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Arsenic exposure alters the expression of genes related to metabolic diseases in differentiated adipocytes and in newborns and children

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ABSTRACT

The mechanisms underlying the association between prenatal arsenic exposure and the development of metabolic diseases remain unclear. Aberrant adipogenesis and adipokine production are associated with increased risk for the development of metabolic diseases in susceptible populations. Generation of mature adipocytes is tightly regulated by the expression of genes encoding: peroxisome proliferator-activated receptor γ (*PPARG*), fatty acid-binding protein (*FABP4*), and glucose transporter-4 (*SLC2A4*), and adipokines such as leptin (*LEP*) and adiponectin (*ADIPOQ*). This study aimed to investigate the expression of these genes, which are associated with the pathogenesis of metabolic diseases in newborns and children exposed to arsenic *in utero*. A high arsenic exposed group showed significantly decreased *PPARG* and *FABP4* expression in cord blood samples from newborns and in saliva samples from children. By contrast, the expression of the *SLC2A4* and *ADIPOQ* mRNA was significantly decreased in high-arsenic exposed children. Furthermore, the levels of toenail arsenic were negatively correlated with the salivary mRNA expression levels of *PPARG* ($r = -0.412$, $p < 0.01$), *aP2* ($r = -0.329$, $p < 0.05$), and *SLC2A4* ($r = -0.528$, $p < 0.01$). *In vitro* studies utilizing umbilical cord derived mesenchymal stem cells (UC-MSCs) as a surrogate for fetal MSCs showed that arsenite treatment (0.5 μM and 1 μM) significantly impaired adipogenic differentiation in a concentration dependent manner. Such impairment may be related to a significant decrease in the expression of: *PPARγ*, *FABP4,* and *SLC2A4* observed at 1 μM arsenite. Arsenite treatment also promoted inflammation through a significant increase in the mRNA expression levels of the pro-inflammatory adipokine, *LEP*, and the inflammatory cytokines: *CXCL6, IL-1β*, and *CXCL8*. Collectively, our results suggests that such alterations may be a consequence of the effects of arsenic exposure on fetal MSCs eventually leading to impaired adipogenic differentiation and the promotion of inflammation, both of which contribute to the development of metabolic diseases later in life.

1. Introduction

Prenatal and early childhood exposure to arsenic has been associated with a wide range of chronic diseases, including cancer and metabolic diseases, later in adult life (Tokar et al., 2011; Hawkesworth et al., 2013). Large population-based prospective studies of children in rural Bangladesh (Hawkesworth et al., 2013) and young adults in Chile (Yuan et al., 2007) showed that *in utero* and early-life exposure to arsenic via contaminated water was associated with increased risk of cardiovascular diseases. In addition, studies from Taiwan, Bangladesh, Mexico, and the United States demonstrated that chronic exposure to arsenic altered several cardiometabolic parameters resulting in increased risk of developing metabolic diseases during adulthood (Maull et al., 2012; Grau-Perez et al., 2017). At present, the mechanisms underlying the pathogenesis of metabolic diseases in relation to prenatal exposure to arsenic exposure have not been characterized and are poorly understood.

Prenatal exposure to toxicants, especially arsenic, could also be a factor contributing to the early-life origin of metabolic diseases possibly by altering fetal developmental processes that regulate the homeostasis of adipose tissue (Wang et al., 2014). During fetal development, mature adipocytes are differentiated from mesenchymal stem cells (MSCs)

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(Shao et al., 2017). Accumulated data demonstrated that arsenic exposure suppressed adipogenesis of both human bone marrow MSCs (Yadav et al., 2013) and mouse adipose tissue MSCs (Shearer et al., 2017). This evidence suggests that MSCs could be another target of arsenic toxicity. The process of adipogenesis involves sequential changes in the expression of specific genes that determine the adipocyte-specific phenotype. Several transcription factors that regulate adipocyte differentiation have been identified. Peroxisome proliferator-activated receptor γ (PPARγ), a master regulator, is necessary to induce adipogenic differentiation by inducing the expression of adipocyte-specific genes including, but not limited to, fatty acid-binding protein (aP2) and glucose transporter-4 (GLUT4).

Accumulated evidence from both preclinical and clinical studies indicated that adipose tissue inflammation is initiated and sustained over time by dysfunctional adipocytes that secrete inflammatory adipokines. Thus, an imbalance in the adipokine profile mediated by dysfunctional adipocytes that leads to chronic inflammation is the major etiologic component of the pathogenesis of metabolic diseases. Among different adipokines, the levels of Leptin (a pro-inflammatory adipokine) and Adiponectin (an anti-inflammatory adipokine) are widely used for predicting the incidence and severity of metabolic diseases (Falahi et al., 2015; Liu et al., 2020). In addition, hypersecretion of inflammatory cytokines by dysfunctional adipocytes, such as the interleukin (IL): IL-6, -8, and -1β, leads to insulin resistance, which could facilitate both the onset and progression of metabolic and vascular diseases (Veljić et al., 2018). When compared to non-obese subjects, a significant decrease in the expression of genes encoding Adiponectin (*ADIPOQ*), along with the increased expression of genes encoding Leptin (*LEP*) and inflammatory genes such as IL-6 (*CXCL6*) and IL-1β (*IL1β*), was observed in visceral adipose tissue from obese subjects (Coín-Aragüez et al., 2018). Analysis of the production and release of IL-8 from human adipose tissue suggests that the increased expression of the gene encoding IL-8 (*CXCL8*) may account for the observed increased plasma IL-8 levels in obese subjects (Bruun et al., 2004). Furthermore, increased levels of plasma IL-6 and IL-8 may contribute to the activation of monocytes, another key effector cell which initiates the formation of atherosclerotic plaques leading to the development of atherosclerosis (Reddy et al., 2019).

Arsenic toxicity is very complex and affects multiple organs throughout the body. One possible mechanism of arsenic toxicity involves alterations in DNA methylation profiles leading to aberrant gene expression (Bailey et al., 2016). Our previous study showed that both newborns and young children who were continuously exposed to arsenic starting *in utero* showed increased expression of several inflammatory genes (*COX2*, *EGR1*, and *SOCS3*) resulting from significant hypomethylation in their promoter regions (Phookphan et al., 2017). These effects may be linked to the mechanisms of arsenic-induced chronic inflammation later in life. Chronic inflammation is recognized as another important mechanism involved in pathogenesis of several chronic diseases. Thus, increased expression of genes related to inflammation mediated by arsenic exposure could contribute to the development of metabolic diseases. In addition, alterations in the expression levels of genes encoding PPARγ (*PPARG*), aP2 (*FABP4*), GLUT4 (*SLC2A4*), Leptin (*LEP*), Adiponectin (*ADIPOQ*), IL-6 (*CXCL6*), IL-8 (*CXCL8*), and IL-1β (*IL1β*) are known to play a crucial role in the pathogenesis of metabolic diseases (Veljić et al., 2018; Trojnar et al., 2019; Al-Hamodi et al., 2014). It is, however, unclear whether arsenic exposure, starting *in utero* and continuing throughout childhood, can alter the expression of these genes.

This study aims to examine the effect of arsenic exposure *in utero* on the expression of genes associated with the pathogenesis of metabolic diseases (*PPARG*, *FABP4*, *SLC2A4*, *LEP*, and *ADIPOQ*) in the newborns and children from our birth cohort. These results were then confirmed by *in vitro* studies using an umbilical cord derived MSC cells (UC-MSCs) as a surrogate for fetal stem cells. The direct effect of arsenite treatment on adipogenic differentiation of fetal MSCs was assessed. Further, the expression pattern of the aforementioned genes that are known to be associated with metabolic diseases was determined in differentiated adipocytes along with the expression levels of inflammatory related genes (*CXCL6*, *CXCL8*, and *IL1β*).

2. Materials and methods

2.1. Study subjects and sample collection

This study utilized 22 cord blood specimens from a birth cohort comprised of 55 pregnant women and their newborns from known arsenic contaminated areas in Ron Pibul district in the southern Thailand (Intarasunanont et al., 2012). The levels of arsenic in both drinking and non-drinking water from the study areas were routinely monitored. The geometric mean and median of household water arsenic concentrations in the exposed population from delivery until saliva collection were 7.07 and 1.45 μg/L (range, 0.43–76.31 μg/L), respectively, for drinking and 80.66 and 14.44 μg/L (range, 1.25–1944 μg/L), respectively, for non-drinking water.

