samples were loaded onto Oasis WAX columns (150 mg, Waters, Eschborn, Germany), pre-conditioned with 24 ml 1.2% ammonia in methanol, 4 ml of methanol and 4 ml of water. The columns were washed with 4 ml 25 mM ammonium acetate buffer (pH 4), 4 ml of methanol and dried for 15 s with a water jet pump. PFBA was eluted using 8 ml 1.2% ammonia in methanol and the samples were dried under a stream of nitrogen. The samples were reconstituted with 100 µl methanol and 100 µl 2 mM aqueous ammonium acetate buffer (pH 4).

2.4. UHPLC–HRMS analysis

The samples were analyzed using an UltiMate 3000 UHPLC connected to a QExactive Focus mass spectrometer (Thermo Fisher, Dreieich, Germany) equipped with a heated electrospray ionization (HESI) source. Samples of 5 µl were injected onto a Hypersil GOLD column (1.9 µm, 2.1 × 150 mm; Thermo Fisher) and eluted with 2 mM ammonium acetate in water/methanol (95:5, solvent A) and 2 mM ammonium acetate in methanol (solvent B). The pH values were not adjusted. The gradient applied at a flow rate of 0.3 ml/min was: 0 min (1% B), 4 min (20% B), 10 min (100% B), 11 min (100% B), 11.5 min (1% B), 13 min (1% B). The column oven temperature was set to 40 °C. High-resolution mass spectrometry (HRMS) was performed in negative ionization mode. The HESI temperature was set at 220 °C, the capillary temperature at 300 °C, the electrospray voltage at 3.7 kV, S-Lens RF level at 60. Sheath and auxiliary gas flow rates were 42 and 5 L/min, respectively. All data in this study were acquired using a full scan mode covering the mass range from 80 to 500 m/z with resolution of 70,000

and automatic gain control setting of 3×10^6 with a maximum injection time of 100 ms. For confirmation, data-dependent MS^2 (dd-MS^2) was triggered by the detection of the accurate masses of PFBA and [$^{13}C_4$]PFBA (inclusion list), where precursor ions are selected by the quadrupole and then sent to the higher-energy collisional dissociation (HCD) cell for ion fragmentation and finally to the Orbitrap mass analyzer for detection. The dd-MS^2 was performed at mass resolution of 17,500, isolation width of 1.0 *m/z* and collision energy (CE) of 10 eV.

3. Results

PFBA levels in human tissue samples were quantified using a specific detection by HRMS and the internal standard [$^{13}C_4$]PFBA (Fig. 1). After extraction with methanol and purification using OASIS WAX columns, PFBA was detectable in all samples. Importantly, typical sources of PFBA contamination (from HPLC water, septum vials, glassware, HPLC tubing etc.) were eliminated from the sample preparation and the mass spectrometric analysis. However, it turned out that PFBA could not be completely removed from the solid-phase extraction columns. Even after pre-conditioning with high volumes of the eluent (24 ml 1.2% ammonia in methanol), little amounts of PFBA were eluted from the OASIS WAX columns. This signal (termed 'analytical background signal') corresponded to a mean amount of about 0.009 ng PFBA per column (n = 5). The Supplemental Table S1 shows the peak areas of PFBA residuals (m/z 212.9792) washed from the OASIS WAX columns ('water') together with the peak areas of PFBA in the samples.

Ignoring the basic contamination released from the solid-phase extraction columns, the tissue PFBA levels were between 0.08 and 0.24 ng/g in lung tissue (n = 7) and between 0.04 and 0.19 ng/g in kidney tissue (n = 9). The signal-to-noise ratios (S/N) were between 26 and 170, reflecting the excellent detection (compare also Fig. 1).

If the analytical background of PFBA released from the columns was subtracted, it became evident that most of the human tissue samples did not contain PFBA. The only human lung sample with a quantifiable amount of PFBA (0.022 ng, more than twice above the analytical background of 0.009 ng) contained approximately 0.17 ng/g tissue (Table S1).

4. Discussion

In case of lung tissue, the PFBA levels were found to be more than 1000-fold lower compared to those of Pérez et al. (2013) who reported a median value of 807 ng/g. As the paper did not report any specific oral or inhalational exposure to PFAS and especially to PFBA in the subjects investigated, they are likely to be people with background exposure in Spain in the year 2008, resulting from the ubiquitous environmental PFAS contamination. In the light of our results and considering the analytical problems with the short-chain compounds PFBA and perfluoropentanoic acid (PFPeA) exhibiting only one mass fragmentation (see below), the PFBA levels reported by Pérez et al. (2013) might not be

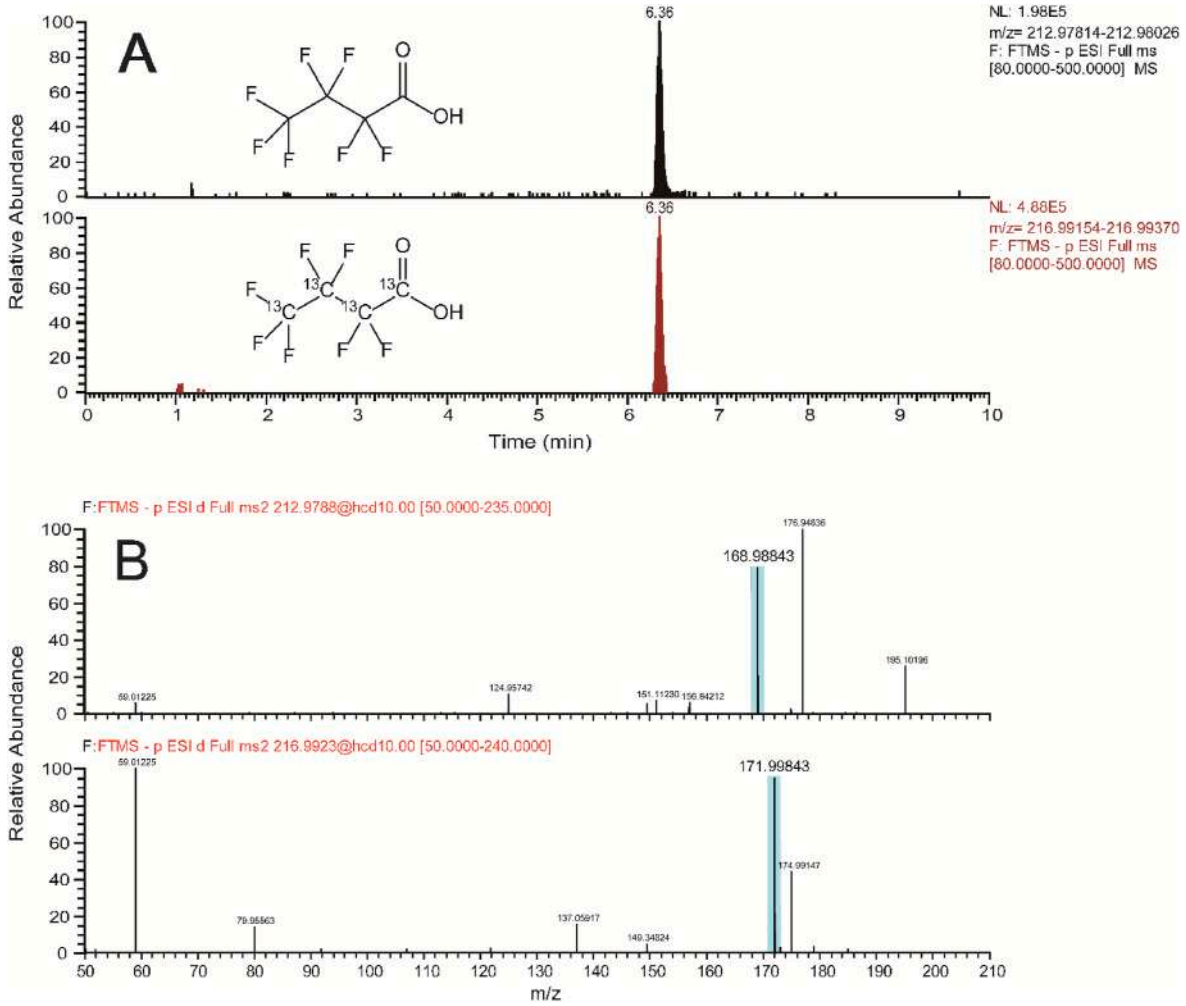

Fig. 1. A) Extracted ion chromatograms of PFBA (*m/z* 212.9792, top) and the internal standard [$^{13}C_4$]PFBA (*m/z* 216.9926, bottom) recorded by high-resolution mass spectrometry (HRMS) from an alkaline extract of human lung tissue. B) The detection was verified by recording of the fragments $C_3F_7^-$ (theoretical *m/z* 168.9894) and [$^{13}C_3$]F_7^- (theoretical *m/z* 171.9994) formed from decarboxylation of PFBA and [$^{13}C_4$]PFBA, respectively.

quantified correctly. In the view of all available data including those on the PFBA kinetics in human serum samples (Chang et al., 2008) it appears to be likely that PFBA is not accumulating on a high level in human lung and kidney tissue. Further investigations are needed to confirm these results.

The current work on PFBA levels in human tissues highlights two important issues of mass spectrometric PFAS analysis in general and PFBA quantification in particular regarding the specificity of the analyses and possible contamination in the sample preparation process. The specificity of PFBA detection by quadrupole mass spectrometers is relatively low because only one fragmentation in the negative mode is available (m/z 213 → 169, decarboxylation of the deprotonated carboxylic acid). Thus, quantification of PFBA by isotope-dilution LC-MS/MS multiple reaction monitoring (MRM) using a tandem-quadrupole mass spectrometer is only feasible with extremely clean matrices such as drinking water. In case of complex matrices (tissues, food), the presence of many other substances with similar molecular masses of parent ions/fragments and retention times may cause a dense background of signals in the vicinity of the PFBA peak due to the low resolution. The resulting lack of specificity using a tandem-quadrupole mass spectrometer and m/z 213 → 169 as sole fragmentation for PFBA analysis in extracts of methanol and sodium hydroxide was highlighted in analytical papers (e.g. Lacina et al., 2011). Analyzing PFBA in human tissues samples, Pérez et al. (2013) refer to the EU directive 2002/657/EC (*concerning the performance of analytical methods and the interpretation of results*) and define three criteria for the identification of the analyte using low resolution mass spectrometry, i.e. retention time, two daughter ions and the ratio between the two peak areas of the daughter ions. However, they did not note that compliance with these specificity criteria is not possible for PFBA and PFPeA, because these short-chain PFAS exhibit only one fragmentation.

The method used in the current study ensured the specificity by mass spectrometric monitoring of the accurate mass of PFBA and its isotope-labeled standard. The S/N values showed that the sensitivity of mass spectrometric detection is sufficient to quantify also the lowest concentration of PFBA in our samples; however, it also led to the detection of PFBA as a contamination released from the OASIS WAX columns, which could not be completely avoided by pre-conditioning the columns with the elution solvent. A possible solution to this inadequacy is the subtraction of the amount of PFBA contamination from the analytical results; this led to the impression that most of the tissue samples, except one lung sample with approximately 0.17 ng PFBA/g tissue, contained only non-quantifiable amounts of PFBA or no PFBA at all (Table S1). Further work may lead to the elimination of PFBA contamination. Tests of other SPE columns were not successful so far.

Apart from the analytical issues of PFBA, the compound has recently been discussed in the context of PFAS effects on the immune system. In general, the clinical relevance of the effects observed in terms of a possibly diminished effectiveness of vaccinations (on a population level or individually) is under discussion, likewise an increased risk or severity of infectious diseases in relation to the exposure to PFAS. The current pandemic with the corona virus SARS-CoV-2 and coronavirus disease 2019 (COVID-19) provides the opportunity to study immune response and clinical severity, respectively, in relation to the internal exposure to PFAS. In a first paper, Grandjean et al. (2020) reported on an association between plasma levels of PFBA and the COVID-19 outcome in a group of 323 Danish persons aged 30–70 years with a diagnosis of Covid-19. Of the study population, 108 (33%) had not been hospitalized, and of those hospitalized, 53 (16%) had been in intensive care or were deceased. PFAS analysis in plasma revealed relatively low median levels of PFOS, PFOA, PFHxS, and PFNA of 4.7, 0.77, 0.48 and 0.38 ng/ml, respectively. In case of PFBA, most samples were below the limit of detection of 0.03 ng/ml (75th percentile: 0.04 ng/ml). For the congeners proven to be immunotoxic at least in experimental animals, unadjusted odds ratios for increasing severities of the disease were calculated to be 1.00 (PFOS) and 0.99 (PFOA). Surprisingly, an unadjusted odds ratio of 2.19 (95% confidence interval: 1.39–3.46) was reported for PFBA, despite the very low levels measured near to or below the LOQ. Grandjean et al. (2020) stated that the unique retention of PFBA in lung tissue reported by Pérez et al. (2013) may offer a clue to interpreting the findings.

In view of our results, a high accumulation of PFBA is unlikely to occur in human lungs and therefore is unsuitable as possible explanation of a higher severity of COVID-19 due to higher PFAS/PFBA exposure. This also applies to a recent article of Catelan et al. (2021) who reported a higher COVID-19 mortality rate in a part of the Italian region Veneto where residents were exposed for decades to drinking water with an unusually high PFBA content; individual data on the internal exposure were not available. In general, the role PFBA in COVID-19 severity has gained a high attention (e.g. Beans 2021), but this link loses plausibility in the light of our findings.

5. Conclusions

The analysis of short-chain PFAS in complex matrices like food or tissue is very challenging with respect to unambiguous identification and sensitive instrumental quantification. Furthermore, the issue of possible external contamination of samples in analytics and pre-analytics leading to relevant background signals has to be considered.

In general, plasma/serum data on PFAS levels are very helpful in epidemiological studies, as they allow the correlation of individual doses and a possible effect. However, an important requirement is that PFAS plasma/serum levels are qualified markers for the internal exposure. As the compounds primarily bind to protein structures, congener- and tissue-specific accumulation may principally occur. Therefore, further investigations of PFAS distribution in human tissues including plasma/serum of the same subjects are highly required to confirm PFAS plasma/serum levels as markers representing the internal exposure.

Declaration of competing interest

On behalf of all authors, the corresponding author states that there are no competing interests to declare.

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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.2021.113830.

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Self-reported oil spill exposure and birth outcomes among southern Louisiana women at the time of the Gulf oil spill: The GROWH study

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ABSTRACT

Background: The chemical, physical, economic, and social effects of a major oil spill might adversely affect pregnancy health.
Objectives: To examine the relationship between oil spill exposure and birth outcomes in a cohort of women living near the Gulf of Mexico at the time of the 2010 oil spill.
Methods: Between 2012 and 2016, 1375 women reported their exposure to the oil spill, and at least one livebirth. Five hundred and three had births both before and after the oil spill. Indicators of oil spill exposure included self-reported financial consequences, direct contact with oil, traumatic experiences, loss of use of the coast, and involvement in litigation. Birth outcomes were low birthweight (LBW; birthweight <2500 g) and preterm birth (PTB; >3 weeks early). Women who were not pregnant at the time of the interview (n = 1001) self-reported outcomes, while women who were pregnant (n = 374) primarily had them abstracted from medical records (n = 374). All pregnancies prior to the oil spill were considered unexposed; those after the oil spill were considered exposed or unexposed depending on interview responses. Generalized estimating equations were used to control for clustering within women, with control for confounders.
Results: The most common type of exposure was economic (49%), but 302 women (22.0%) reported some degree of direct contact with the oil. Associations between most indicators of oil spill exposure and pregnancy outcomes were null, although when all pregnancies were examined, associations were seen with high levels of contact with oil for LBW (adjusted Odds Ratio [aOR] 2.19, 95% CI, 1.29–3.71) and PTB (aOR 2.27, 1.34–3.87).
Discussion: In this community-based cohort, we did not find associations between report of exposure to the oil spill, with the possible exception of high oil contact in some analyses, and birth outcomes. Research incorporating specific biomarkers of oil spill exposure and stress biomarkers would be valuable, to allow for assessing both perceived and actual exposure, especially when direct toxicant exposure is minimal.

1. Introduction

The Deepwater Horizon disaster was the largest marine oil spill in history (Mascarelli, 2010). The effects of the oil spill included not only direct health effects of environmental contamination, but also the anxiety and concern about adverse environmental and health effects, and the economic effects of the fishing closures and the drilling moratorium. The Institute of Medicine, currently the National Academy of Medicine (IOM (Institute of Medicine), 2010), as well as local community groups, identified pregnant women as a particular population of concern. The location and restrictions around the spill itself meant that relatively few people outside the clean-up workers were directly adjacent to the spill

Abbreviations: LBW, low birthweight; PTB, preterm birth.

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(Goldstein et al., 2011). However, the social and financial effects were extensive, due to the drilling and fishing moratoria and effects on tourism, and there was widespread concern about secondary environmental exposures, such as oil and tar balls on beaches (Jonsson, 2010), and exposure via seafood (Rotkin-Ellman et al., 2012).

An oil spill may expose people directly exposed to oil, volatile organic compounds (VOCs), tar balls, or fine particulate matter (PM) (Nance et al., 2016), whose effects may be modified by use of dispersants (Afshar-Mohajer et al., 2019; National Academies of Science, 2020). The combination of evaporation, burning, and clean-up operation emissions can also result in air pollution in the form of increased ozone, peroxyacetyl nitrate, NO_x, carbon monoxide, and black carbon (Middlebrook et al., 2012). Fish and shellfish may take up contaminants, and oil compounds may enter the food supply (Rotkin-Ellman et al., 2012). For the Deepwater Horizon spill specifically, the drilling moratorium and closure of the fishing grounds were financial strains on many families, while social factors such as concern about the environmental destruction and involvement in litigation were also sources of stress. Several studies indicate associations between oil spill exposure and worsened mental health, especially among those with significant direct exposure to the oil spill, financial effects, or disruption of life (Laffon et al., 2016).

As pregnant women are a relatively small proportion of the population and do not normally work in environmental disaster clean-up, few studies have addressed the effects on this group of environmental disasters, outside of Chernobyl and the environmental effects of 9/11 (e.g., (Ericson and Kallen, 1994; Spratlen et al., 2018). One study in Nigeria found a doubling of risk of neonatal mortality in areas near oil spills (Bruederle and Hodler, 2019). A study after the Hebei Spirit (South Korea) oil spill found higher rates of skin and abdominal pain among pregnant women but does not appear to have assessed birth outcomes (Kim et al., 2009). Considering more general exposures to petroleum, a review of exposure to oil and gas extraction activities and pregnancy outcomes reported that several relatively high-quality studies have found a relationship between unconventional oil/gas activities (i.e., hydraulic fracturing) and preterm birth (PTB), while the studies of low birthweight (LBW) were insufficient to draw conclusions (Balise et al., 2016). Another study found that flaring from such activities was associated with PTB but not fetal growth (Cushing et al., 2020). The objective of this analysis was to estimate the association between aspects of exposure to the Gulf oil spill and adverse birth outcomes.

2. Material and methods

The Gulf of Mexico oil spill occurred between April and September 2010; the GROWH (Gulf Resilience on Women's Health; U19 ES020677) Study was conducted between 2011 and 2016 (Harville et al., 2018; Lichtveld et al., 2016). Data were available on 1638 participants recruited from prenatal, health, and Women, Infants, and Children (WIC) clinics; day care centers; and community events and gathering places in southeastern Louisiana (targeting West Bank of Jefferson and Orleans Parishes, and Lafourche, Plaquemines, St. Bernard, and Terrebonne Parishes). Eligibility criteria included: aged 18–45 at enrollment, living in the Gulf area during the oil spill, and, if pregnant, carrying a singleton gestation. 1375 women had information on at least one oil spill exposure and birth outcome with information about pregnancy timing relative to the spill, while 263 either had no pregnancies or were missing data on exposure or outcome. Excluded women had lower ages and BMIs; were less likely to be smokers, Black, or White; and were less likely to reside in a coastal ZIP code (Table S1).

2.1. Exposures

Oil spill experience was measured with questions from several sources, including questions about: (1) a participant's involvement in work on the clean-up and contact with oil, taken from the Gulf Long-Term Follow-Up Study (National Institute of Environmental Health Sciences); (2) direct exposure to the oil spill, taken from studies performed after the Exxon Valdez spill (Palinkas et al., 1993); and (3) the social and economic effects of the oil spill, from a previous study (GUMBO, R03 NR012052). Factor analysis was used to see if the patterns of grouping of similar response questions matched the underlying hypothesized latent constructs, and results were then grouped for power: financial/income consequences (dichotomized as 0–1 indicators/more and categorized as 0–1 indicator/2–3 indicator/4+ indicators); direct contact with oil (handled wildlife, came into contact with oil during cleanup, spent time in an area were oil or oily materials were used, came into contact with oil during activities such as fishing or hunting, or reported coming into contact with oil between April 2010 and April 2011; categorized as none or $\leq$ 1 month of exposure/1 indicator or $>$ 1 exposure/2+ indicators); oil spill-related trauma (damage to people or own property; dichotomized as any/none, categorized as yes to both, yes to one, none); loss of use of the coast (damage to areas where one or one's family fishes, boats, or goes to the coast or beach); and involvement in litigation (Table S2). In addition, a summary variable was created across categories for total exposure to the oil spill (sum of the above individual experiences coded as 3-level variables: financial, direct contact, trauma, loss of use, and litigation, weighted equally; theoretical range was 0–10; range in this sample was 0–9; recategorized to 0, 1–2, 3–4, and 5+).

2.2. Outcomes

Each woman was also asked for a reproductive history including up to 8 pregnancies. Questions for participants included date of/age at each pregnancy, outcome of each pregnancy (livebirth, stillbirth, miscarriage, induced abortion, ectopic/molar/other), birthweight of each child, and whether the pregnancy was early, late, or on time (and, if not on time, by how much). LBW was defined as birthweight $<$2500 g and PTB as birth more than 3 weeks early. If a woman was still pregnant at the time of the interview, her medical records were abstracted for birthweight and gestational age (characteristics of women by outcome are provided in Table S3).

2.3. Analytic methods

Each pregnancy (based on estimated last menstrual period and delivery date) was determined to have occurred before (prior to April 20, 2010), or during or after the oil spill (on or after April 20, 2010 through 2016). If the precise date of the start or end of pregnancy was not known and it was estimated to have occurred within 6 months of the oil spill, it was omitted from the analysis because it could not be categorized as exposed or unexposed (n = 41 pregnancies; usually this occurred when a woman reported age at a pregnancy rather than date). Results were analyzed with generalized estimating equations, using each woman as a cluster, with a logit link; zip code was also incorporated to allow for any spatial autocorrelation. Multiple imputation was used to account for missing covariate data (166 observations with missing data for covariates included in modeling; 10 imputations with Markov chain Monte Carlo methods).

The first set of models includes only pregnancies after the oil spill. In order to address possible biases due to correlated over-reporting or unmeasured confounding by woman, several additional analyses were run. Women were categorized based on their reported exposure to the oil spill, with an interaction between pregnancies occurring prior to and those occurring after the oil spill. If exposure was equally or more predictive of outcomes prior to the oil spill (i.e., the interaction was not significant), it was considered that any associations were likely due to unmeasured confounding. Thus, the pregnancies before the oil spill serve as negative controls (Lipsitch et al., 2010). A sensitivity analysis was also performed stratifying the analysis by time since the oil spill (less than and greater than 2 years); in most cases the sample size was too small to allow for model convergence, but where results were

possible they have been added to the text. A separate analysis also incorporated all pregnancies before the oil spill as unexposed, with those after the oil spill are categorized as exposed or unexposed as defined above. All analysis was conducted using SAS version 9.4 (SAS Institute Inc.).

The study was approved by the Institutional Review Boards of Tulane University, Ochsner, and Louisiana WIC, and all participants provided written informed consent (Tulane IRB # 239911).

3. Results

Analysis was limited to women with a history of at least one birth or who were pregnant at the time of the interview (Table 1). The analyzed population was about two-thirds black, predominantly low-income, and of high BMI. Approximately one-third were exposed to the oil spill in some way, with 302 (22.0%) reporting some degree of direct contact with the oil, and about 10% being significantly affected by the spill (3+ indicators of exposure). The 503 women who had pregnancies both before and after the spill were similar in profile to the overall cohort, with the exception of being older on average and all having two or more births.

The only associations seen among pregnancies after the oil spill (Table 2) were with high levels of contact with oil for LBW (aOR 2.19, 95% CI, 1.29–3.71) and PTB (aOR 2.27, 1.34–3.87), and for loss of use of the coast and PTB (aOR 1.52, 1.01–2.30). When limited to those with a birth before and after the spill (Table 3), the corresponding OR for LBW and high oil contact exposure was 1.88, 0.88–4.02 and for high levels of contact and PTB was 2.12, 0.90–4.99; for loss of use of the coast and PTB it was 1.71, 0.84–3.46. Associations were also examined with pregnancies that occurred before the oil spill, to assess possible unmeasured confounding or correlated measurement error, and the interaction with time was examined. Only the interaction for contact with oil and PTB was statistically significant (Table 3), although the associations for contact with oil were stronger for both outcomes for pregnancies occurring after the oil spill than before it. However, mid-level contact with oil was also associated with higher risk of PTB in the pre-oil spill analysis. When stratified by time relative to the oil spill, the ORs for high contact with oil were similar for pregnancies occurring within two years after the spill and those occurring later, for both LBW (<2 years, aOR 2.01, 95% CI 0.91–4.41 and > 2 years, aOR 2.30, 95% CI 1.20–4.41) and PTB (aOR 2.30, 95% CI 0.99–5.35 and aOR 2.25, 95% CI 1.09–4.65). Results were similar when pregnancies before the oil spill were included in the unexposed group (Table S3).

4. Discussion

Although pregnant women are considered a vulnerable population, few previous studies have addressed the adverse effects of oil spills on birth outcomes (Bruederle and Hodler, 2019). In this analysis of a community-based cohort, we did not find strong associations between report of social, economic, or physical exposure to the oil spill and birth outcomes. Most estimated associations were null or in the direction of protective association. The exception was contact with oil, which was associated with higher risk of LBW and PTB in some analyses. While the imprecision of the estimates and the measurement error inherent in self-report of exposure mean that no conclusion of effect can be drawn, this is worth following up in studies with more detailed measures of contact with oil compounds.

The most directly relevant previous study is from Nigeria, and indicated oil spills that occurred before conception were associated with a doubling of risk of neonatal mortality in nearby areas (Bruederle and Hodler, 2019; Nriagu et al., 2016). Both early birth and reduced fetal growth are strong risk factors for neonatal mortality, and some of the odds ratios we estimated were in the range of 2.0. However, our results are not as strong or definitive as the Nigerian study. In other environmental disasters, previous studies found that psychological effects were

Table 1
Participants in the GROWH study with information on birth outcomes, oil spill exposure, and timing of pregnancy.

	all women (n = 1375)	birth before and after oil spill (n = 503)
	N (%)	N (%)
age		
18-25	385 (28.5)	80 (16.3)
>25-30	391 (28.9)	180 (36.6)
>30-35	297 (22.0)	145 (29.5)
>35	279 (20.6)	87 (17.7)
parity at interview		
nulliparous	65 (4.8)	
multiparous	1297 (95.2)	503 (100.0)
race		
black	866 (63.3)	331 (66.3)
white	377 (27.6)	129 (25.9)
some other race[a]	125 (9.1)	39 (7.8)
married or living with partner		
yes	547 (40.6)	202 (41.1)
no	799 (59.4)	289 (58.9)
income		
<$15K	623 (47.0)	239 (48.9)
$15K–35K	443 (33.4)	165 (33.7)
≥$35K	260 (19.6)	85 (17.4)
smoker		
current	364 (26.6)	131 (26.2)
former	72 (5.3)	23 (4.6)
never	931 (68.1)	347 (69.3)
BMI		
≤20	73 (5.5)	30 (6.3)
20-25	295 (22.4)	99 (20.7)
>25-30	334 (25.4)	133 (27.8)
>30	615 (46.7)	216 (45.2)
pregnant at time of interview		
yes	374 (27.2)	157 (31.2)
no	1001 (72.8)	346 (68.8)
ZIP code of residence		
coastal	491 (36.9)	184 (37.6)
non-coastal	839 (63.1)	305 (62.4)
any exposure to oil spill		
yes	1000 (68.5)	324 (64.4)
no	460 (31.5)	179 (35.6)
financial loss		
yes	667 (48.8)	222 (44.4)
no	700 (51.2)	278 (55.6)
direct contact with oil		
yes	302 (22.0)	99 (19.7)
no	1073 (78.0)	404 (80.3)
direct impact to people or property		
yes	79 (5.8)	29 (5.8)
no	1291 (94.2)	473 (94.2)
loss of use of coast		
yes	560 (41.0)	174 (37.4)
no	807 (59.0)	327 (65.3)
litigation		
yes	342 (24.9)	122 (24.3)
no	1030 (75.1)	380 (75.7)
total number of indicators of experience		

(continued on next page)

Table 1 (*continued*)

	all women (n = 1375)	birth before and after oil spill (n = 503)
	N (%)	N (%)
0	388 (28.2)	158 (31.4)
1	367 (26.7)	134 (26.6)
2	449 (32.7)	156 (31.0)
3+	171 (12.4)	55 (10.9)
time between oil spill (may include more than one pregnancy per woman)		
>5 years before	924 (30.9)	361 (24.8)
2–5 years before	440 (14.7)	287 (19.7)
within 2 years before	335 (11.2)	201 (13.8)
2 years after	448 (15.0)	228 (15.6)
>2 years after	843 (28.2)	381 (26.1)

[a] Includes those self-identifying as Asian, Hispanic with no other race, or Middle Eastern with no other race.

as strong as physical ones. Anxiety due to Chernobyl, but not the environmental threat itself, was associated with earlier births in a sample of Swedish women (Levi et al., 1989). The increased mental and emotional problems in children exposed to Chernobyl prenatally did not correlate with radiation dose, indicating that these deficits were likely due to difficulties of adaptation and relocation (Kolominsky et al., 1999). After 9/11, post-traumatic stress was associated with reduced infant head circumference (Engel et al., 2005), while results of studies of environmental exposure were mixed, often finding effects only in subgroup analyses (Choi et al., 2008; Lederman et al., 2004; Perera et al., 2005). However, in the current analysis, effects of social and financial stress due to the oil spill were not found. This may be due to the fact that most of the population had few direct ties to industries affected by the oil spill and was fairly homogeneous with respect to income. Negative effects on fetal growth and birthweight have been seen fairly consistently after disaster, but associations with gestational age are very mixed (Harville et al., 2010).