For the prenatal cohort, pregnant women were approached in the clinic waiting rooms at the time of their first prenatal visit at the participating hospital. Almost every pregnant woman interviewed (approximately 90%) agreed to participate. Pregnant women with the following criteria were excluded from the study: 1) pregnant women who intended to leave the studied areas within 1 year of initial interview, 2) pregnant women with prior history of premature labor, and 3) pregnant women who had undergone labor induction or cesarean section. In children cohort at follow-up, parents of eligible children got a packet with a detailed explanation of the project as well as questionnaire and consent form. Approximately 85% of eligible children participated. Children who had not continuously lived in the studied areas since at birth were excluded from the study.

Cord blood samples collected at the time of delivery were aliquoted and stored in an RNA preserving reagent as previously described until analysis was performed (Fry et al., 2007). For a follow-up study, saliva specimens from 55 children aged 6–9 years from the same newborn cohort that had been continuously exposed to arsenic beginning *in utero* were utilized. Saliva samples were aliquoted and stored in an RNA preserving reagent as previously described until analysis was performed (Hinhumpatch et al., 2013). All of the subjects' parents were requested to complete a questionnaire to obtain personal information and allow identification of potential confounding factors. Samples were analyzed for the expression levels of genes related to metabolic diseases including: *PPARG*, *FABP4*, *SLC2A4*, *LEP*, and *ADIPOQ*. This study was conducted according to the recommendations of the Declaration of Helsinki (World Medical Association, 1989) for international health research. All the children's parents gave written consent for participation in this study.

2.2. Measurement of arsenic concentration

2.2.1. Analysis of total arsenic concentration

Determination of arsenic concentration in nails, saliva and cord blood were previously described (Hinhumpatch et al., 2013; Intarasunanont et al., 2012). Briefly, all nail samples were washed by sonication with acetone and 1% (v/v) TritonX-100 for 10 min then digested in Teflon vessels using a microwave oven (Milestone ETHOS) before analysis for total arsenic concentrations by inductively coupled plasma mass spectrometry (ICP-MS). Saliva and cord blood samples were digested with 1 ml suprapure nitric acid using a microwave oven. The digested samples were analyzed for total arsenic concentration by ICP-MS.

2.2.2. Analysis of arsenic speciation

Arsenic speciation was determined according to a modification of a previously described method (Hinhumpatch et al., 2013). Briefly, samples were determined by diluting sample with deionized water (3-fold dilution), then centrifuging at 1200 g for 10 min and the supernatant

filtering through a 0.45 μm syringe filter. The filtered samples were subjected to analysis with HPLC/ICP-MS.

2.3. Cell culture and sodium arsenite treatment

UC-MSCs were obtained from American Type Culture Collection (ATCC, USA). The cells were maintained according to the manufacturer's recommendations in complete medium [α-minimum essential medium (α-MEM, Gibco, USA) supplemented with 2.2 g $NaHCO_3$, 10% fetal bovine serum (Millipor, USA), L-glutamine (Gibco), and 100 U Penicillin/Streptomycin (Gibco) at 37 °C with 5% CO_2. For treatment with sodium arsenite, UC-MSCs were seeded overnight in appropriate culture vessels at 5000 cells/cm^2 to reach approximately 80% confluence before starting the experiments. UC-MSCs were treated with arsenite at various concentrations (0, 1, 5, and 10 μM) for periods ranging from 1 to 15 days. Solutions of arsenite at different concentrations were freshly prepared in sterile distilled water and dissolved thoroughly before treatment.

To study the effect of arsenite treatment on adipogenic differentiation of UC-MSCs, UC-MSCs (5000 cells/cm^2) were seeded in T25 tissue culture flasks and placed at 37 °C in a 5% CO_2 humidified incubator overnight. UC-MSCs were then cultured in adipogenic differentiation media (α-MEM, 10% FBS, 0.5 μM dexamethasone, 0.5 μM isobutylmethyl-xanthine and 50 μM indomethacin) in combination with treatment with arsenite at various concentrations (0, 0.5 and 1 μM). The differentiation medium containing arsenite was changed every 3 days until the UC-MSCs differentiated into mature adipocytes (15 days). Cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay (Sigma-Aldrich, USA). No significant decrease in cell viability (>95% viability) was observed in cells treated with 0.5 and 1 μM arsenite for 15 days compared to the control (untreated adipocytes).

2.4. RNA extraction

Total RNA was isolated from UC-MSCs using RNeasy® mini kit (Qiagen, Germany) according to the manufacturer's protocol. RNA isolation from cord blood or saliva samples was carried out using the PAXgene Blood RNA kit (Qiagen) or Qiagen RNeasy Micro Kit (Qiagen), respectively. The amount of total RNA was measured using an ND-1000 spectrophotometer, Nanodrop (NanoDropTechnologies, Inc., USA).

2.5. Quantitative real time PCR (qRT-PCR)

Total RNA (100 ng) was reverse-transcribed to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) according to the manufacturer's instruction with Eppendorf Mastercycler pro PCR system (Eppendorf, USA). The expression of genes related to adipogenesis, adipokines, and inflammation was performed using THUNDERBIRD® SYBR®qPCR mix (TOYOBO, Japan) or ABI-Step One Plus real time RT-PCR (Applied Biosystems). The primer sequences are shown as follows from 5′ to 3′ end:

PPARG (F: TCAGGTTTGGGCGGATGC, R: TCAGCGGGAAGGACTTTATGTATG), *FABP4* (F: ATGGGATGGAAAATCAACCA, R: GTGGAAGTGACGCCTTTCAT), *SLC2A4* (F: ATCCTTGGATTCCTCATTGG, R: CAGGTGAGTGGGAGCAATCT), *LEP* (F: GAACCCTGTGCGGATTCTTGT, R: TCCATCTTGGATAAGGTCAGGAT), *ADIPOQ* (F: CATGATCAGGAAACCACGACT), R: TGAATGCTGAGCGGTAT), *CXCL8* (F: TTTTGCCAAGGAGTGCTAAAGA, R: AACCCTCTGCACCCAGTTTTC) and *PPIA* (F: TCCTGGCATCTTGTCCATG, R: CCATCCAACCACTCAGTCTTG). The thermo cycling condition was 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s, annealing at 60 °C for 45 s and finally at 60 °C for 1 min. Each sample was run in duplicate and normalized to the endogenous reference gene, *PPIA*. The expression of *CXCL6* (Hs00985639_m1) and *IL1β* (Hs00174097_m1) was determined in relation to the expression of *GAPDH* (Hs03929097_ml) by using Taqman Gene Expression Assay (Applied Biosystems). qRT-PCR was performed using ABI-Step One Plus real time RT-PCR (Applied Biosystems) and the thermo cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and annealing at 60 °C for 1 min and finally at 60 °C for 1 min. Each sample was run in duplicate and normalized to an endogenous reference gene, *GADPH*. Relative gene expression was calculated using the comparative CT method.

Cord blood RNA (1000 ng) or salivary RNA (700 ng) was reverse transcribed to cDNA with the qScript cDNA SuperMix kit (QuantaBio, USA) or High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. The relative expression of genes related to adipogenesis, adipokines, and inflammation was assessed as mentioned earlier.

2.6. Measurement of PPPAR-γ, aP2, Leptin, and Adiponectin concentration

The protein levels of PPPAR-γ, aP2, Leptin and Adiponectin were measured in cord blood serum, saliva, and cell supernatants using ELISA kits; Human PPPAR-γ ELISA Kit (CUSABIO, USA), Human FABP4/A-FABP (Quantikine R&D system, USA), Human Leptin (Quantikine R&D system), and Human Adiponectin/Acrp30 according to the manufacturer's guidelines. The absorbance was measured at 450 nm with a background subtraction at 570 nm using a SpectraMax®i3X microplate reader (Molecular Devices, USA). Standard curve and the protein level were analyzed using SolfMax Pro version 7.0 (Molecular Devices).

2.7. Statistical analysis

Statistical analysis was performed with Stata software (version 10.0, StataCorp LP, USA). Data are expressed as the mean ± SE as mentioned. For *in vitro* studies, one-way analysis of variance (ANOVA) with a Dunnett's test was used to determine the differences between the treatment groups relative to the control. In human studies, the Mann-Whitney *U* test was used to determine statistically significant differences between high and low arsenic exposure groups. A bivariate regression model was used to assess the relationships among the study parameters. A multivariate adjusted regression model was also used to assess the association between exposure variables and the expression of genes related to adipogenesis and adipokines among arsenic exposed children. The potential confounders including BMI and the levels of toenail and saliva arsenic were adjusted in the model. Arsenic concentrations were assessed for normality using the Skewness/Kurtosis test. All data were z-scored prior to performing multivariate regression. The regression coefficients were reported as standardized (z-transformed) β coefficients with 95% confidence interval (CI). For all tests, p-values of <0.05, <0.01, and<0.001 were considered to indicate a statistically significant difference.

3. Results

3.1. Prenatal arsenic exposure alters the expression of genes involved in metabolic diseases in the newborn and in children

3.1.1. Demographic characteristics

Arsenic exposure and demographic characteristics of newborns and children are shown in Table 1. According to ATSDR, a level of arsenic in unexposed individuals is set as 1 μg/g in the nail (ToxGuide, 2007). In this study, the level of toenail arsenic, which is widely accepted as a biomarker for arsenic exposure in human, is used to classify low- and high-exposure groups. Toenail arsenic levels below 1 μg/g were classified as "low exposure". From the 22 recruited newborns, the high exposure group ($n = 10$) had higher levels of arsenic exposure as indicated by significantly increased arsenic concentrations in both toenails (a biomarker of long-term arsenic exposure) and cord blood (a biomarker of recent arsenic exposure) by 14.6- ($p < 0.001$) and 1.6-fold

Table 1
Arsenic exposure and demographic characteristics in the newborn and children.

Parameter	Arsenic exposure by toenail arsenic concentrations[a]		*p*-value[b]
	Low exposure	High exposure	
Newborn			
Gender			
Male [n (%)]	5 (41.67%)	6 (60.00%)	0.392
Female [n (%)]	7 (58.33%)	4 (40.00%)	
Birth weight	3.35 ± 0.11	3.18 ± 0.14	0.329
	3.25 (2.90–4.00)	3.30 (2.40–3.85)	
Arsenic level			
- Arsenic in drinking water (μg/L)	0.58 ± 0.65	2.48 ± 0.65	0.214
	0.69 (nd-5.85)	2.23 (0.47–5.48)	
- Arsenic in non-drinking water (μg/L)	3.10 ± 0.01	37.73 ± 18.47	**0.009**
	6.72 (0.01–61.63)	13.49 (1.14–147.60)	
Biomarker of arsenic exposure			
-Cord blood (μg/L)	0.19 ± 0.03	2.77 ± 0.78	**0.000**
	0.07 (nd-0.60)	1.47 (1.08–8.23)	
iAs	0.98 ± 0.16	2.13 ± 0.79	0.137
	0.94 (0.69–1.35)	1.64 (0.90–4.35)	
MMA	0.26 ± 0.21	0.32 ± 0.29	0.858
	nd (nd-0.90)	nd (nd-1.20)	
DMA	0.78 ± 0.17	0.97 ± 0.30	0.426
	0.73 (0.45–1.20)	0.91 (nd-1.77)	
Children			
Gender			
Male [n (%)]	10 (45.45%)	19 (57.57%)	0.244
Female [n (%)]	12 (54.55%)	14 (42.42%)	
Age (years)	8.04 ± 0.55	8.13 ± 0.57	0.541
Min-max	7.8 (7.0-9.0)	8.30 (6.8–9.2)	
BMI (kg/m^2)	16.05 ± 2.75	17.0 ± 3.71	0.312
Min-max	15.27 (13.57–25.63)	15.82 (11.75–26.52)	
Arsenic level (μg/L)			
- Arsenic in drinking water (μg/L)	0.85 ± 0.34	3.11 ± 1.20	**0.028**
	0.30 (nd-7.18)	0.60 (0.06–37.71)	
- Arsenic in non-drinking water (μg/L)	1.65 ± 0.59	10.42 ± 2.79	**0.000**
	0.37 (0.03–11.05)	2.92 (0.47–60.82)	
Biomarkers of arsenic exposure			
-Salivary arsenic (μg/L)	0.50 ± 0.04	2.73 ± 0.52	**0.000**
	0.50 (0.20–0.93)	1.99 (1.01–18.43)	
iAs	0.32 ± 0.05	0.72 ± 0.08	**0.000**
	0.23 (nd-0.73)	0.60 (0.15–2.54)	
MMA	0.08 ± 0.01	0.15 ± 0.06	0.270
	0.05 (nd-0.16)	0.06 (nd-1.10)	
DMA	0.12 ± 0.02	0.16 ± 0.05	0.577
	0.09 (nd-0.40)	0.10 (nd-1.07)	
-Toenail arsenic (μg/g)	0.44 ± 0.07	1.08 ± 0.14	**0.000**
	0.46 (0.03–1.13)	0.79 (0.27–3.62)	

Values are expressed as the Mean ± SE on the first line and the Median (minimum - maximum) on the second line.
iAS = inorganic arsenic; MMA = monomethylarsonic acid; DMA = dimethylarsinic acid.
[a] Arsenic exposure in both newborns and children was stratified according to their toenail arsenic levels as low (<1 μg/g, n = 12 for newborn or n = 22 for children) and high (≥1 μg/g, n = 10 for newborn or n = 33 for children) exposure groups according to the cutoff level of arsenic in unexposed individuals set by ATSDR (Trojnar et al., 2019).
[b] Statistically significant differences among groups at $p < 0.05$ and $p < 0.001$ by One-way ANOVA and Chi-square are highlighted in bold.

($p < 0.01$), respectively, compared to those of the low exposure group (n = 12). Analysis of arsenic speciation in cord blood showed that the inorganic arsenic (iAs) is the major form followed by monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA). The levels of iAs, MMA, and DMA in cord blood obtained from the high-arsenic exposed group were not significantly higher than those found in low-arsenic exposed group. No statistically significant differences were observed with respect to the median levels of gender and birth weight between the low- and high-arsenic exposed groups.

In children, the high exposure group (n = 33) had been exposed to significantly higher levels of arsenic in both drinking and non-drinking water by 3.7- ($p < 0.05$) and 6.3-fold ($p < 0.001$), respectively, compared to those of the low exposure group (n = 22). In addition, significantly higher levels of arsenic exposure as determined from arsenic concentrations in both toenail and saliva samples were observed in the high-arsenic exposed group by 5.5- and 2.5-fold ($p < 0.001$), respectively, compared to those of the low exposure group. Analysis of arsenic speciation in saliva samples also showed that iAs is the major form followed by MMA and DMA. The levels of iAs in saliva obtained from the high-arsenic exposed group were significantly higher than that of low-exposed group (0.72 ± 0.08 vs 0.32 ± 0.05 μg/L, $p < 0.001$). The levels of MMA and DMA in saliva obtained from low- and high-exposed children were not significant difference. No statistically significant differences were observed in the median levels of age, gender, and BMI between low- and high-arsenic exposed groups.

3.1.2. *Expression of genes involved in pathogenesis of metabolic diseases*

To investigate whether exposure to arsenic *in utero* through early-childhood alters the expression of genes involved in the pathogenesis of metabolic diseases, we first determined the expression of genes involved in adipogenesis (*PPARG, FABP4,* and *SLC2A4*) and genes encoding adipokines (*LEP* and *ADIPOQ*) in cord blood samples from the newborns. As shown in Fig. 1a and b, the expression levels of *PPARG* and *FABP4* mRNA in newborn cord blood were significantly lower in the high-arsenic exposed group by approximately 1.6- and 2.2-fold ($p < 0.05$), respectively. A slight but not statistically significant decrease in the expression of *SLC2A4* mRNA was observed between the high- and low-arsenic exposed groups (Fig. 1c). Meanwhile, the expression of *LEP* and *ADIPOQ* mRNA was not detectable in these cord blood samples.

The effect of continued exposure to arsenic throughout early childhood on the expression profile of genes related to metabolic diseases was further evaluated in children from the same cohort using saliva samples. Similar to what was observed in arsenic exposed newborns, a significant decrease ($p < 0.01$) in the mRNA expression levels of *PPARG* (3.4-fold) and *FABP4* (6.7-fold) was found in the high-arsenic exposed group (Fig. 1d–e). Interestingly, the high-arsenic exposed children showed a significant decrease in salivary mRNA expression level of *SLC2A4* by 6-fold compared to that of the low-arsenic exposed group (Fig. 1f). Further study revealed a significant decrease in salivary *ADIPOQ* mRNA level (3-fold; $p < 0.05$, Fig. 2b) with no alteration in *LEP* mRNA level (Fig. 2a) in the high-arsenic exposed group relative to the low-exposed group.