Pollution due to oil spills has many potential adverse health effects. While little mechanistic work has directly addressed pregnant women's exposure to oil spills, air pollution, oxidative stress, and genetic damage

Table 2
Self-reported exposure to the Gulf oil spill and birth outcomes in southern Louisiana women, 2011–2016 (n = 960 women/1255 pregnancies).

	low birthweight				preterm birth			
	unadjusted		adjusted		unadjusted		adjusted	
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
any oil spill exposure	0.99	(0.68, 1.44)	1.06	0.73-1.54	1.13	(0.74, 1.72)	1.12	0.72-1.74
oil money	0.75	(0.52, 1.08)	0.81	0.56-1.17	0.77	(0.52, 1.15)	0.76	0.50-1.14
trauma	1.19	(0.55, 2.58)	1.19	0.56-2.51	0.93	(0.40, 2.16)	0.84	0.34-2.07
coast	1.18	(0.82, 1.71)	1.31	0.90-1.90	1.50	(1.01, 2.23)	1.52	1.01–2.30
litigation	1.15	(0.76, 1.72)	1.15	0.76-1.72	0.72	(0.44, 1.18)	0.74	0.45-1.20
contact	1.00		1.00		1.00		1.00	
1 indicator	0.88	0.40-1.95	0.92	0.42-2.01	1.02	0.50-2.07	1.02	0.49-2.12
2+ indicators	2.10	1.26-3.50	2.19	1.29-3.71	2.25	1.34-3.77	2.27	1.34-3.87
overall	1		1.00		1.00		1.00	
1–2 indicators	0.99	0.62-1.56	1.01	0.63-1.62	0.95	0.56-1.63	0.95	0.54-1.65
3–5 indicators	0.85	0.53-1.39	0.94	0.58-1.54	1.04	0.61-1.78	1.01	0.60-1.83
>5 indicator	1.25	0.73-2.16	1.37	0.79-2.35	1.48	0.83-2.64	1.46	0.80-2.66

Table 3
Oil spill exposure and birth outcomes, women with pregnancies both before and after the oil spill, stratified by timing of pregnancy (n = 503/1471 pregnancies).

	low birthweight				p for interaction	preterm birth				p for interaction
	post oil spill		pre oil spill			post oil spill		pre oil spill		
	OR	95% CI	OR	95% CI		OR	95% CI	OR	95% CI	
any oil spill exposure	0.94	0.55, 1.59	0.90	0.54, 1.50	0.81	1.55	0.74, 3.27	1.29	0.74, 2.23	0.77
income loss	0.78	0.45, 1.35	1.05	0.64, 1.71	0.44	1.04	0.52, 2.05	0.91	0.54, 1.52	0.89
trauma	1.52	0.56, 4.12	2.09	0.81, 5.44	0.79	0.87	0.18, 4.15	2.06	0.80, 5.29	0.24
loss of use of the coast	1.24	0.71, 2.17	0.93	0.55, 1.57	0.49	1.71	0.84, 3.46	1.11	0.65, 1.87	0.28
litigation	1.27	0.70, 2.29	1.59	0.96, 2.62	0.56	0.79	0.36, 1.75	1.40	0.84, 2.35	0.20
contact	1.00		1.00		0.12	1.00		1.00		0.02
1 indicator	1.24	0.38, 4.08	1.35	0.58, 3.15		1.03	0.28, 3.78	2.29	1.03, 5.09	
2+ indicators	1.88	0.88, 4.02	0.84	0.40, 1.78		2.12	0.90, 4.99	0.83	0.39, 1.75	
overall experiences					0.70					0.30
1–2 indicators	0.77	0.35, 1.66	0.90	0.49, 1.64		1.21	0.46, 3.14	1.53	0.78, 3.00	
3-5	1.09	0.56, 2.11	0.86	0.43, 1.73		1.89	0.78, 4.57	1.25	0.66, 2.48	
>5	1.12	0.54, 2.34	1.05	0.49, 2.24		1.47	0.52, 4.12	0.96	0.44, 2.08	

Adjusted for age, gravidity, weight gain, BMI, race, income, education, smoking.

have been linked to adverse birth outcomes, though not always conclusively (Li et al., 2019; Patelarou and Kelly, 2014; Protano et al., 2012; Sultana et al., 2017). These exposures have been found after oil spills: air benzene and PM2.5 levels were higher in the affected parishes after the oil spill (Nance et al., 2016), and coastal sediments near oil spills have shown long-term endocrine disruption potential (Liu et al., 2018). Long-term increased oxidative stress and genetic damage have been demonstrated post-oil spill in those exposed to Hebei Spirit and Prestige oil spills (Kim et al., 2017; Perez-Cadahia et al., 2008). Studies also indicate severe health effects of oil spills on other outcomes: many post-oil spill studies show adverse respiratory effects (Laffon et al., 2016), and there was an increased risk of nonfatal myocardial infarction among long-term workers on the Gulf oil spill clean-up (Strelitz et al., 2018). That said, risk assessment for children exposed to affected beaches in Louisiana was estimated to be quite low (Black et al., 2016), and most analyses of seafood and consumption risk analysis indicated no increased risk due to the spill (Wickliffe et al., 2018). Despite this, many women reported avoiding seafood or eating less fish, and may have replaced seafood with less healthy alternatives (Simon-Friedt et al., 2016), which could have influenced pregnancy health. Most of the women in this study lived in parishes adjacent to the coast but not in the coastal zip codes, nor did they work on the clean-up, and levels of exposure to the oil-spill-related pollutants were likely low.

Strengths of the study include the systematic assessment of reproductive history, recruitment at a range of community-based locations, and inclusion of a large number of women who may be vulnerable due to income, race, or geography. While analysis can never fully remove bias in an observational study, we performed several analyses to address possible biases. Presenting the results from before the oil spill addresses the possible bias due to overreporting; performing the analysis within-woman controls for potential unmeasured confounding; and stratifying by time allows for focusing on the time period when the strongest effects would be expected. Limitations include the lack of direct biological measures of exposure to oil spill chemicals and the fact that many of the included births took place substantially later than the oil spill (although this would be consistent with the Nigerian study that found the strongest risk with preconception exposure (Nriagu et al., 2016).) Most birth outcomes were self-reported. Overall, maternal recall of these outcomes is quite good (Harville et al., 2019; Rice et al., 2007; Troude et al., 2008), and women were asked pregnancy by pregnancy with absolute measures ("How much did the baby weigh?") rather than potentially subjective ones ("Was the baby born too small?"). A concern could be that the attention paid to the oil spill caused over-reporting of complications; due to the objective outcome, the lack of an overall increase in adverse outcomes after the spill, and the results of the within-woman analysis, we do not think this is a major source of bias. We considered low birthweight, which is not an ideal outcome due to its incorporating both length of gestation and reduced fetal growth. However, we find that women remember birthweight more precisely than gestational age (Harville et al., 2019). As these women were predominantly already at high social and financial risk, and therefore at higher risk for adverse outcomes, the potential excess risk conferred by the oil spill may have been limited.

To conclude, this study found no effects of the social or economic consequences of the Gulf oil spill on adverse birth outcomes. Research incorporating specific biomarkers of oil spill exposure would be valuable (Huang et al., 2017). Such measures may be needed at the time of exposure, given the volatile nature of oil-related compounds, and would be assisted by programs such as Disaster Research Response 2 (DR2) (National Institute of Environmental Health Sciences, 2021) and other efforts to streamline the study design, approval, and funding possibilities post-disaster. It would also be useful to incorporate more stress biomarkers into environmental epidemiological research, to allow for assessing perceived and actual exposure, especially when direct toxicant exposure is minimal.

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Appendix A. Supplementary data

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Semen quality and sperm DNA integrity in city policemen exposed to polluted air in an urban industrial agglomeration

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ABSTRACT

Background: We examined sperm quality in a cohort of city policemen in Ostrava at the end of a period with high concentrations of air pollutants (winter) and in the same cohort at the end of a relatively low exposure period (summer).

Methods: The study group was comprised of 54 nonsmoking city policemen living and working in Ostrava, Czech Republic. Average daily air-pollutant concentrations recorded by stationary monitoring for 90 days preceding the collection of semen samples were evaluated for different city districts of Ostrava. Standard semen parameters were assessed according to the guidelines of the World Health Organization (2010). The parameters were semen volume, sperm concentration, sperm morphology, sperm motility, acrosome reaction and sperm plasma membrane integrity. Sperm DNA damage was analysed by the sperm chromatin structure assay (SCSA). Sperm motion characteristics were determined by Computer Assisted Semen Analysis (CASA).

Results: The concentrations of all monitored pollutants (particulate matter, sulphur dioxide, nitrogen oxides, carbon monoxide, benzo[a]pyrene, benzene) were significantly increased during winter ($p < 0.001$), except for ozone, the concentration of which was significantly higher during summer. Sperm volume, concentration, % vitality, % sperm morphology (normal form) and % acrosome-intact sperm did not differ significantly between the monitoring periods. The percentages of total motility and progressive motility were significantly higher in March, i.e. at the end of winter ($p = 0.001$). However, CASA testing showed differences in sperm motion kinetics between spring and autumn samples. In the spring samples, we found a significantly lower % of straightness ($p = 0.044$) and the length of straight-line path ($p = 0.01$), while linearity and straight-line velocity were near the borderline value ($p = 0.064$; $p = 0.054$, respectively). As compared to summer, high exposure to air pollution during winter significantly increased the extent of sperm chromatin integrity damage (median 22.6 vs. 18.6%) ($p = 0.003$) and the proportion of immature spermatozoa (median 11.2 vs. 9.9%, $p = 0.001$). Sperm DNA damage negatively correlated with total motility and progressive motility ($r = -0.611, -0.299$; $p < 0.001$). The negative correlation with vitality, normal morphology and acrosome-intact sperm ($r = -0.522, -0.550$ and -0.511, respectively) was also significant ($p < 0.001$).

Conclusion: The examination of the same cohort of city policemen at the end of a period of high air pollution and at the end of relatively low exposure reduced the effects of age, different lifestyles, different occupational exposures, localities and genetic polymorphism on sperm quality impairment associated with air pollution. This study did not demonstrate impaired standard semen parameters in association with exposure. It was shown that sperm chromatin damage and the percentage of immature sperm were highly sensitive to air pollution.

1. Introduction

The city of Ostrava and its surrounding area is an important industrial centre in the Czech Republic with a long-term high environmental burden. There are various air pollution sources in this region, such as traditional steelworks and coke ovens, local heating, transport and local transmission of polluted air from the industrial regions in Poland. The most harmful pollutants contributing to health risks are airborne dust, benzen and benzo[a]pyren (B[a]P). Ostrava is the city with the highest B[a]P concentrations in the Czech Republic and in Europe (Jirik et al., 2016; Tomaskova et al., 2016; Hunova, 2020).

The exposure to air pollution is associated with a range of adverse

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health effects, including reproductive toxicity. Several studies have investigated association between outdoor air pollution and semen quality. High levels of air pollution were linked to a large decrease in semen volume, sperm concentration, progressive motility, total motility and the percentage of morphologically normal sperm. In addition, an increase in the sperm DNA fragmentation index has been reported (summarized by Zhang et al., 2020). A number of studies have shown that human semen is an early and sensitive environmental and reproductive health marker and can be used as an early signal of environmental stress (summarized by Montano et al., 2018). The utility of human semen as an early biomarker of pollution in men living in different regions of Campania was shown by Bergamo et al. (2016).

However, many lifestyle factors can affect sperm quality, such as smoking, alcohol consumption and drug addiction, diet, obesity, psychological stress (summarized by Sansone et al., 2018; Nassan et al., 2018a; Aboulmaouahib et al., 2018; Ranganathan et al., 2019; Leisegang et al., 2020). Furthermore, age, occupational exposure and health condition also play an important role (Evenson et al., 2020; Rubes et al., 2021). The effect of polymorphism in metabolic and DNA repair genes has also been described (Rubes et al., 2005, 2010; Llavanera et al., 2020). Due to the fact that there are many different factors which could affect the development of sperm in the cohorts under study, the results obtained by the investigation of the polluted air impact on sperm quality are inconsistent.

The aim of this study was to assess sperm quality in a cohort of city policemen in Ostrava at the end of a period of high concentrations of airborne pollutants (winter) and in the same cohort at the end of a relatively low-level exposure period (summer). This approach reduced the role of lifestyle factors, different occupational exposures, localities and genetic polymorphism in the resulting sperm quality deterioration due to air pollution. City policemen spend most of their working time outdoors walking around the city, where they are exposed to exhaust fumes from car traffic and pollutants from local industry. Therefore, they are a particularly suitable group for studying the effect of air pollution in large urban agglomerations.

2. Methods

2.1. Study design and population

The study group consisted of 54 nonsmoking city policemen living and working in Ostrava, Czech Republic. The average age of the policemen was 40.4 ± 9.4 years (range 21–61 years). All of them attained at least secondary education. The average length of employment with the Ostrava City Police Force is 14.1 ± 7.99 years. Data on each participant's reproductive and general health and on factors that might impact his semen quality were collected by questionnaire. Policemen with chronic or andrological diseases and long-term treatment were excluded from the study. Special attention was paid to diabetes, varicocele, accessory gland infection and chlamydial infection. These diagnoses were not established in any study participant. Alcohol or drug abuse was also monitored. Drug addiction was not detected, only 3 policemen reported regular alcohol consumption. In addition, reproductive history, the number of children and possible fertility problems were recorded. A total of 39 (72%) policemen included in the study fathered at least one child. Two-thirds of childless policemen were still single. All participants signed an informed consent form and could cancel their participation at any time during the study, in accord with the Helsinki II declaration. The study was approved by the ethical committee of the Institute of Experimental Medicine AS CR in Prague, approval number: 2018/09.