To address whether arsenic-mediated changes in mRNA levels reflect the protein expression, the protein levels of PPAR-γ, aP2, Leptin, and Adiponectin were measured by ELISA. No statistically significance was observed in the protein levels of both PPAR-γ and aP2 in cord-blood serum between low- and high-arsenic exposed groups (Fig. 1g and h, respectively). In saliva, the protein levels of PPAR-γ (Fig. 1i) and Leptin (Fig. 2c) did not change significantly in the low- and high-arsenic exposed groups. In contrast, significant decreases in the protein levels of aP2 (Fig. 1j) and Adiponectin (Fig. 2d) were observed in the high-arsenic exposed group compared to that in the low-arsenic exposed group.

The reduction in the expression of *PPARG* and *FABP4* mRNA found in high-arsenic exposed newborns was exacerbated in high-arsenic exposed children coupled with an additional decrease in the expression of *SCL2A4* and *ADIPOQ* mRNA. For the protein expression profile, only high-arsenic exposed children showed a significant decreased of aP2 and Adiponectin. These results suggest that continuous exposure to arsenic disrupts adipocyte development and adipokine production particularly in children.

3.1.3. *Association between arsenic exposure and the expression of genes involved in metabolic diseases*

In order to examine the relationship between arsenic exposure and the studied parameters, bivariate analysis was initially performed. As shown in Table 2, a significant negative correlation was only observed between the levels of arsenic in non-drinking water and the mRNA

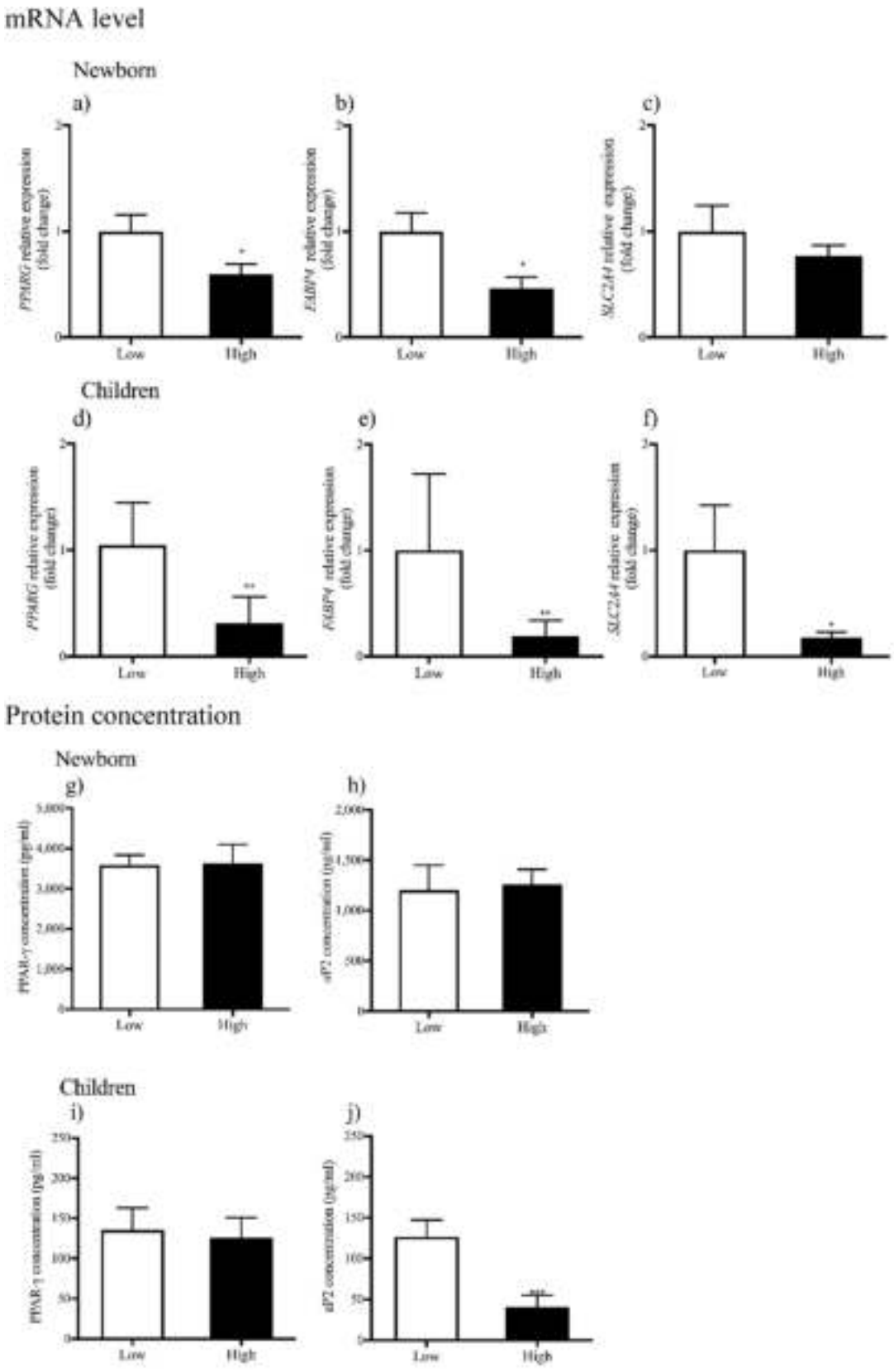

Fig. 1. Expression of genes related to metabolic disease in arsenic-exposed newborn and children who continuously exposed to arsenic since *in utero*
Total RNA was isolated from cord blood obtained from low-arsenic exposed (Low, n = 10) or high-arsenic exposed newborns (High, n = 12) (a–c) or from saliva obtained from low-arsenic exposed (Low, n = 22) or high-arsenic exposed (High, n = 33) children (d–f). The mRNA levels of *PPARG* (a and d), *FABP4* (b and e), and *SLC2A4* (c and f) were assessed by qRT-PCR using *PPIA* as an endogenous reference. The protein levels of PPAR-γ and aP2 were measured by ELISA in cord blood (g and h) and saliva (i and j), respectively. Each bar represents the mean ± SE. *, **, *** indicate a statistically significant difference from the low-arsenic exposed group at $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively.

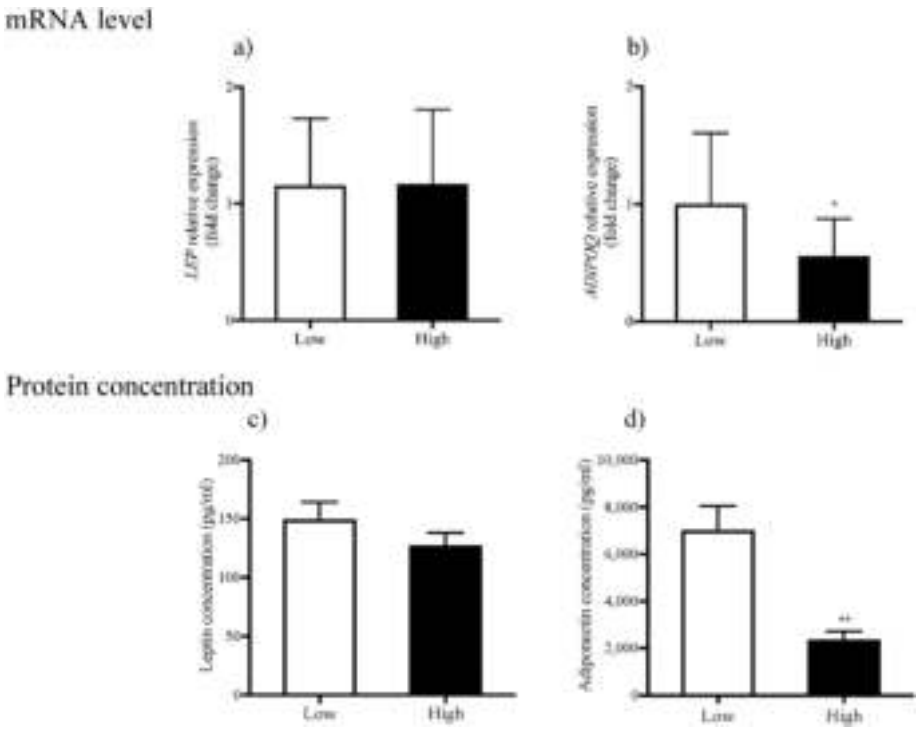

Fig. 2. Salivary expression of genes involved in adipokine in children continuously exposed to arsenic since *in utero*
Total RNA was isolated from saliva obtained from low-arsenic exposed (Low, n = 22) or high-arsenic exposed children (High, n = 33). The mRNA levels of *LEP* (a) and *ADIPOQ* (b) were assessed by real-time PCR using *PPIA* as an endogenous reference. The protein levels of Leptin (c) and Adiponectin (d) in saliva were determined by ELISA. Each bar represents the mean ± SE. * and ** indicate a statistically significant difference from the low-arsenic exposed group at $p < 0.05$ and $p < 0.01$, respectively.

expression levels of *SLC2A4* in newborns ($r = -0.437$, $p < 0.05$). However, in children, significant correlations were observed between the levels of arsenic in drinking water and the mRNA expression level of *PPARG* ($r = -0.262$, $p < 0.05$) and *FABP4* ($r = -0.244$, $p < 0.05$); the levels of toenail arsenic and the mRNA expression levels of *PPARG* ($r = -0.453$, $p < 0.01$), *FABP4* ($r = -0.346$, $p < 0.01$), and *SLC2A4* ($r = -0.268$, $p < 0.05$); as well as the levels of salivary arsenic and the expression level of *FABP4* mRNA ($r = -0.248$, $p < 0.05$). In contrast, arsenic levels in non-drinking water did not significantly correlate with the expression of any of the genes studied. Interestingly, the children's BMI showed a significant negative correlation only to the mRNA expression levels of *LEP* ($r = -0.357$, $p < 0.01$) and *ADIPOQ* ($r = -0.297$, $p < 0.05$).

Multivariate-adjusted regression analysis was additionally performed by adjusting covariates of toenail arsenic levels, salivary arsenic levels, and children's BMI. As shown in Table 3, the levels of toenail

Table 2
Bivariate analysis of association among the study parameters.

a) Newborn					
Metabolic genes	Correlation coefficient (r)				
	Arsenic concentration				Birth weight
	Drinking water	Non-drinking water	Toenail	Cord blood	
PPARG	−0.188 (p = 0.201)	−0.017 (p = 0.473)	0.188 (p = 0.521)	0.015 (p = 0.948)	−0.306 (p = 0.083)
FABP4	−0.034 (p = 0.440)	0.049 (p = 0.421)	−0.166 (p = 0.572)	−0.032 (p = 0.889)	−0.179 (p = 0.212)
SLC2A4	0.068 (p = 0.382)	−0.255 (p = 0.146)	−0.157 (p = 0.593)	0.086 (p = 0.702)	−0.290 (p = 0.096)
b) Children					
Metabolic genes	Correlation coefficient (r)				
	Arsenic concentration				BMI
	Drinking water	Non-drinking water	Toenail	Salivary	
PPARG	**−0.262 (p = 0.029)**	−0.125 (p = 0.191)	**−0.453 (p = 0.000)**	−0.161 (p = 0.129)	0.039 (p = 0.396)
FABP4	**−0.244 (p = 0.036)**	−0.214 (p = 0.062)	**−0.346 (p = 0.005)**	**−0.248 (p = 0.037)**	0.08 (p = 0.288)
SLC2A4	−0.016 (p = 0.453)	−0.121 (p = 0.189)	**−0.268 (p = 0.012)**	0.097 (p = 0.241)	0.053 (p = 0.355)
LEP	0.087 (p = 0.270)	−0.148 (p = 0.153)	0.034 (p = 0.406)	0.089 (p = 0.270)	**−0.357 (p = 0.007)**
ADIPOQ	−0.114 (p = 0.201)	−0.113 (p = 0.209)	−0.021 (p = 0.438)	0.048 (p = 0.365)	**−0.297 (p = 0.017)**

r represents correlation coefficient.
A p-value (p) of <0.05 was considered to be a statistically significant difference.

Table 3
Multivariate regression analysis between arsenic exposure and salivary gene expression in children

Metabolic genes	Coefficient β [95% CI]		
	Toenail arsenic	Salivary arsenic	BMI
PPARG	**-0.412** [-0.798, -0.165] **(p = 0.004)**	0.106 [-0.232, 0.484] (p =0.483)	0.041 [-0.233, 0.314] (p =0.766)
FABP4	**-0.329** [-0.697, -0.074] **(p = 0.016)**	-0.140 [-0.515, 0.184] (p =0.346)	0.154 [-0.111, 0.420] (p =0.248)
SLC2A4	**-0.334** [-0.656, -0.076] **(p = 0.001)**	0.288 [-0.010, 0.648] (p =0.057)	0.125 [-0.121, 0.378] (p =0.323)
LEP	0.095 [-0.202, 0.406] (p =0.502)	0.111 [-0.186, -0.428] (p =0.431)	**-0.348** [-0.577, -0.061] **(p = 0.017)**
ADIPOQ	0.036 [-0.285, 0.369] (p =0.795)	0.114 [-0.197, 0.468] (p =0.417)	**-0.301** [-0.581, -0.022] **(p = 0.035)**

β represents coefficient; 95% CI represents 95% confidence interval
A p-value (p) of <0.05, <0.01, and <0.001 were considered as a statistically significant difference

arsenic were inversely associated with the mRNA expression levels of *PPARG* (β = −0.412, 95% CI: −0.798, −0.165, P < 0.01), *FABP4* (β = −0.329, 95% CI: −0.697, −0.074, P < 0.05), and also *SLC2A4* (β = −0.334, 95% CI: −0.656, −0.076, P < 0.01). There was no significant association between children's BMI and arsenic exposure; however, children's BMI showed a significant negative association with the mRNA expression levels of *LEP* (β = −0.348, 95% CI: −0.557, −0.061, P < 0.05) and *ADIPOQ* (β = −0.301, 95% CI: −0.581, −0.022, P < 0.05).

Taken together, the significant negative association between the expression of genes related to adipogenesis (*PPARG*, *FABP4,* and *SCL2A4* mRNA) and toenail arsenic levels, revealed in both bivariate and multivariate analyses, indicating that children who have been exposed to arsenic starting *in utero* have impairment in the development of adipose tissue.

3.2. Effect of arsenite treatment on adipogenesis of UC-MSCs and gene expression in differentiated adipocytes, in vitro

To investigate whether dysfunction of fetal MSCs is involved in alteration of the expression of genes related to metabolic diseases in children exposed to arsenic since *in utero*, *in vitro* studies using UC-MSCs as a surrogate for fetal MSCs were carried out. The concentrations of arsenite used in this study, although higher than the level indicated by WHO guidelines at 10 μg/L in drinking water, were within a reasonable range for possible exposure because the levels of arsenic in non-drinking water in the Ron Piboon area varied from 0.01 to 147 μg/L. Furthermore, arsenite concentration at 0.5 μM used in this study is expected to be relevant given that pregnant Egyptian women who were passively exposed to tobacco smoke with a mean concentration of iAs of 59.7 ± 9.2 μg/L (~0.8 μM) in the maternal blood (Abdel Hameed et al., 2020).

3.2.1. Adipogenic differentiation

During prenatal adipogenesis, fetal adipocytes develop from fetal MSCs. Thus, UC-MSCs were used as a representative model to investigate whether prenatal exposure to arsenic impairs adipogenesis and the function of differentiated adipocytes. As shown in Fig. 3a, adipocytes differentiated from UC-MSCs showed a more rounded morphology compared to the undifferentiated UC-MSCs, which have a more fibroblast-like spindle shape. Along with the different morphology, an increased amount of lipid, as determined by LipidTox staining (green), was observed in differentiated adipocytes indicating that there is a larger number of adipocytes in the adipocyte differentiation group (Fig. 3a). In addition, the expression level of genes related to adipogenesis was significantly increased in differentiated adipocytes compared to that in undifferentiated UC-MSCs. Observed increases were 5.9-fold (p < 0.001) for *PPARG*, 55.7-fold (p < 0.001) for *FABP4*, and 27.6-fold (p < 0.01) for *SLC2A4* (Fig. 3b).

The effect of arsenite treatment on adipogenic differentiation of UC-MSCs was then investigated. UC-MSCs were cultured in adipocyte differentiation medium and treated with arsenite at 0, 0.5, and 1 μM for 15 days. This period is required for UC-MSCs to differentiate into mature adipocytes. Images of LipidTox staining demonstrated a significant decrease in lipid content as shown in green in arsenite treated group compared to that of the control group (Fig. 4). In order to elucidate the possible molecular mechanisms involved in arsenite-mediated suppression of adipogenic differentiation, the expression of genes involved in adipogenesis, *PPARG*, *FABP4*, and *SLC2A4,* were determined in differentiated adipocytes. As shown in Fig. 5a–c, arsenite treatment at 0.5 and 1 μM significantly decreased the mRNA expression levels of all studied genes when compared to those in untreated cells, by 3.1- and 6.3-fold for *PPARG,* 16.7- and 33.3-fold for *FABP4*, and by 4- and 4.8-fold for *SLC2A4* (p < 0.001). In concordance with the mRNA levels, the protein levels of PPAR-γ and aP2 were significantly decreased in arsenite treatment at 1 μM (Fig. 5f and g, respectively).