2.2. Inhalation exposure

The Czech Hydrometeorological Institute, Ostrava, performed stationary monitoring. Average daily air-pollutant concentrations recorded by stationary monitoring for 90 days preceding the collection of the semen samples were evaluated for different city districts and the whole territory of Ostrava.

2.3. Semen collection and analysis

Semen samples were collected on site by masturbation into clean glass containers in March and September 2019. Abstinence interval of 2–7 days was requested. After liquefaction at room temperature, standard semen parameters were assessed according to the guidelines of the World Health Organization (WHO, 2010). The parameters included the semen volume, sperm concentration, sperm morphology (head shape, midpiece and tail defects), sperm motility, acrosomal reaction and sperm plasma membrane integrity. Sperm counts were determined using a Neubauer chamber. Sperm motility was evaluated under a light microscope at 200x magnification. Acrosome-intact sperm rates were analysed by the *Pisum sativum* (PSA) lectin staining of fixed semen smears (Mortimer, 1994). Sperm vitality was estimated by assessing the percentage of sperm with plasma membrane damage detected by staining with eosin-nigrosin (WHO, 2010). The percentage of morphologically normal sperm was determined by examining 200 sperm per sample stained with a Diff-Quik rapid staining kit at 1000 × magnification under oil immersion and classifying them according to strict criteria as described by the WHO guideline (WHO, 2010).

Sperm DNA damage was analysed after acridine orange staining by the sperm chromatin structure assay (SCSA) using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) as previously described (Rubes et al., 2010). Semen samples were exposed to 488 nm monochromatic laser light, and red (ssDNA) and green (dsDNA) fluorescence values were recorded in 5000 spermatozoa per sample. SCSA-Soft software (SCSA Diagnostics, Inc., Brookings, SD, USA) was used to assess the rates of sperm with fragmented DNA (DNA fragmentation index, DFI) and high-density staining (representing mainly immature sperm, HDS).

2.4. Computer Assisted Semen Analysis (CASA)

The collected ejaculates were allowed to undergo liquefaction at room temperature for 45 min prior to analysis. Leja standard 20 μm depth chambers in format of 8 chambers per slide (Leja Products B.V., GN Nieuw Vennep, The Netherlands) were used for the analysis. Two μL per sample with concentration of 30 million sperm/mL was placed into the chambers. Dense samples were diluted with saline buffer at 37 °C and pH 7.2. Sperm motion characteristics were determined in at least 300 sperm using CEROS II Version 1.0 hardware and evaluated by HT CASA II Version 1.3 software (Hamilton Throne, Inc., Beverly, MA). The analysed standard parameters included total sperm motility, progressive sperm motility and sperm concentration. Sperm kinetics were assessed by means of length of average path (DAP), length of straight line path (DSL) and length of curvilinear path (DCL). Sperm velocity over specific paths was expressed by average path velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL), and sperm trajectories were represented by straightness VSL/VAP (STR), linearity VSL/VCL (LIN), amplitude of lateral head displacement (ALH), beat cross frequency (BCF) and wobble VAP/VCL (WOB).

2.5. Cotinine assay

The level of tobacco smoke exposure reported in the lifestyle questionnaires was verified by detection of the urinary levels of cotinine as the major nicotine metabolite by radioimmunoassay (Langone and Van Vunakis, 1982). Subjects with cotinine levels higher than 200 ng/mg of creatinine were considered active smokers and were excluded.

2.6. Statistical analysis

Because not all variables are normally distributed (Kolmogorov-Smirnov test) nonparametric analyses were applied. Statistical analysis was performed by nonparametric exact tests using the SPSS software package, version 18 for Windows (SPSS, Inc. Chicago, IL, USA). Wilcoxon matched pairs test for dependent samples was used. Mann-Whitney Sum *U* test was used for bivariate comparison between environmental pollution values obtained in the 90-day period before sampling. Correlation analyses with continuous variables were performed using Spearman's rank correlation.

3. Results

3.1. Air pollution

Quarterly average concentrations of major traffic-associated pollutants ($PM_{2.5}$, NO_2, benzene and benzo[a]pyrene) related to the whole territory of Ostrava are shown in Table 1. Median concentrations of different air pollutants, detected at stationary stations in Ostrava for the monitoring periods are presented in Table 2. The concentrations of all monitored pollutants ($PM_{2.5}$, SO_2, NO, NO_2, NO_x, CO, B[a]P) were significantly higher during the winter season ($p < 0.001$), except for O_3, which was significantly higher during the summer months season ($p < 0.01$). Average concentrations of B[a]P detected at six stationary stations placed in different districts of Ostrava differed significantly according to the location of industry: in winter from 2.4 to 15.8 ng/m^3 and in summer from 0.3 to 5.5 ng/m^3 (Table 3). The difference between winter and summer was highly significant at all stations ($p < 0.001$).

3.2. Semen outcomes

The values of different semen parameters detected at the end of winter and summer, respectively, are shown in Table 4. The analysis of semen samples revealed that this cohort of policemen consisted largely of normospermic subjects (WHO, 2010). Sperm volume, concentration, % vitality, % sperm morphology (normal form) and % acrosome-intact sperm did not differ significantly between the monitoring periods. The percentages of total motility and progressive motility were the only standard parameters which were significantly increased in March, i.e. following the winter season ($p = 0.001$).

However, Computer Assisted Semen Analysis (CASA) showed differences in sperm motion kinetics between the spring and autumn samples. In the spring samples, we found a significantly lower % of straightness ($p = 0.044$) and length of straight-line path ($p = 0.01$), while linearity and straight-line velocity (VSL) were near the borderline value ($p = 0.064$; $p = 0.054$, respectively) (Table 5). High exposure to air pollution during winter significantly increased the extent of sperm chromatin integrity damage compared to summer ($p = 0.003$) and also the proportion of immature spermatozoa (HDS, $p = 0.001$).

Many air pollutants cause oxidative stress, which has detrimental effect not only on DNA but also on the cell and mitochondrial membranes. Therefore, we established a correlation between the sperm DNA fragmentation index and other sperm quality parameters following high exposure, particularly to B[a]P. DFI negatively correlated with total motility and progressive motility ($r = -0.611$, -0.299; $p < 0.001$), but also with a number of motility characteristics, i.e. VCL, VSL, DSL and ALH measured with CASA ($r = -0.580$, -0.541, -.357 and -0.481, respectively; $p < 0.001$). The high negative correlation with vitality, normal morphology and acrosome-intact sperm ($r = -0.522$, -0.550 and -0.511, respectively) was also significant ($p < 0.001$).

Table 1
Air pollution levels for the first and the third quarter of 2019.

Variable	First quarter	Third quarter
PM_{10} ($\mu g/m^3$)	32.8	19.5
$PM_{2.5}$ ($\mu g/m^3$)	27.7	14.5
NO_2 ($\mu g/m^3$)	19.70	12.5
Benzene ($\mu g/m^3$)	2.40	1.3
Benzo[a]pyrene (ng/m^3)	4.60	0.6

Average concentrations related to the whole territory of Ostrava.
Abbreviations: PM, particulate matter.

Table 2
Air pollution recorded by monitoring stations for 90 days preceding the collection of the semen samples.

Variable	Season	Median	Minimum	Maximum
$PM_{2.5}$ ($\mu g/m^3$)	Winter	25.0	2.6	247.6
	Summer	11.2	3.6	44.5
SO_2 ($\mu g/m^3$)	Winter	7.4	1.3	69.5
	Summer	5.6	1.3	45.9
NO_x ($\mu g/m^3$)	Winter	33.0	4.7	185.1
	Summer	21.1	5.4	101.4
NO ($\mu g/m^3$)	Winter	5.3	0.5	84.0
	Summer	3.8	0.5	36.7
NO_2 ($\mu g/m^3$)	Winter	23.4	4.0	66.1
	Summer	16.0	4.5	46.1
CO ($\mu g/m^3$)	Winter	0.5	0.1	2.2
	Summer	0.3	0.1	1.6
O_3 ($\mu g/m^3$)	Winter	40.9	5.2	79.1
	Summer	64.8	18.8	97.8
B[a]P (ng/m^3)	Winter	2.8	0.2	49.0
	Summer	0.3	0.0	24.0

Abbreviations: PM, particulate matter; B[a]P, benzo[a]pyrene.

Table 3
Concentrations of benzo[a]pyrene measured at individual monitoring stations.

Monitoring station	Winter	Summer
1	0.9 (0.2, 11)	0.2 (0.0, 1.3)
2	1.3 (0.2, 10)	0.1 (0.0, 1.6)
3	2.1 (0.3, 14)	0.1 (0.0, 1.3)
4	2.3 (0.3,17)	0.3 (0.0, 2.7)
5	2.9 (0.3, 28)	1.4 (0.2, 6.3)
6	14 (1.7, 49)	4.0 (0.1, 24)

Note: Data presented as median (min, max) in ng/m^3.

Table 4
Results of the semen analysis.

Variable	March	September	p-value
Sperm volume (mL)	3.4 ± 1.17 3.2 (0.7, 7.0)	3.2 ± 1.11 3.4 (1.0, 6.2)	0.079
Sperm concentration (x10^6/mL)	101.5 ± 78.14 93.5 (8.0, 345.0)	95.1 ± 63.09 96.3 (10.0, 255.0)	0.549
Total motility (%)	56.1 ± 6.51 57.0 (30.0, 69.0)	51.4 ± 7.85 52.5 (32.0, 64.0)	0.001
Progressive motility (%)	49.7 ± 8.72 51.0 (6.0, 64.0)	45.6 ± 7.85 47.0 (27.0, 58.0)	0.001
Vitality (%)	72.1 ± 9.70 73.5 (47.0, 90.0)	70.5 ± 8.90 71.5 (50.5, 88.5)	0.201
Sperm morphology, normal form (%)	9.6 ± 4.48 8.8 (2.5, 22.0)	9.0 ± 4.79 7.5 (1.5, 22.0)	0.132
Sperm head morphology, normal form (%)	14.0 ± 6.26 13.8 (3.0, 22.0)	12.4 ± 6.39 11.0 (2.0, 28.5)	0.109
Acrosome-intact sperm (%)	82.1 ± 6.83 82.5 (64.0, 92.0)	81.3 ± 6.36 81.5 (67.5, 93.5)	0.225
DFI (%)	24.4 ± 11.56 22.6 (7.5, 58.6)	21.6 ± 13.07 18.6 (5.5, 62.4)	0.003
HDS (%)	12.7 ± 6.19 11.2 (4.6, 32.5)	11.3 ± 5.23 9.9 (3.6, 27.1)	0.001

Note: Data presented as mean ± standard deviation and median (min, max).
Abbreviations: DFI, DNA fragmentation index; HDS, high DNA stainability.

Table 5
Range of computer-assisted semen analysis parameters in the study group.

Variable	March	September	p-value
VCL (μm/s)	73.0 (46.6–120.7)	73.7 (33.3–114.9)	0.740
VSL (μm/s)	39.9 (21.4–55.3)	41.0 (16.2–66.3)	0.054
VAP (μm/s)	48.0 (25.7–69.5)	49.3 (23.1–70.7)	0.877
DSL (μm)	16.6 (10.4–23.8)	17.9 (7.7–28.6)	0.010
ALH (μm)	4.5 (2.5–7.9)	4.5 (1.8–7.1)	0.192
LIN (%)	53.6 (31.4–76.1)	56.8 (33.1–77.1)	0.064
STR (%)	80.4 (59.9–94.2)	82.9 (58.9–94.4)	0.044
BCF (Hz)	18.6 (14.1–27.0)	18.9 (9.9–25.7)	0.231

Note: Data presented as median (min,max).
Abbreviations: VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; DSL, length of straight line path; ALH, amplitude of lateral head displacement; LIN, linearity VSL/VCL; STR, straightness VSL/VAP; BCF, beat cross frequency.

4. Discussion

In a number of studies, there are discrepancies in the effects of air pollution on sperm quality. In our study, we tried to reduce the impact of lifestyle factors as much as possible by the examination of our study participants after a period of high exposure to air pollutants and again after a period of low exposure during one year. It was shown that seasonal effects should also be taken into account. The majority of standard parameters of sperm quality (sperm volume, concentration, vitality, % normal morphology sperm or % acrosome-intact sperm) did not differ significantly between spring and autumn collection. However, we found a significantly higher percentage of motile and progressively motile sperm in ejaculates collected in spring ($p < 0.001$). This is in accordance with the findings of some authors who described seasonal variations in sperm parameters related to temperature fluctuations and daylight duration, namely higher sperm motility and concentration in spring (Santi et al., 2018, Kabukçu et al., 2020). Kabukçu et al. (2020) in a comprehensive study lasting for 8 years and involving over 6000 sperm samples found that progressively motile sperm count in October was 23.6% lower than the value of May ($p = 0.026$).

Even though there is a seasonal increase in sperm motility in the spring, CASA showed impaired sperm motion kinetics. In the spring samples, the values for straightness and the length of straight line path were significantly lower. This can be attributed to the high concentration of B[a]P in winter. Mukhopadhyay et al. (2010a) used CASA to evaluate the *in vitro* effect of B[a]P on sperm hyperactivation and acrosome status in normozoospermic semen samples from non-smokers. B[a]P statistically significantly affected sperm motility, which manifested itself by increased hyperactivation and a significant decrease in STR proportion. A decreased proportion of STR sperm was also found in smokers with high exposure to B[a]P and in men exposed to heavy metals (Mukhopadhyay et al., 2010b).

The only air pollutant the concentration of which was significantly increased in our study in summer was O_3. Ozone induces oxidative stress and may disrupt sperm development. The effect of ozone on sperm quality is still not exactly known. Sokol et al. (2005) found a significant negative correlation between ozone levels and sperm concentrations. Qiu et al. (2020) studied the relationship between environmental exposure (PM_2, PM_{10}, SO_2, NO_2, CO, O_3) and qualitative semen parameters (semen volume, concentration and motility). They found that chronic exposure to O_3, in contrast to other pollutants, positively correlated with forward motility. Wdowiak et al. (2019) found a significant negative correlation between the atmospheric ozone levels and the percentage of sperm with normal morphology. Hansen et al. (2010) did not observe any statistically significant relationship between exposure to O_3 and DNA integrity and chromatin maturity. In our study, the average sperm count and the percentage of sperm with normal morphology were decreased in summer, but not significantly. The potential effect of ozone on motility was probably obscured by seasonal effects.