The significant decrease in the mRNA expression level of *PPARG*, which is a key transcription factor involved in adipogenesis, may in turn decrease the mRNA expression of its downstream target genes (*FABP4* and *SLC2A4*). Thus, the significant decreases in the expression of *PPARG* and *FABP4* at both the mRNA and protein levels after arsenite treatment suggests that arsenic exposure would impair the formation of adipocytes during differentiation of UC-MSCs.

3.2.2. Expression of genes encoding adipokines and inflammatory cytokines in differentiated adipocytes

Given that it is well recognized that adipokines play a critical role in modulating both local and systemic inflammation, the effects of arsenite treatment on the expression of the genes encoding adipokines (*LEP* and *ADIPOQ*) and inflammatory cytokines (*CXCL6, CXCL8,* and *IL-1β*) were further investigated in differentiated adipocytes. Treatment with 1 μM arsenite resulted in a significant increase in *LEP* mRNA, which is a pro-inflammatory adipokine, by approximately 6.2-fold (p < 0.01) compared to that in the untreated adipocytes (Fig. 5d). The mRNA expression of *ADIPOQ*, which is an anti-inflammatory adipokine, did not significantly decrease in response to arsentite treatment (Fig. 5e). For the protein expression, arsenite treatment significantly increased the protein level of Leptin (Fig. 5h), but significantly decreased the protein level of Adiponectin, when compared to the untreated adipocytes (Fig. 5i).

In addition, treatment with arsenite significantly increased *CXCL6, CXCL8*, and *IL-1β* mRNA expression levels in differentiated adipocytes in a concentration-dependent manner. A significant increase (p < 0.001) was observed in differentiated adipocytes treated with 0.5 and 1 μM arsenite by 3.9- and 7.4-fold for *CXCL6* (Fig. 6a), 4.1- and 5.5-fold for *CXCL8* (Figs. 6b), and 2.7-and 3.3-fold for *IL-1β* (Fig. 6c).

Taken together, the alterations in the expression of genes that are associated with adipocyte differentiation and adipokine production

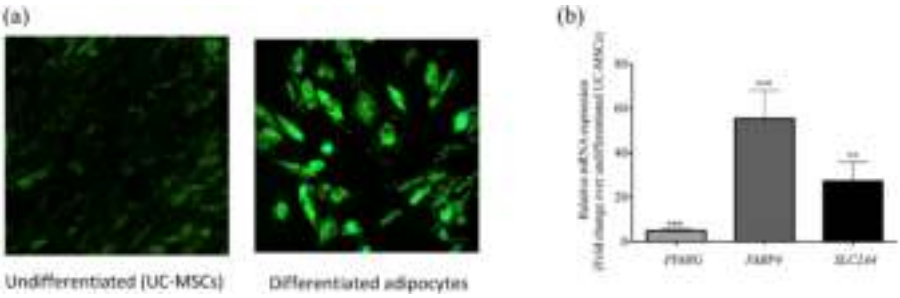

Fig. 3. Characterization of adipogenic differentiation of UC-MSCs
UC-MSCs (5000 cell/cm^2) were cultured in adipogenic differentiation medium (Stempro, USA) for 15 days. The medium was changed every 3 days throughout the process of differentiation. (a) Cells were fixed and stained for lipid droplets (lipidTOX, Green). (b) The mRNA levels of: *PPARG*, *FABP4*, and *SLC2A4*, were assessed by real-time PCR using *PPIA* as an endogenous reference. Each bar represents the mean ± SE from three independent experiments. ** and *** indicate a statistically significant difference from the control (undifferentiated UC-MSCs) at $p < 0.01$ and $p < 0.001$, respectively based on unpaired *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

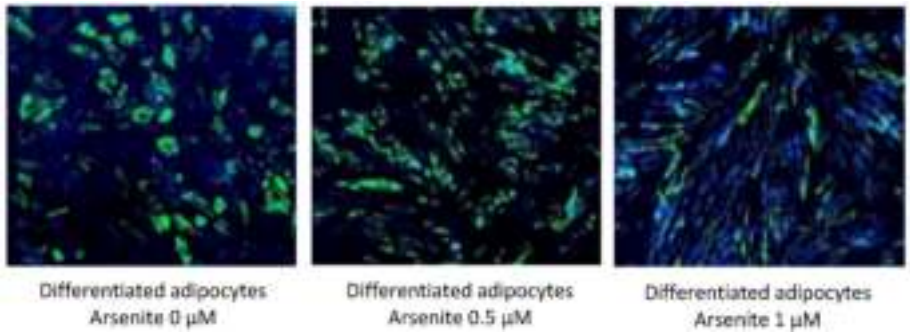

Fig. 4. Effect of arsenite treatment on adipogenic differentiation of UC-MSCs
UC-MSCs (5000 cell/cm^2) were cultured in adipogenic differentiation medium with various concentrations of arsenite (0, 0.5 and l μM) for 15 days. Cells were fixed and counter-stained for lipid droplets (LipidTOX, Green) and nucleus (DAPI, Blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

observed in our *in vitro* studies support the findings, which showed the decreased expression of *FABP4* and *ADIPOQ* at both mRNA and protein levels in children exposed to arsenic *in utero*.

4. Discussion

This study investigated how early-life arsenic exposure can lead to development of metabolic diseases later in life by studying the expression of selected genes involved in metabolic diseases in newborns and children that were continuously exposed to arsenic from the prenatal period. Although decreased expression of genes associated with adipogenesis (*PPARG*, *FABP4*, and *SLC2A4*) was observed in both high-arsenic exposed newborns and children, the effect was more pronounced in exposed children in which increasing toenail arsenic level was negatively associated with the expression of those genes. In addition, significantly decreased expression of *ADIPOQ* mRNA, an anti-inflammatory adipokine, was observed only in high-arsenic exposed children. Studies using UC-MSCs confirmed that the arsenic impaired adipogenic differentiation observed in newborns and children coincided with decreased expression of the aforementioned genes.

Elevated BMI in association with other risk factors such as chronic inflammation and unhealthy lifestyle play a crucial role in the pathophysiology of several metabolic diseases (Galicia-Garcia et al., 2020). In our study, no significant difference was observed in birth weight and BMI between the low- and high-arsenic exposed groups. However, according to WHO guidelines, the BMI of high-arsenic exposed children observed in this present study could be classified as overweight (Abuawad et al., 2021). This finding is in line with epidemiological studies conducted in Bangladesh, New Mexico and United States which demonstrated that exposure to high levels of arsenic in drinking water (>50 μg/L) is positively associated with BMI in exposed adolescents (median age = 14 years) (Abuawad et al., 2021) and adults (Gomez--Rubio et al., 2011). These results suggested that the high-arsenic exposed children may develop obesity and related diseases later in life.

It is well established that impairment of proper adipocyte development (adipogenesis) and function plays an important role in the development of metabolic diseases. As a master regulator of adipocyte differentiation, the decreased expression of *PPARG* as well as its downstream target genes such as *FABP4* and *SLC2A4* would seriously disrupt the generation of adipose tissue. The existing data demonstrated that lower expression of *PPARG* at both mRNA and protein levels was observed in patients with several metabolic diseases such as type II diabetes (Sun et al., 2021) and fatty-liver disease (Liss and Finck, 2017). Furthermore, the decreased gene expression of *SLC2A4*, the major glucose transporter in adipose tissue, promotes insulin resistance and hypertension, which leads to the development of cardiovascular diseases (Leguisamo et al., 2012). Interestingly, increased plasma levels of aP2, encoded by *FABP4*, has been used as a predictive marker for obesity and insulin resistance in adult populations with increased risk of developing metabolic diseases (Trojnar et al., 2019). A recent finding showed an inverse association between mRNA expression of *FABP4* and obesity. This discrepancy between plasma level and mRNA expression of *FABP4* in adipose tissue observed in obese subjects may be due to dysfunction of adipocytes (Queipo-Ortuño et al., 2012). In addition, the low expression of *FABP4* mRNA and protein led to increased risk of cardiovascular diseases in diabetic patients (Dahlström et al., 2021). Therefore, decreased mRNA expression of *PPARG*, *FABP4*, and *SLC2A4* observed in high-arsenic exposed children suggests that these children may be at higher risk of developing metabolic diseases. In this study, the mRNA levels of *PPARG*, *FABP4* and *SLC2A4* were decreased in both newborns and children exposed to high levels of arsenic (toenail arsenic level > 1 μg/g) suggesting that the effect of arsenic-mediated alteration in the expression of genes related to adipogenesis in children was initiated during exposure *in utero*. Further reduction in the expression of those genes observed in children compared to that in newborns could be due to a longer period of continuous exposure. The newborns were exposed to arsenic only *in utero* (9 months), while these children were continuously exposed to arsenic for approximately 8 years (the median age of the participating children). This observation indicated that the continuous exposure to arsenic during childhood may aggravate arsenic's adverse effects.

Alteration in the expression of adipokines (e.g. high leptin and/or

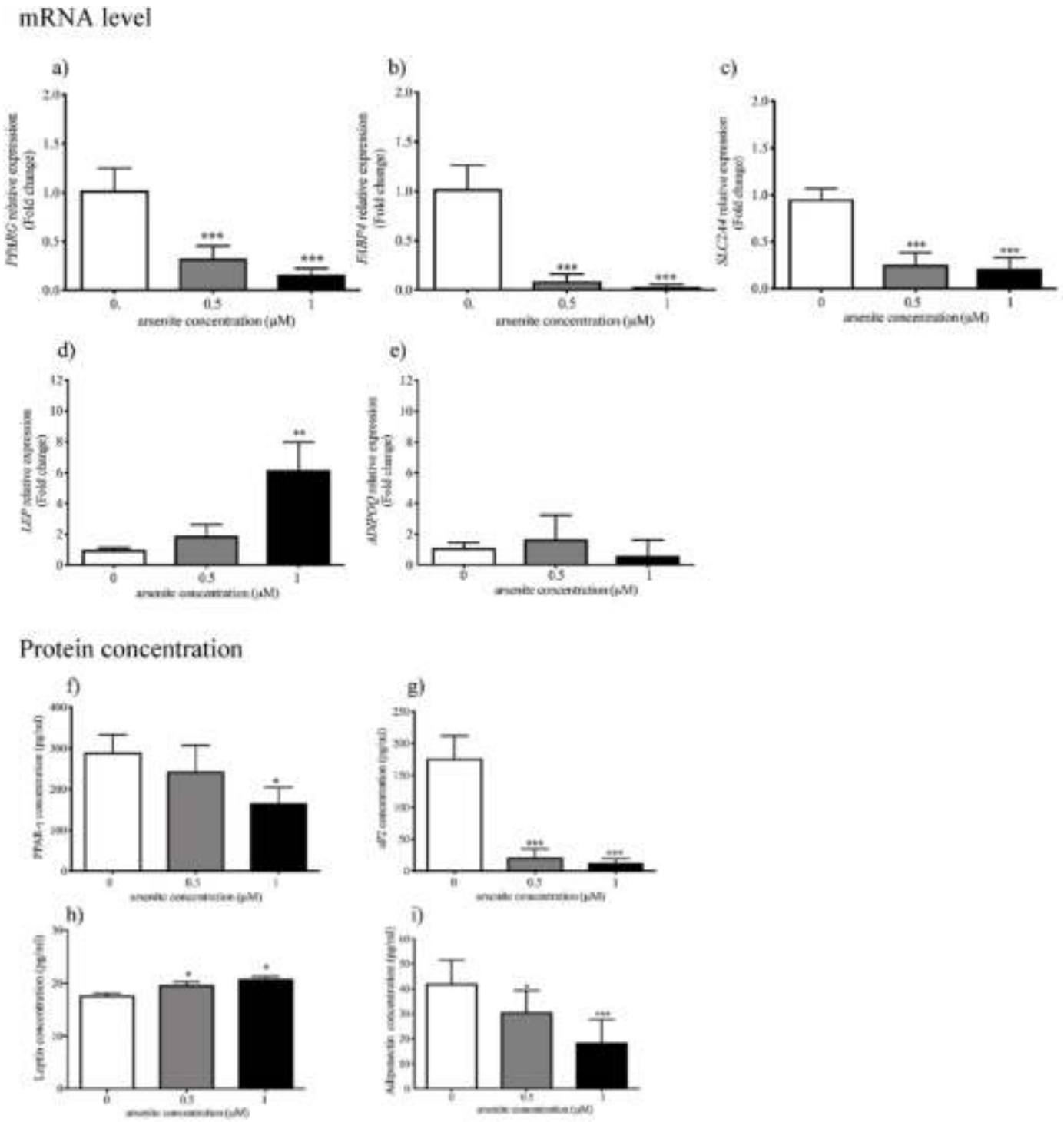

Fig. 5. Effect of arsenite treatment on the expression of genes related to metabolic diseases *(PPARG, FABP4, SLC2A4)* and adipokine production (*LEP* and *ADIPOQ*) UC-MSCs (5000 cell/cm^2) were cultured in adipogenic differentiation medium with various concentrations of arsenite (0, 0.5 and l μM) for 15 days. The mRNA level of *PPARG* (a), *FABP4* (b), *SLC2A4* (c), *LEP* (d), and *ADIPOQ* (e), were assessed by qRT-PCR using *PPIA* for *PPARG*, *FABP4*, *SLC2A4*, *LEP*, and *ADIPOQ*, an endogenous reference. The protein levels of PPAR-γ (f), aP2 (g), Leptin (h), and Adiponectin (i) were assessed by ELISA. Each bar represents the mean ± SE from three independent experiments. *, **, and *** represent statistically significant difference from the control (arsenite 0 μM) at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

low adiponectin expression in both serum and saliva) has been recognized as early diagnostic tools for monitoring the onset and progression of metabolic diseases (Thanakun et al., 2014). In this study, the expression of genes encoding adipokines was not detectable in cord blood samples. The low expression of *ADIPOQ* and *LEP* mRNA in cord blood might be due to the fact that adipose tissue of the fetus usually remains dormant (Symonds et al., 2003). However, in children, a significantly lower expression of *ADIPOQ* at both mRNA and protein level was observed in the high-arsenic exposed children compared to that found in the low-arsenic exposed group. Altered expression of *ADIPOQ* mRNA, which results in low serum adiponectin levels, was associated with increased risk of developing metabolic diseases in Mexican children (He et al., 2019) and in other adult populations (Mente et al., 2013). Thus, high-arsenic exposed children with decreased

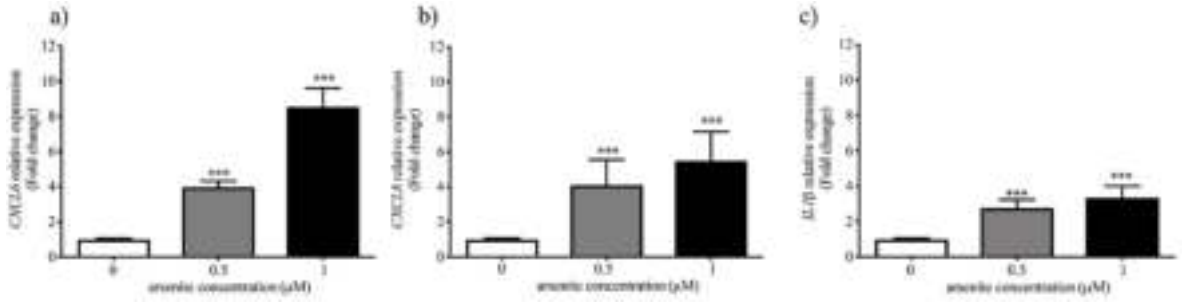

Fig. 6. Effect of arsenite treatment on the expression of genes related to inflammatory cytokines *(CXCL6, CXCL8, IL1β)* in differentiated adipocytes
UC-MSCs (5000 cell/cm^2) were cultured in adipogenic differentiation medium with various concentrations of arsenite (0, 0.5 and l μM) for 15 days. The mRNA level of *CXCL6* (a), *CXCL8* (b), and *IL1β* (c), were assessed by qRT-PCR using *GAPDH* for *CXCL6* and *IL1β* or *PPIA* for *CXCL8* as an endogenous reference. Each bar represents the mean ± SE from three independent experiments. *** represents a statistically significant difference from the control (arsenite 0 μM).

expression of both *ADIPOQ* mRNA and protein level should have a higher risk of developing metabolic diseases.