Seasonal variations of atmospheric temperature also affect sperm development. Santi et al. (2018) described seasonal variation in sperm parameters related to temperature fluctuations. This variation was observed only for parameters related to sperm quantity. As temperature increases, total sperm count, concentration, non-progressive motility, immotile sperm and normal forms are at the lowest values. The authors stated that ambient temperature predominantly exerts effect in the final stage of spermatogenesis. In the month preceding sampling, the average daily temperature in our study was 5.2 °C in spring and 13.3 °C in autumn. The highest temperatures recorded in September were 26.9 ± 3.3 °C. We did not find any extreme values that would have a significant effect on spermiogenesis. Some of the above effects may contribute to the fact that sperm volume, concentration, motility, vitality and normal morphology were not significantly higher in autumn, i.e. after a period of low-level air pollution. These effects can also contribute to discrepancies in the published results.

Based on this and our previous studies, chromatin integrity in sperm appears to be a sensitive indicator of air pollution less affected by other

factors (Rubes et al., 2005). We found a highly significantly increased sperm chromatin integrity damag and the proportion of immature sperm with defects in the histone-to-protamine transition (HDS) in the city policemen after the winter season with high air pollution levels compared to the autumn sampling. We assume that the main cause of sperm chromatin integrity damage in policemen from Ostrava after the winter season is the high ambient concentration of B[a]P. Saad et al. (2019) demonstrated that high concentrations of PAH metabolites in urine cause high levels of sperm DNA fragmentation in men with idiopathic infertility. Calogero et al. (2011) studied the adverse role of traffic-related pollution substances on sperm in men working at motorway tollgates. The men at motorway tollgates had a significantly higher ($P < 0.001$) percentage of sperm chromatin damage ($18.3 \pm 1.4\%$) compared to controls ($11.3 \pm 1.2\%$). They also found a significant positive correlation between sperm chromatin damage and the length of occupational exposure, which suggests a time-dependent relationship. Bosco et al. (2018) found that men living near the steelworks in the province of Taranto showed a significantly higher percentage of sperm DNA fragmentation compared to those in a control region (25% vs. 16.8%, $p < 0.01$). In fact, steelworks are one of the air pollution sources in Ostrava. Nassan et al. (2018b) investigated associations between residential distances to major roadways and various sperm characteristics in order to determine their quality deterioration in connection with public transport. They concluded that residential proximity to major roads was not associated with impaired sperm quality parameters and sperm DNA integrity. However, the actual exposure of different participants in the study is not clear and the neutral comet assay method used to determine chromatin damage is less sensitive than SCSA.

Many traffic-related pollutants have high oxidative activity and induce oxidative stress causing damage not only to sperm DNA, but also to cell and mitochondrial membranes. This explains the high degree of correlation between sperm DNA fragmentation and motility impairment. Aghazarian et al. (2021) studied the relationship between motion kinetics and standard sperm parameters with sperm DNA damage. They found a significant relationship among a number of characteristics of sperm motion kinetics: VAP, VSL and VCL, which is in accordance with our results. We detected a highly significant negative correlation of these sperm motility characteristics with chromatin fragmentation. Regarding the standard semen parameters, we found a highly significant negative correlation of DFI with motility, normal morphology and vitality in both spring ($r = -0.399, -0.550, -0.522$) and autumn ($r = -0.543, -0.610, -0.639$) in agreement with the mentioned authors. In addition, a negative correlation with the percentage of acrosome-intact sperm was observed ($r = -0.511$, $p < 0.001$). The above-mentioned authors considered sperm vitality as the most accurate indicator of sperm DNA damage, which corresponds to our study demonstrating a high correlation between DFI and vitality. Negative correlation between standard semen parameters (concentration, motility, morphology) and DFI was also described by Gao et al. (2021). However, unlike them, we did not find a significant correlation between standard parameters and HDS. Zini et al. (2009) reported that HDS values are essential for morphology of the sperm head with incomplete sperm chromatin condensation, and contribute to sperm head defects. In our study, we did not find a correlation between HDS and the percentage of normal sperm heads, but the correlation between the percentage of normal heads and DFI was -0.399 ($p = 0.003$).

5. Conclusion

When comparing the occurrence of sperm abnormalities caused by air pollution in different localities, it should be borne in mind that a number of external and internal factors may influence the result. By examining the same cohort of men during a period of high-level and a period of low-level air pollution, we minimized internal factors, such as age, lifestyle, profession etc. We did not find significant deterioration of standard sperm quality parameters after the period of high-level air pollution. In some of the parameters such as motility, the seasonal effect could have masked the exposure effect. The most sensitive bioindicator of air pollution is impaired sperm chromatin integrity and the percentage of immature sperm with all their implications for male fertility. As opposed to standard parameters of sperm quality, assisted reproductive technology is unable to cope with these abnormalities.

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Conflict of competing interest

The authors declared no conflicts of interest.

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The German Environmental Survey for Children and Adolescents 2014–2017 (GerES V) – Study population, response rates and representativeness

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ABSTRACT

The German Environmental Survey (GerES) is a population-representative, cross-sectional study on environmental exposures of the general population of Germany. GerES has repeatedly been conducted since 1985 by the German Environment Agency (UBA) in close collaboration with the Health Interview and Examination Surveys of the Robert Koch Institute (RKI). In the German Environmental Survey for Children and adolescents 2014–2017 (GerES V) pollutants and other environmental stressors were measured in human samples as well as in the homes of 3- to 17-year-old children and adolescents. Interviews were conducted about health-related behaviors and living conditions. The GerES V basic program encompassed examinations of whole blood, blood plasma, morning urine and drinking water samples, measurements of ultrafine particles and noise levels, comprehensive standardized interviews, and self-administrated questionnaires. Additional modules on volatile organic compounds and aldehydes, particulate matter ($PM_{2.5}$) in indoor air, organic compounds in drinking water and pollutants in house dust were conducted in subsamples. Potential GerES V participants were identified and attained by the RKI from those participants who were examined and interviewed for the cross-sectional component of the second follow-up to the German Health Interview and Examination Survey for Children and Adolescents (KiGGS Wave 2). The gross sample of GerES V comprised 3031 children and adolescents of which 2294 finally took part in the survey. This equals a total response rate of 75.7 %. Response rates varied, depending on region, type of municipality, age and sex, from 66.0 % to 78.3 %. By calculating individual case weights, discrepancies due to sample design and non-response between the GerES V sample and the whole population could be considered in statistical analysis. Therefore, the representativeness of the GerES V results with regard to age, sex, community size and region was assured.

1. Introduction

The German Environmental Survey (GerES) is part of the German Federal Environmental Health Monitoring (German name: *Gesundheitsbezogene Umweltbeobachtung des Bundes*) (Federal Ministry for the Environment, 2020) and has been performed on behalf of the Federal Government since 1985. GerES is the most comprehensive cross-sectional population survey obtaining data on the exposure to environmental pollutants in Germany. The survey aims to collect, update, and evaluate representative data on the exposure of people living in Germany to environmental pollutants. Participants are visited at home and provide samples of urine, drinking water, and house dust. Moreover, indoor air samples are taken in the participants' home. All these samples as well as the blood samples previously collected by the Robert Koch Institute (RKI) are analyzed for various pollutants. Some GerES cycles have additionally analyzed physical stressors such as noise and airborne particulate matter as well as biological stressors like mould. Furthermore, extensive standardized interviews and self-administered questionnaires are used in GerES to investigate exposure relevant aspects such as the use of products, dietary habits, the amount of time spent in particular surroundings, as well as home furnishings. All GerES participants also took part in the respective Health Interview and Examination Survey of the RKI (Kurth et al., 2002). As a consequence, health condition and exposure to environmental

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pollutants are always simultaneously determined in the participants and could also be combined with extensive sociodemographic study data collected by the RKI. Moreover, parts of the blood sample taken in the health study by RKI could be used in GerES for further analysis. Building upon the experience from four previous GerES cycles (Schulz et al., 2007), GerES V started with a pilot study in 2013. In this pilot study, the German Environment Agency (UBA) tested the feasibility of the survey instruments, timelines, logistics, as well as the timing of the home visits and the related participant burden. The pilot study encompassing 39 home visits was performed on the basis of a randomly chosen sample of children and adolescents and their families living in Berlin (Jesske et al., 2015). Based on the experiences of the pilot study, all survey instruments, the schedule for fieldwork, sample and data management were evaluated and improved for the main study of GerES V. The study comprised the basic program and four additional modules. The basic program was performed with all participants and included Human Biomonitoring (HBM) with sampling of morning urine, whole blood and blood plasma, as well as the examination of drinking water samples, measurement of ultra-fine particles, noise levels self-administrated questionnaires and standardized interviews. The additional modules on indoor air volatile organic compounds and aldehydes, particulate matter (PM2.5), organic compounds in drinking water and pollutants in house dust were conducted in subsamples (cf. Section 2.2-2.5).

Like the pilot study, also the main study of GerES V was conducted in close collaboration with RKI's German Health Interview and Examination Survey for Children and Adolescents (KiGGS Wave 2) (Mauz et al., 2017), which is the second follow-up of the KiGGS baseline study from 2003 to 2006.

The Ethics Committee of the Berlin Chamber of Physicians (Eth-14/14) and the Federal Officer for Data Protection and Freedom of Information (III-425/009#0018) had approved GerES V. Approval was also given for KiGGS Wave 2 (Hoffmann et al., 2018).

The tasks of the RKI in GerES V were to acquire potential participants from individuals who have participated in KiGGS Wave 2 by asking them for written consent for data (address data and contact details) as well as blood sample transfer to UBA, to provide KiGGS Wave 2 data of GerES V participants to UBA for a joint data evaluation, and to calculate weighting factors reducing discrepancies of distribution between the sample and the overall population (Kurth et al., 2018). On behalf of UBA, Kantar Health Germany GmbH (KH) carried out and evaluated the GerES V fieldwork (starting January 21, 2015 and ending June 21, 2017) by subcontracting the Face2Face department (F2F-Line) of TNS Infratest (Cholmakow-Bodechtel et al., 2018).

In the following, the process of gaining participants for GerES V, the sample design, response rates, and data weighting are described.

2. Sample design

GerES V is a cross-sectional study based on a representative sample of children and adolescents aged 3–17 years with main residence in Germany. The sampling procedure was based on a two-stage protocol developed by the RKI and in cooperation with GESIS (Leibniz Institute for Social Sciences) (Hoffmann et al., 2018). In the following, sample selection criteria for the basic program and the four additional modules of the GerES V main study are described.

2.1. Sample selection criteria for the basic program of GerES V

The sample selection for KiGGS Wave 2 and GerES V has been described by Hoffmann et al. (2018): For KiGGS Wave 2, the RKI selected a representative sample of 0- to 17-year-old children and adolescents living in Germany. The addresses of children and adolescents were drawn from the municipal population registries for each of the 167 sample points originally used in the KiGGS baseline study (Hoffmann et al., 2018; Kamtsiuris et al., 2007; Kurth et al., 2008). Predominantly one child or adolescent per household address was selected to participate in the survey and in few cases more than one were chosen. RKI researchers asked all 3- to 17-year-old children and adolescents as well as their parents (or legal representatives) who participated in interviews and examinations of the cross-sectional component of KiGGS Wave 2 whether they would be willing to also participate in GerES V. For acquisition of potential participants for GerES V and asking for written consents for data as well as blood sample transfer (cf. 3.1) the following inclusion and exclusion criteria were applied by RKI.

2.1.1. Inclusion criteria

- Participation in KiGGS Wave 2 (interviews and examinations of the cross-sectional component)
- Aged 3–17 years at the time of the KiGGS Wave 2 examination
- Live at least 16 days per month at the current address (same address during GerES V and KiGGS Wave 2)

2.1.2. Exclusion criteria

- Inability to fully understand the aims of the survey and hence to provide informed consent
- Communication with the family not possible due to language barriers

Potential participants fulfilling the above-mentioned criteria and for who written consent was available have been contacted for the recruitment by KH (cf. Section 3.2). After being recruited by KH and the successful arrangement of a home visit, all participants underwent the same basic program of GerES V. This program comprised the following parts:

- Parental interview (computer assisted personal interview – CAPI)
- Child/adolescent interview (CAPI), from the age of 11 years
- Questionnaire on diseases and health problems suffered by the child and adolescent (self-administered questionnaire – SAQ)
- Documentation of residential environment (CAPI)
- Anonymized satisfaction questionnaire (SAQ)
- Human biomonitoring (blood sample, morning urine sample including documentations (CAPI))
- Drinking water monitoring (S0: sample taken from the running water, S1: sample taken after stagnation, both samples were taken from the tap from which water is usually drawn for consumption (German Environment Agency, 2018) including documentation (CAPI))
- Noise level measurement including documentation (CAPI)
- Indoor air measurement of ultra-fine particles including documentation (CAPI)

For certain pollutants sampling and analysis were particularly expensive and time-consuming. For these, UBA selected appropriate subsamples from all GerES V participants. Inclusion criteria for the selection of participants for these subsamples were defined for each of the four additional modules separately (see Section 2.2 to 2.5).

2.2. Sample selection criteria for module "indoor air"

In this module of GerES V, contaminants in indoor air were analyzed for various volatile organic compounds (VOC) and aldehydes by passive sampling (Birmili et al. under review). In order to achieve the targeted sample size of 668, four GerES V participants from each of the total 167 sample points were selected for the module "Indoor air" like this: For each of the four age groups (3–5 years, 6–10 years, 11–13 years, and 14–17 years), the first family visited in each sample point that was deemed eligible by interviewers was asked whether they would like to participate in this module. If the family declined, the second visited and deemed eligible was asked, and so forth. The eligibility of a family for this subsample was determined by the interviewers of KH during the

home visit according to the following criteria:

- After conducting the basic program, the interviewers had to be convinced that the family was capable of completing the module on their own.
- During sampling the home had to be inhabited.
- At the end of the sampling (after approximately one week), at least one person needed to be at home. The family could not be travelling at that time, at least one family member would have to be at home for the period of five to eight days of sampling. If the family was away for 2 days maximum (e.g. at the weekend) but home on the final day, participation was still possible.
- No residential refurbishing was planned to be undertaken during the sampling period.
- It had to be possible to hang the passive samplers at a height of 2 m in the room selected for sampling.

2.3. Sample selection criteria for module "house dust"

Aim of this module in GerES V was to analyze house dust for pollutants, such as plasticizers and flame retardants (Daniels, 2019). In total 668 vacuum cleaner bags from the households of four participants per sample point were aimed to be collected. Therefore, five GerES V participants per point were randomly pre-selected and asked for a house dust sample during the home visit. A house dust sample was considered useable if.

- the contents of the bag originated solely from the participant's household and
- the vacuum cleaner bag was at least one third full.

Already after visits in the first 15 sample points, it became evident that strict adherence to the pre-selected participants would make it impossible to collect four useable samples per point. This was because many households used vacuum cleaners without bags (e.g., water vacuum cleaners), bags were less than one third full, or the vacuum cleaners were used also outside the dwelling. In order to nonetheless collect 668 samples, the selection criteria were subsequently modified as follows:

- The number of pre-selected families being asked for a vacuum cleaner bag was increased to seven.
- Later, the useable samples were selected depending on availability and willingness to participate without pre-selection, spread evenly across the four age groups. To compensate for the too low number of samples in previous points, the target of house dust samples to be taken per point was finally increased to six.

2.4. Sample selection criteria for module "organic pollutants in drinking water"

Organic contamination of drinking water, usually arising from plastic material, were analyzed for a subsample of the GerES V participants. The target was to take cold and warm water sample pairs at two households of each of the 167 sample points. After visiting 15 sample points, the number of sample pairs taken per sample point was reduced to one due to logistical restraints. This sample pair was taken at homes of those families, which were visited early at the respective sample point because of technical needs during water analyses.

2.5. Sample selection criteria for module "particulate matter ($PM_{2.5}$)"

In the smallest module of GerES V, particulate matter ($PM_{2.5}$) was measured and also analyzed for polycyclic aromatic hydrocarbons. $PM_{2.5}$ samples were taken in homes of 80 participants. The selection of this non-population representative sample was based primarily on their proximity to the UBA in Berlin. Hence, only GerES V participants from sample points in Berlin, Brandenburg and Mecklenburg-Western Pomerania were eligible for participation in this module. In summary, 16 sample points were included, from each of which up to five families participated who fulfilled the following criteria:

- A suitable place for the installation of equipment for measuring levels of particulate matter (protected outside space, power supply, stable ground etc.) must exist.
- The family has to be at home during the two to three weeks following the GerES V home visit (no planned absences such as holidays).
- Residential refurbishing must not be carried out during the measurement period of one week.

3. Process of gaining participants for GerES V

The process of gaining participants for GerES V out of the KiGGS Wave 2 participants involved two steps: In the first step done by RKI, potential participants were acquired from individuals who have participated in KiGGS Wave 2 and met all other inclusion criteria (cf. Section 2.1). From this group, GerES V participants were recruited in the second step by KH.

3.1. Acquisition of potential GerES V participants in KiGGS Wave 2

RKI fieldworkers provided information about GerES V to all children and adolescents participating in interviews and examinations of the cross-sectional component of KiGGS Wave 2 and their parents (or legal guardians) during their visit in the KiGGS Wave 2 examination centers. Parents agreeing to the participation of their children were asked to sign a consent form allowing the RKI to pass on their addresses and contact details to the UBA, and agreeing to be contacted by the UBA or UBA's contractor. They were also asked to provide consent for parts of their blood samples donated in KiGGS Wave 2 examination to be passed on to the UBA to be analyzed for environmental chemicals. Children and adolescents aged 14 years and older had to co-sign both consent forms.

At the beginning of the KiGGS Wave 2 fieldwork, the interest in becoming a potential participant for GerES V was lower than expected. Therefore, RKI and UBA took measures to increase the number of given consents. In addition to refresher courses provided for RKI staff, such measures included.

- Incentives in the form of five Euro in cash for potential participants and the GerES mascot, a cartoon squirrel as key ring and for the younger participants as cuddly toy
- Survey information, invitation letter, and consent forms being translated in four additional languages (English, Russian, Serbian, and Turkish)
- A comprehensive list of arguments to help RKI fieldworkers convincing people and to overcome their reservations, e.g., by explaining the advantages of home visits in GerES V
- Since GerES V examinations and interviews were taking place at home, some parents wanted to consult other family members before giving consent. These people were called later by the RKI to obtain their consent. However, blood samples from KiGGS Wave 2 were not available for these potential participants of GerES V due to the missing consent at the time of the KiGGS Wave 2 examination.

3.2. Recruitment of GerES V participants

All KiGGS Wave 2 participants having agreed that their address and contact details are transferred to UBA and UBA's contractor were contacted by KH. The time gap between the fieldwork of KiGGS Wave 2 and GerES V was kept as short as possible and reached 4 months exceptionally. KH arranged appointments for home visits with the families. Ideally, this was done about three weeks before the start of the GerES V fieldwork in the respective sample point, either by post or telephone.

Ten days before the home visit, KH sent to the recruited families the appointment confirmation, the consent form for the GerES V survey participation, and further survey materials. As an additional incentive, the consent form also included the option to permit the individual reporting of the study results in HBM and in the samples from the home of the participants. A detailed description of procedures regarding the GerES V fieldwork can be found in Cholmakow-Bodechtel et al. (2018). At the beginning of each home visit, interviewers first collected the signed consent forms for participation in GerES V before carrying on with the home visit.

Due to the initially low participation rate during the GerES V fieldwork, optimizing measures were taken by UBA and KH to increase the response rate and the completeness of the survey instruments. KH staff and F2F-Line interviewers e.g. received refresher courses and text blocks used in postal communication with families were improved. Therefore the focus in these documents was shifted from the importance for research to the individual benefits for participants (Cholmakow-Bodechtel et al., 2018).

4. Results

4.1. Initial gross sample, net sample, and response rate of GerES V

Table S1 in the supplemental file provides an overview of initial sample size, non-participants, participation and response rates of the examination and interview group of KiGGS Wave 2 (Hoffmann et al., 2018). 3567 children and adolescents aged 3–17 years participated in interviews and examinations of the cross-sectional component of KiGGS Wave 2. From these 3567 individuals, 107 were excluded as quality neutral losses (QNL) with regard to the inclusion/exclusion criteria (cf. Section 2.1). Evaluation of the KiGGS Wave 2 data of six further potential participants revealed incomplete information (cf. Section 4.2). Hence, these individuals were subsequently removed from the group of potential participants. The gross sample from which potential participants for GerES V were identified thus comprised 3454 children and adolescents. Of these, 3109 children and adolescents (90 %) were successfully attained by the RKI fieldworkers as potential participants and could therefore be contacted by UBA and KH (cf. Table 1). This rate was increased by different optimization measures taken over the course of KiGGS Wave 2 (cf. Section 3.1) from 88.6 % in the first third to 93.5 % in the last third of the fieldwork period. Finally, a total of 2386 families participated in GerES V and the net sample comprised 2294 cases. The overall response rate was 75.7 % (cf. Table 1). Taking account of the KiGGS Wave 2 initial gross sample of 9230 participants (cf. Table S1 Supplemental file) and 818 QNL, KiGGS Wave 2 and GerES V had a combined response rate of 27.6 %.

Table 1
Development from the KiGGS Wave 2 participants to the net samle of GerES V.

Study	Samples	N	%
KiGGS Wave 2	**Initial gross sample for acquisition of potential GerES V participants** (KiGGS Wave 2 participants)	**3567**	
	Incomplete KiGGS Wave2 data by GerES V case definition	6	
	Quality neutral losses (QNL) at the time of acquiring potential GerES V participants at the KiGGS Wave 2 examination center	107	
	Gross sample for acquisition of potential GerES V participants	**3454**	**100.0**
	Individuals not interested in GerES V	345	
	Initial gross sample for recruitment of GerES V participants (acquired potential GerES participants)	**3109**	**90.0**
GerES V	Quality neutral losses (QNL) at the time recruitment	78	
	Gross sample for GerES V	**3031**	**100.0**
	Refusals and non-contacts	651	
	Participants	2386	
	Incomplete GerES V data by case definition	92	
	Net sample (cases)	**2294**	**75.7**

N = sample size.

4.2. Case definition and non-participation

Table 1 contains absolute numbers and percentages of non-participants. 78 of the potential participants (2.5 %) had to be excluded as QNL by KH when appointments were made. Further 651 potential participants (21.5 %) who had stated their interest in participating in GerES V during KiGGS Wave 2 ultimately did not take part. In sum, 743 of the potential participants did not participate in GerES V. Data sets of 92 participants (3.0 %) were incomplete and did not meet the requirements of the definition of a case that included:

- Completed KiGGS Wave 2 data set
- Completed GerES V documentation on residential environment
- Completed GerES V parental interview and completed child/adolescent interviews for participants aged 11 years and older
- S0 running water sample including documentation collected in GerES V
- Morning urine sample including documentation for participants aged five and older collected in GerES V

4.2.1. Quality-neutral losses

QNL at the time of the recruitment for GerES V were defined by following reasons:

- Child or adolescent no longer lives at the address provided
- Child or adolescent living at the address provided for less than 16 days every month
- Child or adolescent not living at the address provided during the fieldwork due to extended period of absence such as year abroad for professional, educational or other reasons
- Communication impossible due to language barriers
- Child or adolescent deceased

Table S2 in the supplemental file shows the relative frequencies of these disqualifications. A substantial proportion of QNL (70.5 %) were due to families moving home between KiGGS Wave 2 and GerES V. QNL were observed to be distributed evenly across age groups, sex, and interviewer teams (Cholmakow-Bodechtel et al., 2018).

4.2.2. Other reasons for non-participation

The 651 participants who stated an interest in taking part in GerES V but ultimately did not, declined for a number of reasons. Fig. 1 shows the relative frequencies of reasons for not participating. The most frequently mentioned reasons were ‘lack of time’ (21.5 %), ‘unwilling (no reason given)’ (18.3 %) and ‘not interested, not convinced of the survey’s importance’ (8.4 %). The reason ‘home visit not wanted’ was given for 6.1 % of individuals. For the first 18 sample points visited by KH, the latter reason for not participating was given for a much higher percentage of 17.7 %. Optimization measures (cf. Section 3.2) helped reduce this fraction. There was no identifiable clustering of these other reasons for non-participation according to age group, sex or interviewer team (Cholmakow-Bodechtel et al., 2018).

4.3. Distribution of the gross and net sample by sample selection criteria

Table 2 presents absolute numbers of participants and response rates by region, community size, age group and sex of the participants (Kurth et al., 2018). The highest response rate was achieved among people living in West Germany (78.3 %), being almost eight percentage points higher than that for East Germany (70.5 %). The lowest response rate (66.0 %) was recorded in mid-sized communities. Considering age, the

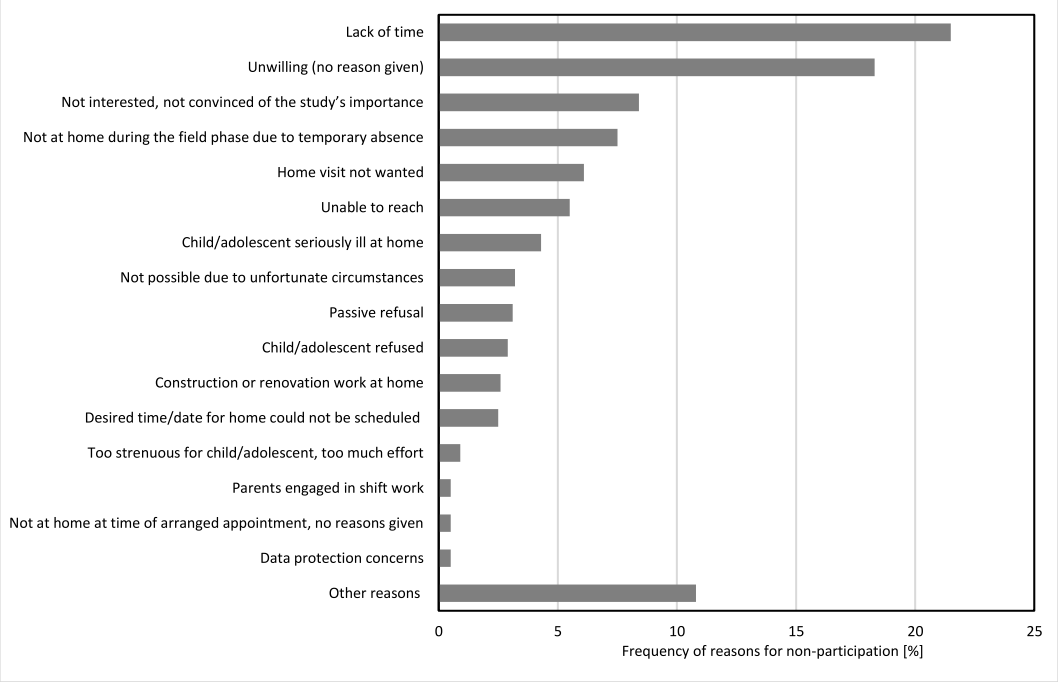

Fig. 1. Overview of reasons for not participating in GerES V and relative frequencies.

Table 2
Overview of gross sample, net sample and response rate for GerES V, stratified by region, community size, age group and sex.

	Initial gross sample		Quality neutral losses		Gross sample		Refusals, non-contact, incomplete data		Net sample (cases)	Response rate
	N	%	N	%	N	%	N	%	N	%
Total	**3109**	**100**	**78**	**2.5**	**3031**	**100**	**743**	**24.3**	**2294**	**75.7**
Region										
West Germany	2032	100	38	1.9	1994	100	433	21.7	1561	78.3
East Germany	974	100	33	3.4	941	100	278	29.5	663	70.5
Berlin	103	100	7	6.8	96	100	26	27.1	70	72.9
Community size (number of inhabitants)										
<50.000	949	100	20	2.1	929	100	223	24.0	706	76.0
50.000 to < 100.0000	319	100	10	3.1	309	100	105	34.0	204	66.0
≥100.000	1841	100	48	2.6	1793	100	409	22.8	1384	77.2
Age group (in years)										
3–5	569	100	21	3.7	548	100	124	22.6	424	77.4
6–10	1004	100	19	1.9	985	100	233	23.7	752	76.3
11–13	720	100	16	2.2	704	100	161	22.9	543	77.1
14–17	816	100	22	2.7	794	100	219	27.6	575	72.4
Sex										
male	1537	100	36	2.3	1501	100	372	24.8	1129	75.2
female	1572	100	42	2.7	1530	100	365	23.9	1165	76.1

For abbreviations see footnote of Table 1.

response rate for the 14- to 17-year-olds (72.4 %) was the lowest of all age groups. The response rate for females (76.1 %) was slightly higher than that for males (75.2 %). In the KiGGS Wave 2 gross sample, similar higher response rates for females (females: 43.4 %, males: 39.7 %) were reported by Hoffmann et al. (2018). In 2015, the first year of GerES V fieldwork, total participant response rates ranged from 71.1 % to 75.0 %. This rate increased in 2016 to 79.1% and to 86.8% in 2017. This steady increase of the response rate can be attributed to numerous optimization measures (cf. Section 3.2) as well as to increasing routine of GerES V fieldworkers to recruit participants over the course of the survey.

4.4. Completeness of survey instruments of the basic program

The number and proportion of complete and useable data obtained by the specific survey instruments of the basic program, stratified by region, age group, and sex are shown in Table 3. Since the documentation on residential environment, the CAPI parents, and the CAPI child/adolescent as well as the S0 running water sample were required by the case definition, the completeness was 100 %. The most frequent reasons for failure to provide a morning urine sample for younger children were 'child uses nappies at night' or 'child wetted bed at night'. Furthermore, some infants simply refused to provide the sample. 97.8 % of parents filled out the SAQ 'Diseases and health problems suffered by the child or adolescent'. The proportion of useable SAQ was spread approximately evenly across all groups. 82.0 % of cases provided blood samples, whereas the share increased with age, from 67.0 % (3–5 years) to 92.2 % (14–17 years). Fewer girls (80.1 %) provided blood samples than boys (83.9 %). The S1 stagnation water sample was obtained correctly in 95.4 % of the cases. It was possible to measure noise levels outside children's bedroom windows in 92.6 % of the cases. Failures were generally less due to architectural features, for example, 'room had a

Table 3
Overview of complete and useable data (number and proportion) obtained by the specific survey instruments of the basic program of GerES V, stratified by region, age group and sex.

			West Germany	East Germany	3–5	6–10	11–13	14–17	Female	Male
Documentation of residential environment	N	2294	1595	699	424	752	543	575	1165	1129
	%	100	100	100	100	100	100	100	100	100
CAPI parents	N	2294	1595	699	424	752	543	575	1165	1129
	%	100	100	100	100	100	100	100	100	100
CAPI children/adolescents	N	1118	771	347			543	575	583	535
	%	100	100	100			100	100	100	100
Self-administered questionnaire on diseases and health problems suffered by the child/adolescent	N	2244	1559	685	418	738	523	565	1134	1110
	%	97.8	97.7	98.0	98.6	98.1	96.3	98.3	97.3	98.3
Morning urine sample incl. CAPI documentation	N	2259	1565	694	389	752	543	575	1143	1116
	%	98.5	98.1	99.3	91.8	100	100	100	98.1	98.9
Blood sample incl. CAPI documentation	N	1880	1327	553	284	593	473	530	933	947
	%	82.0	83.2	79.1	67.0	78.9	87.1	92.2	80.1	83.9
S0 running water sample incl. CAPI documentation	N	2294	1595	699	424	752	543	575	1165	1129
	%	100	100	100	100	100	100	100	100	100
S1 stagnation water sample incl. CAPI documentation	N	2189	1523	666	411	719	514	545	1113	1076
	%	95.4	95.5	95.3	97.0	95.6	94.7	94.8	95.5	95.3
Noise level measurement incl. CAPI documentation	N	2125	1484	641	389	706	502	528	1079	1046
	%	92.6	93.0	91.7	91.8	93.9	92.5	91.8	92.6	92.7
Ultra-fine particles measurement incl. CAPI-documentation	N	2235	1567	668	412	734	533	556	1135	1100
	%	97.4	98.2	95.6	97.2	97.6	98.2	96.7	97.4	97.4

For abbreviations see footnote of Table 1.

roof window', than defect measurement instruments. The proportion of valid noise measurements was spread approximately evenly across all groups, with values ranging between 91.7 % and 93.9 %. Measurements and documentation of the ultrafine particles were obtained successfully in 2235 cases (97.4 %). All requirements of the definition of case especially the completeness of KiGGS participation were verified repeatedly after merging KiGGS Wave 2 data and GerES V data. Few participants were excluded retrospectively from the sample sizes, because they did not meet the requirements of the definition of a case. Consequently, the sample sizes and response rates, described here, slightly differ from those showed in Cholmakow-Bodechtel et al. (2018). Since the information shown here, better describe the GerES V data set, it was applied for following data evaluations.

4.5. Completeness of survey instruments of the additional modules

Table S3 in the supplemental file shows the absolute and relative frequencies of completed participation in the additional modules by age group and sex. Participation rates in the individual modules correspond to those of the basic program: The participation of girls in the additional modules is slightly higher than that of boys, and participation is higher in the 6- to 10-year-old age group than in the other age groups. The target number of participants for the additional module "Indoor air" was 668. Measurements were obtained for 642 participants (96.1 %). The target for collecting 668 useable house dust samples was also almost achieved (96.5 %). Additional drinking water samples (cold and hot drinking water samples for analyzing organic pollutants) were to be taken for 176 participants. Useable samples could be taken from 99.4 % of homes (175 participants). 74 of the 80 planned measurements in the module "Particulate matter" were obtained (92.5 %).

5. Representativeness and weighting

The total population of Germany targeted by GerES V comprised approximately 11 million 3- to 17-year-old children and adolescents living in Germany from 2014 to 2017 who were listed in municipal population registries. Table 4 compares the stratification of the overall population (as of December 31, 2015) with that of the GerES V and KiGGS Wave 2 net samples. The composition of the KiGGS Wave 2 net sample also influences the GerES V net sample. In the following, numbers in brackets represent the difference of percent points (pp) between GerES V and the total population. Due to the sampling design, participants from former East Germany were overrepresented in KiGGS Wave 2 and GerES V (+15.1 pp) when compared to the overall population. Communities in East Germany were selected with higher probability in order to gain more precise data for this region separately (Hoffmann et al., 2018). Children and adolescents living in small communities with up to 50,000 inhabitants (+6.4 pp) and the 11-to13-year--old age group (+3.8 pp) are also overrepresented. Children and adolescents from municipalities with over 100,000 inhabitants (−5.1 pp) as well as those in the 14-to17-year-old age group (−3.7 pp) are underrepresented. Male participants are slightly underrepresented

Table 4
Overview of stratification of KiGGS Wave 2 and GerES V and net samples and the overall population (as of December 31, 2015).

	KiGGS Wave 2		GerES V		Proportion of total population	Difference between GerES V and total population
	N	%	N	%	%	% points
Total	**3567**	**100**	**2294**	**100**	**100**	
Region of residence						
West Germany	2338	65.6	1561	68.0	82.2	−14.2
East Germany	1111	31.1	663	28.9	13.8	+15.1
Berlin	118	3.3	70	3.1	4.0	−0.9
Size of municipality (number of inhabitants)						
< 50,000	1083	30.4	706	30.8	24.42	+6.4
50,000 to < 100,000	358	10.0	204	8.9	10.22	−1.3
> 100,000	2126	59.6	1384	60.3	65.42	−5.1
Age (in years)						
3-5	653	18.3	424	18.5	19.1	−0.6
6-10	1143	32.0	752	32.8	32.1	+0.7
11-13	816	22.9	543	23.7	19.9	+3.8
14-17	955	26.8	575	25.1	28.8	−3.7
Sex						
male	1766	49.5	1129	49.2	51.5	−2.3
female	1801	50.5	1165	50.8	48.5	+2.3

For abbreviations see footnote of Table 1.

(−2.3 pp) in comparison to the overall population.

The RKI analyzed and reported the distribution of participants in the KiGGS Wave 2 sample across education level groups of participants' parents (Hoffmann et al., 2018). The values are based on the highest level of education achieved according to the Comparative Analysis of Social Mobility in Industrial Nations (CASIM). Groups with lower education are under- and groups with higher education are slightly over-represented in the net sample of KiGGS Wave 2. As expected, such a distribution is also observed in the GerES V net sample. For reducing the effects of the above mentioned and other discrepancies of distribution between the sample and the overall population, the RKI calculated weighting factors for KiGGS Wave 2 and GerES V (Hoffmann et al., 2018; Mauz et al., 2017). These weighting factors are used in statistical data analyses for rebalancing frequencies in terms of age, sex, federal state and German citizenship (yes/no). Furthermore, the distribution of the education levels of participants' parents according to the CASMIN categorization were adjusted to the respective education level distribution of the heads of household in Microcensus data (Hoffmann et al., 2018).

A set of weighting variables were calculated for the total GerES V sample for the module subsamples as well as for subsamples designated for specific chemical analysis:

- Subsample I: morning urine to measure metabolites of plasticizers and polycyclic aromatic hydrocarbons, phenols, and 2-Mercaptobenzothiazole (2-MBT)
- Subsample II: morning urine to measure chlorophenols and selenium species
- Subsample III: whole blood to measure metals
- Subsample IV: blood plasma to measure polychlorinated biphenyls (PCB), organochlorine pesticides, and per- and polyfluoroalkyl substances (PFAS)
- Subsample V: indoor air samples to measure volatile organic compounds (VOC) and aldehydes
- Subsample VI: house dust to measure flame retardants and plasticizers
- Subsample VII: blood plasma for later chemical analyses for additional pollutants

6. Discussion and conclusions

The total response rate of 75.7 % in GerES V is quite similar to the response rate achieved more than ten years ago in GerES IV (77.3 %) (Schulz et al., 2012). Morton et al. (2005) compared response rates of studies in analytic epidemiological research, and identified response rates ranging from 41 % to 88 % with a median of 74 %. Relevant literature indicates that response rates in population-based cross-sectional studies have generally fallen in recent years (Galea and Tracy, 2007; Latza et al., 2004; Mindell et al., 2015) and that willingness to participate is influenced by the kinds of questions addressed by research (Groves et al., 2004). Against this background, the response rate realized in GerES V can be considered satisfactory and is in line with the results of other population-based studies such as NHANES (response rates of 63.8%–65.2 % in the examined sample of 1- to 19-year-olds in 2015–2016) (Centers for Disease Control and Prevention, 2020). Nonetheless, the response rates for the two surveys KiGGS Wave 2 and GerES V leave room for improvement: Future joint surveys should continue to emphasize ongoing quality control during participant recruitment and data collection phases to ensure that both processes are optimized over the course of the survey. For instance, families were more willing to participate in the study if the focus was more on personal benefit, e.g. receiving an individual reporting of the study results, which was also confirmed by the evaluation of the satisfaction questionnaire (Moldenhauer et al., 2018).

The composition of the net sample of GerES V showed only minor deviations from the composition of the target population, which could be compensated by weighting the collected data. GerES V results are therefore representative for the entire population of 3- to 17-year-olds living in Germany and allow us to make qualified statements regarding the environmental pollutants affecting children and adolescents in general, as well as stratified by age group, sex, and region. Study results on pollutants measured in morning urine samples have been published regarding plasticizers (Schwedler et al., 2020a, 2020b 2020c), parabens (Murawski et al., 2020f), polycyclic aromatic hydrocarbons (PAH) (Murawski et al., 2020a), pyrrolidones (Schmied-Tobies et al., 2021a), bisphenol A and benzophenones (Tschersich et al., 2021), chlorophenols (Schmied-Tobies et al., 2021b), 2-MBT (Murawski et al., 2020e), CIT/MIT (Murawski et al., 2020c), lysmeral (Murawski et al., 2020b), benzene and acrylamide (Schwedler et al., 2021), TOTM, BHT and 4-MBC (Murawski et al., 2020d), whereas the publication of cotinine, glyphosate and metals like cadmium, mercury are planned. Additionally, first results have been released on traffic noise (Tobollik et al., 2019) and ultrafine particles (Ohlwein et al., 2019) as well as PFAS (Duffek et al., 2020), PCB and organochlorine pesticides (OCP) (Bandow et al., 2020) in the blood samples. Interview data on walking time to public green spaces has also been published (Rehling et al., 2020). In the future further data evaluations are intended for metals and organic substances in drinking water, plasticizers and flame retardants in house dust, VOC, formaldehyde and other carbonyls in indoor air, ultrafine particles and PAH in particulate matter and further interview/questionnaire data. With the completion of GerES V, a comprehensive body of quality-assured monitoring data is now available to support policies such as REACH, the regulation of the European Union for improving the protection of human health and the environment from the risks due to chemicals. An important result of GerES V are reference values representing the exposure of the young generation in Germany to various environmental pollutants. These values serve as a useful benchmark for epidemiological analyses of populations facing particular environmental hazards and eliminate the need to include large control groups. Moreover, reference values are important for identifying individual risks and monitoring the effectiveness of preventative measures undertaken to limit human exposures at the population level. Data and experiences from GerES V substantially contribute to the European Joint Programme HBM4EU for further coordinating and advancing human biomonitoring in Europe (Ganzleben et al., 2017).

Declaration of competing interest

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

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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.2021.113821.

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The U.S. national biomonitoring network – Enhancing capability and capacity to assess human chemical exposures

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ABSTRACT

Background: With the increased use of biomonitoring in public health, biomonitoring networks are forming worldwide. The National Biomonitoring Network (NBN), created in 2018, is an interconnected system of U.S. government laboratories in collaboration with public health partners, to advance human biomonitoring science and practice. The NBN aims to harmonize biomonitoring data for use in routine public health practice.
Methods: The NBN has taken a systems approach to provide high-quality biomonitoring data by establishing quality standards, mentoring nascent programs, and enhancing analytical capability and capacity through technical assistance. Guided by a multi-disciplinary Network Steering Committee (NSC), the NBN has developed an organizational framework, membership criteria, and guidance practices related to study design, quality management and analytical measurements. To facilitate the production of these resources, the NSC established interdisciplinary workgroups of subject matter experts.
Results: To date, 20 state public health laboratories have joined the NBN. Differences in land-use practices, state and local laws and availability of resources resulted in considerable variability in the design and approach of NBN member biomonitoring programs. By contributing technical guidance, technical training, examples and templates for analytical and epidemiological practices and opportunities for collaboration and interaction, the NBN addressed some of these challenges. Important challenges remaining are to define minimum data variables for laboratory measurements, demographic and exposure information, and to identify an appropriate national repository for biomonitoring data.
Conclusion: The current NBN membership has greatly benefited from the resources, collaboration and engagement with other state and federal scientists. The NBN hopes to expand membership and increase interaction with biomonitoring networks internationally. While the objectives of biomonitoring networks around the world may differ, understanding their structures, advantages and limitations inform the NBN and provide opportunity for cross-network collaboration.

1. Introduction

People are exposed to chemicals daily through food, their indoor and outdoor environments, work, lifestyle, and recreational activities. Measurement of chemical toxicants in the environment provides valuable information regarding potential sources and pathways of exposure and the external dose to which individuals may be exposed. These measures estimate the absorption of a contaminant by considering its chemical and physical properties, routes of exposure and uptake kinetics (National Research Council, 2006). By contrast, human biomonitoring, the measurement of chemicals and or their metabolites in biological specimens, quantifies the internal dose of a contaminant by integrating exposure from all sources and routes resulting in an accurate assessment of individual body burden (Sexton et al., 2004). Biomarkers of exposure (e.g. blood lead levels) complement environmental monitoring measures (e.g. lead in drinking water) to provide the most accurate exposure estimates when assessing human health risks from exposures to environmental chemicals. Biomonitoring data are also used by the US Environmental Protection Agency, the federal agency responsible for setting regulatory standards in water, air and environmental media, in

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the risk assessment process. By choosing the appropriate biomarkers of exposure, investigators may assess acute (short-term or high dose) or chronic (long-term or persistent) exposure.

Biomonitoring data are increasingly used in routine public health practice to investigate general and specific populations' exposures, communities' concerns (Daly et al., 2018), guide emergency response activities (Weibrecht et al., 2012), inform public health decision-making, and evaluate the efficacy of public health interventions (Abrams et al., 2006). In the United States, the National Center for Environmental Health (NCEH) at the Centers for Disease Control and Prevention (CDC) through the National Health and Nutrition Examination Survey (NHANES) provides nationally representative estimates of human exposure to select environmental chemicals (Calafat, 2012). NHANES data inform the risk assessment process and protect environmental health. However, within the USA, states vary significantly in demographics, industry (e.g. agricultural vs. manufacturing), geography, exposure sources, and regulation, potentially leading to considerable environmental health disparities. Therefore, state or local population-based surveillance is necessary for public health officials to understand the unique risks faced by their residents and evaluate the effectiveness of mitigation measures.

So far, a relatively small number of U.S. public health laboratories have developed analytical capability and capacity for biomonitoring on occasional basis to investigate environmental health concerns in their respective jurisdictions. These targeted investigations conducted in collaboration with environmental epidemiologists and health officials in their states had typically focused on exposed or affected populations and were time and resource limited (Barton et al., 2020; Daly et al., 2018; Gibson et al., 2020; Graber et al., 2019; Landsteiner et al., 2014; Nair et al., 2021; Rogatsky et al., 2017).

Recognizing the need for biomonitoring capacity at the state level, in 2001, NCEH funded 25 states to design biomonitoring plans (CDC 2001), and select states to develop state-based capacity (CDC 2003, CDC 2009, CDC 2014, CDC 2019). Concurrent with these activities, CDC transferred to the states advanced analytical technology and capacity for high-throughput testing for chemical threat agents in clinical specimens through the Laboratory Response Network for Chemical Threat Agents (LRN-C) (CDC 2002–2020) (https://emergency.cdc.gov/lrn/chemical.aspdone. Emergency response activities provided the opportunity to engage new partners in the clinical and medical toxicology communities directly. Leveraging these analytical capabilities and enhanced collaborations, additional state public health laboratories and their public health partners became engaged in biomonitoring efforts and explored the implementation of population-based environmental health surveillance.

Understanding the increased prominence of biomonitoring for exposure and risk assessment, the Association of Public Health Laboratories (APHL) in collaboration with CDC developed two successive 5-year plans beginning in 2009 which culminated with the development of the National Biomonitoring Network (NBN) in 2018. The NBN goals are to promote quality management directives and opportunities for connection and collaboration among public health programs to produce high quality biomonitoring data for use in public health practice (APHL 2009; APHL 2012; APHL 2014; APHL 2019a; Latshaw et al., 2017). This paper aims to provide an overview of the objectives, structure, governance and implementation of the National Biomonitoring Network.

2. Methods

The implementation of the NBN was modeled upon the structure and development of other laboratory networks (Kirk et al., 2010; Astes et al., 2010, CDC LRN-C 2002; Villanueva et al., 2019). It is a performance based network that affords jurisdictional flexibility to the state biomonitoring programs in designing and operationalizing their work, allowing for programs that meet the current needs for community investigation, environmental health surveillance and evaluation of public health policy and intervention. This approach results in several challenges such as the need to harmonize laboratory measurements, nomenclature, questionnaire development and study design. The NBN provides technical assistance and guidance towards these objectives. Since resource constraints limit the scope of many state programs, APHL also works to educate partners and legislators about the value and uses of biomonitoring data in public health practice.

Recognizing that human biomonitoring is a multi-disciplinary endeavor, the NBN is guided by a Network Steering Committee (NSC) of subject matter experts in analytical chemistry, epidemiology, exposure science, public health, risk communication, and toxicology. These experts represent several CDC programs (Division of Laboratory Sciences, Environmental Public Health Tracking Program, the National Institute of Occupational Safety and Health), the Agency for Toxic Substances and Disease Registry, the Environmental Protection Agency, the National Institute of Environmental Health Sciences, the National Institute of Standards and Technology, and multiple state programs (currently Massachusetts, Minnesota, New Jersey, New York, Texas and Wisconsin; New Hampshire and Arizona were former representatives). NSC members, who are selected based on their subject matter expertise, commit to at least a two-year term, extended as needed to ensure continued engagement. Additional members can be added as specific expertise needs are identified.

In the few years since its inception, the NSC has defined the governance structure, established a tiered network format, and developed a five-year timeline and implementation plan. The NSC has also identified multiple areas requiring focused attention and authorized the establishment of topic-specific work groups to research and draft recommendations that are presented to the NSC for consideration. This is an iterative process designed to maximize stakeholder input and perspective.

- Governance – the NSC is co-chaired by subject matter experts representing the Division of Laboratory Sciences at CDC and a state public health laboratory with considerable biomonitoring experience. The NSC works under the auspices of the APHL Environmental Health Committee and is ultimately accountable to the APHL Board of Directors. The NSC, which meets monthly via teleconference and at least annually in-person, is empowered to establish and dissolve ad hoc work groups and to provide recommendations related to specific topics (e.g., study design and membership.
- Network structure – the NBN has tiered architecture based on the public health laboratory capabilities, demonstration of biomonitoring methods proficiency and experience at the time of application; there is flexibility and opportunity to change tiers as appropriate (Fig. 1). Membership is currently limited to government laboratories working within the public health system. Laboratories reapply for membership in the NBN every three years, at which time their capabilities and proficiencies are reviewed, as is the Tier designation. Applications are reviewed by a panel comprised of one representative each from the NBN Steering Committee, CDC and APHL. The NSC may consider expansion of the NBN membership to include non-government laboratories and/or non-laboratory partners in subsequent years.
- Engagement and scientific exchange opportunities – the NBN provides frequent and regular engagement opportunities for member laboratories to share experiences, successes and challenges via an online electronic platform and quarterly conference calls. Every two years, the network convenes the National Biomonitoring Meeting which affords the opportunity to share progress on analytical methodology, current biomonitoring investigations and a forum for advanced technical training.
- Cross-network collaboration- The NBN works collaboratively and learns from international biomonitoring programs through joint presentations at scientific conferences, analytical performance

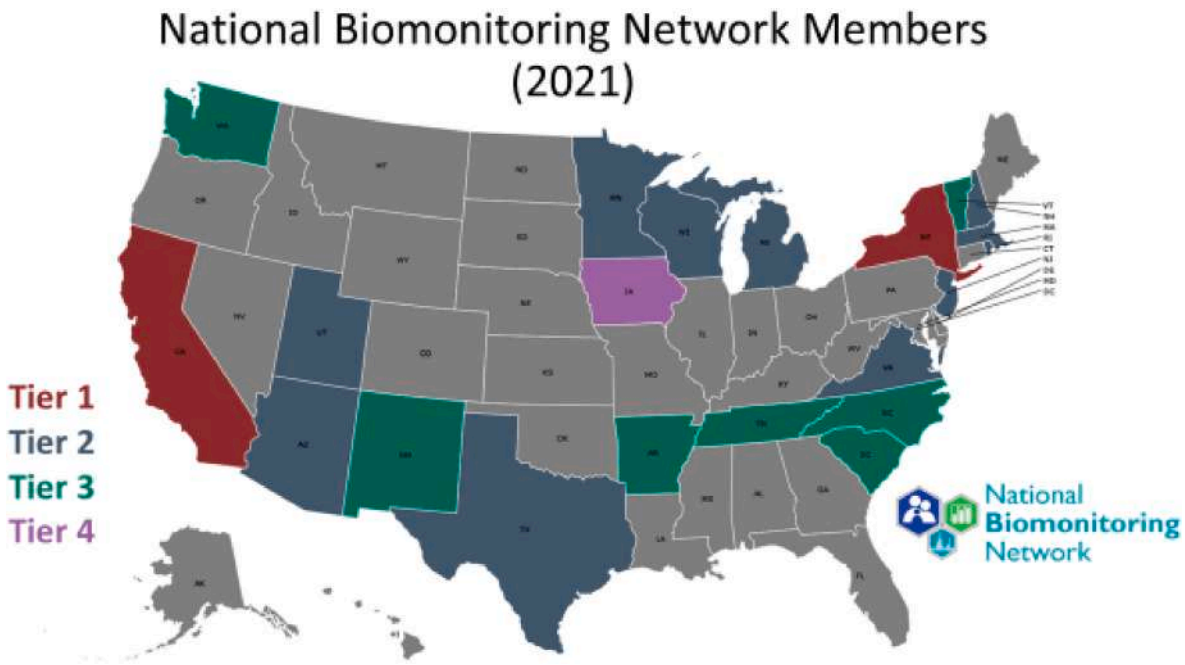

Fig. 1. National biomonitoring network member laboratories.
Tier 1
• Laboratories engaged in biomonitoring activities related to statistically representative population based surveillance, targeted investigations and emergency response.
• Demonstrated successful participation in an established quality assessment program.
• A well-established biomonitoring team integrated within the state public health system
Tier 2
• Laboratories engaged in biomonitoring activities related to targeted and emergency response.
• Demonstrated successful participation in an established quality assessment program.
• A well-established biomonitoring team integrated within the state public health system.
Tier 3
• Laboratories with biomonitoring capabilities and infrastructure but not actively conducting biomonitoring.
• Established implementation plan which includes a biomonitoring team integrated within the state public health system and a project timeline.
Tier 4
• Laboratories considering development of biomonitoring capabilities.

exercises and participation in the newly formed International Biomonitoring Network.

3. Results and discussion

The NBN aims to develop and enhance biomonitoring science through the sharing of quality management practices and tools, technical assistance and training, and peer mentorship. The performance-based network allows for innovation and improvement in recruitment practices, questionnaire development, analytical technology, data analysis and communication. A network toolkit includes resources such as accreditation guidance, method validation templates, study participant recruitment strategies, human subjects review guidelines, examples of questions to be asked to study participants, data analysis practices, and model study participant reports. The toolkit can assist members through the pre-analytical, analytical, and post-analytical phases of a biomonitoring program. Updated technical guidance for laboratory biomonitoring programs, training videos on specimen collection and results reporting and CDC's biomonitoring specimen collection guidance are available to NBN members in the network resource center on the APHL website (www.aphl.org). Defined performance metrics are assessed annually to evaluate the efficacy and impact of the technical resources offered.

As of June 2021, 20 laboratories are NBN members (Fig. 1). The network is currently exploring opportunities for greater network interaction through mentorship and twinning relationships. More experienced laboratories could volunteer to mentor laboratories who would benefit from that experience while honing their own training and technology transfer skills. Laboratory twinning is a concept that has been used internationally for capacity building, networking and to bring communities together (Mills et al., 2019). The NSC is considering a twinning program that would enable members to collaborate in a mutually beneficial way.

Harmonization of human biomonitoring data is exceptionally challenging given differences in individual program design, purpose, and approach (https://www.aphl.org/aboutAPHL/publications/Documents/EH-2020-NBN-Harmonization.pdf). Other programs domestically and internationally strive to harmonize biomonitoring measures by creating analytical centers of specialized excellence (Balshaw et al., 2017; Haines et al., 2017) or standardizing biomarkers and methodology (Hond et al., 2015; Schwendler et al., 2017). These strategies for data harmonization are customized to meet the objectives of the individual biomonitoring programs: surveillance vs targeted investigations, biomarkers common to multiple programs vs biomarkers specific to select jurisdictions, targeted assays vs non-targeted screening.

Data harmonization efforts are dependent upon rigorous quality management of all phases (pre-analytical, analytical, and post-analytical) of testing and data analysis. The NBN membership requires demonstrated capability through documented method validation, demonstration of technical competency, independent certification or accreditation (e.g., Clinical Laboratory Improvement Amendments (CLIA), College of American Pathologists (CAP), and International

Organization for Standardization (ISO)), and successful participation in external quality assessment (EQA) programs at the concentrations expected in the target populations. EQA programs, such as those administered by the Centre de Toxicologie du Québec (CTQ, https://www.inspq.qc.ca/en/ctq/eqas) and the University of Erlangen-Nuremberg (http://www.g-equas.de/default.htm), allow for comparison of a laboratory's testing to a peer group of laboratories or a reference laboratory to assess method accuracy and estimate inter-laboratory bias. As novel analytes of concern emerge, there may be a lag time in the development and implementation of proficiency panels for these biomarkers. As an interim measure, network laboratories may consider alternate ways to demonstrate analytical proficiency such as comparing results obtained from the analysis of the same sample by different laboratories (APHL 2019b).

A significant challenge ahead for the nascent National Biomonitoring Network is to identify a national centralized data repository for state biomonitoring data. Complexities include establishing data standards, incorporating data from known exposed populations as well as population-based surveillance values, defining data access protocols and identifying resources to accomplish these tasks. The NBN began by evaluating existing data platforms such as CDC Environmental Public Health Tracking Network (Kearney et al., 2015), NIEHS Children's Health Exposure Analysis Resource (Balshaw et al., 2017) and Human Health Exposure Analysis Resource (https://www.niehs.nih.gov/research/supported/exposure/hhear/index.cfm), and the APHL Informatics Messaging Services (www.aimsplatform.com) to assess their suitability, limitations, and willingness to accept biomonitoring data. The assessment identified system gaps and resource requirements for implementation. A small pilot effort is planned to assess the practicality of a data lake, a storage repository where vast amounts of raw data are held in its native format until it is needed, for state biomonitoring results which integrate with available resources for data visualization, ideally producing a dashboard similar to the HBM4EU platform (https://www.hbm4eu.eu/eu-hbm-dashboard/).

The establishment and implementation of a National Biomonitoring Network are challenging and ambitious activities that are the culmination of work by many dedicated public health scientists and partners working collaboratively. As with any process that develops organically, from the ground up, diverse approaches and solutions exist. Vast differences in resources, staffing and support for biomonitoring projects have contributed to the diversity of practices in the implementation of biomonitoring programs across the states. By encouraging and facilitating discussions across professional disciplines and among states conducting biomonitoring studies, the NBN has developed tools for chemists, epidemiologists, toxicologists, and risk communication specialists to design biomonitoring studies and programs that are more comparable to one another. Broad stakeholder engagement that contributed diverse expertise and perspective was critical in the crafting of these tools. One of the most important outcomes of the interactions among NBN members has been the development of trusted relationships and mutual respect, the cornerstone of a strong network.

Despite the progress made, work remains to be done. For example, the development of an NBN centralized national repository for state biomonitoring data will be a long-term effort requiring considerable allocation of time and resources. Also, besides strengthening relationships within the NBN, learning from, and collaborating with international biomonitoring networks can further shape the implementation and success of the NBN system. Additionally, strategies for the sustainability and continued growth of the network need to be assessed and a strategic plan developed.

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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.2021.113828.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the CDC. Use of trade names is for identification only and does not imply endorsement by the CDC, the Public Health Service, or the US Department of Health and Human Services.

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