Based on multivariate regression analysis, the expression of genes related to metabolic diseases (*PPARG, FABP4,* and *SLC2A4*) was significantly associated with toenail arsenic levels only, whereas the expression of genes encoding adipokines (*LEP* and *ADIPOQ*) were negatively associated with only BMI. A negative association between prenatal arsenic exposure and the expression of genes involved in adipogenesis suggested that arsenic exposure interrupts adipocyte differentiation, which could interfere with their function and lead to increased risk of developing metabolic diseases in an exposed population. In this study, it was not possible to investigate other parameters associated with metabolic diseases such as plasma glucose (HbA1c), triglycerides, and high-density lipoprotein cholesterol because blood sample collection in children participating in research projects in Thailand is not permitted. Furthermore, these parameters cannot be measured in all sample types used in this study because: the instability of glucose and lipid profiles in frozen cord-blood serum (França et al., 2018). In addition, these parameters are not present in saliva. Besides, the supernatants, which are solely derived from differentiated adipocytes, might not be representative of all of the changes observed in metabolic diseases.

Although the contribution of dysfunctional fetal stem cells to the onset of metabolic diseases mediated by arsenic exposure is not fully understood, the *in vitro* studies using UC-MSCs exposed to arsenite confirmed that arsenic was indeed responsible for alterations in the expression of various genes involved in metabolic diseases observed in the arsenic-exposed newborns and children. Arsenite treatment (at both 0.5 and 1 μM) disrupted adipogenic differentiation of UC-MSCs partly through the decreased mRNA expression of *PPARG*, *FABP4*, and *SLC2A4*. This is well in line with our observations in exposed children and in human MSCs derived from adipose tissue (Munir et al., 2017). In mice, prenatal exposure to arsenic decreased *PPARG* expression in the offspring leading to the development of metabolic diseases (Ditzel et al., 2016). Furthermore, MSCs derived from adipose tissue of arsenic exposed mice show decreased *FABP4* expression, thereby impairing adipocyte differentiation (Shearer et al., 2017). Chronic exposure to arsenite (0–2 μM) for 7 days (Yadav et al., 2013) or up to 8 weeks (Padmaja Divya et al., 2015) significantly decreased the expression of *SLC2A4* mRNA in the mouse preadipocyte cell line, 3T3-L1, resulting in the reduction of insulin-stimulating glucose uptake.

Accumulated evidences revealed that abnormalities in metabolic activity are significantly associated with epigenetic alterations (Cheng et al., 2018), for instance, adipose tissue from obese or diabetic subjects had hypermethylation in the promoter region of *PPARG* (Nilsson et al., 2014) and *ADIPOQ*, but hypomethylation in promoter region of *LEP* (Houde et al., 2015). Thus, the alterations in the expression of genes related to metabolic diseases observed in this study may be mediated by arsenic-induced changes in promoter DNA methylation patterns. Apart from DNA methylation, histone modification is another possible mechanism. Adult male mice exposed to arsenic during gestation showed a significant decreased in the expression of *FABP4* mRNA, which is associated with histone modification, but not DNA methylation (Nohara et al., 2012). Consequently, the mechanistic links between prenatal exposure to arsenic and epigenetic alteration of genes related to metabolic diseases warrant further investigation.

It is now well accepted that the intricate balance of pro- and anti-inflammatory adipokines play a major role in the regulation of both local and systemic inflammatory responses. Most importantly, several adipokines (e.g. Adiponectin and Leptin) and inflammatory cytokines (e.g. TNFα, IL1β, IL6, IL8 and IL10) released from inflamed adipocytes are involved in the development of insulin resistance, which is one of the main pathogenic mechanisms of metabolic diseases (Esser et al., 2014) In addition, treatment with IL6 promoted insulin resistance by inhibiting the expression of genes related to adipogenesis (*PPARG* and *SLC2A4*) in human adipocyte cell line (Rotter et al., 2003). Interestingly, our study demonstrated that arsenic treatment significantly increased the expression of genes encoding a pro-inflammatory adipokine (*LEP*) and inflammatory cytokines (*CXCL6*, *CXCL8* and *IL1β*) in differentiated adipocytes. Although the expression of *ADIPOQ* mRNA, an anti-inflammatory adipokine, in differentiated adipocytes treated with arsenite did not show significant alteration, its protein level was significantly decreased in a concentration-dependent manner by arsenite treatment. Our results were in good agreement with several studies using human MSCs derived from adipose tissue. Arsenite treatment (0.1–2.5 μM) significantly decreased the expression of *ADIPOQ* mRNA in differentiated adipocytes from human MSCs (Garciafigueroa et al., 2013; Klei et al., 2013).

Notably, the present study is the first study to show that arsenite treatment significantly increased the expression of genes related to inflammation (*LEP*, *CXCL6*, *CXCL8* and *IL1β*) in differentiated adipocytes, providing supporting evidence indicating that arsenic-induced inflammation of adipocytes could be another contributor to the development of metabolic diseases in exposed populations (Fuentes et al., 2013; Kang et al., 2016).

In conclusion, the decreased expression of *PPARG*, *FABP4*, and *SLC2A4* observed in newborns and children exposed to arsenic indicated that exposure to arsenic *in utero* that continues throughout childhood may disturb the differentiation and function of fetal MSCs. Moreover, inflammation induced by increased expression of inflammatory genes in differentiated adipocytes treated with arsenite suggested the involvement of adipose tissue inflammation in the pathogenesis of metabolic diseases. Taken together, our data suggests that prenatal arsenic exposure adversely affects fetal MSCs leading to impaired adipogenesis and alters expression of genes involved in metabolic diseases in newborns and children who were continuously exposed to arsenic. Future research is needed to clarify the links between *in utero* arsenic exposure, defective adipogenic differentiation of fetal MSCs, and disease development later in life.

Credit author statement

PS performed experiments, data analyses, and drafting this manuscript. PN coordinated and designed laboratory experiments, data analysis and manuscript preparation. TN made the contributions to the experimental designs, data analyses and drafting this manuscript. KC and PH prepared data for experimental part of the manuscript. MR, as the principal investigator, involved in data revisions, research conclusion and reviewing this manuscript as well as sought funding supports. All authors read and approved the final manuscript.

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Approval for human subjects research

This study was conducted according to the recommendations of the Declaration of Helsinki (World Medical Association, 1989) for international health research. All the children's parents gave written consent for participation in this study.

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References

Abdel Hameed, E.R., Sherif, L.S., Awad, A.H., El Ashry, H.H., Ahmed, H.H., Sallam, M. M., et al., 2020. Arsenic and cadmium levels in maternal and umbilical cord blood and their associations with birth outcomes. Biomed Pharmacol J 13 (1), 61–69.

Abuawad, A., Spratlen, M.J., Parvez, F., Slavkovich, V., Ilievski, V., Lomax-Luu, A.M., et al., 2021. Association between body mass index and arsenic methylation in three studies of Bangladeshi adults and adolescents. Environ Int [Internet 149, 106401. https://doi.org/10.1016/j.envint.2021.106401. Available from:

Al-Hamodi, Z., Al-Habori, M., Al-Meeri, A., 2014. Saif-Ali R. Association of adipokines, leptin/adiponectin ratio and C-reactive protein with obesity and type 2 diabetes mellitus. Diabetol Metab Syndr [Internet] 6 (1), 99. https://doi.org/10.1186/1758-5996-6-99. Available from:

Bailey, K.A., Smith, A.H., Tokar, E.J., Graziano, J.H., Kim, K.W., Navasumrit, P., et al., 2016. Mechanisms underlying latent disease risk associated with early-life arsenic exposure: current research trends and scientific gaps. Environ. Health Perspect. 124 (2), 170–175.

Bruun, J.M., Lihn, A.S., Madan, A.K., Pedersen, S.B., Schiøtt, K.M., Fain, J.N., et al., 2004. Higher production of IL-8 in visceral vs. subcutaneous adipose tissue. Implication of nonadipose cells in adipose tissue. Am. J. Physiol. Endocrinol. Metab. 286 (1 49–1), 8–13.

Cheng, Z., Zheng, L., Almeida, F.A., 2018. Epigenetic reprogramming in metabolic disorders: nutritional factors and beyond. J. Nutr. Biochem. 54, 1–10.

Coín-Aragüez, L., Pavón, F.J., Contreras, A., Gentile, A.M., Lhamyani, S., De Diego-Otero, Y., et al., 2018. Inflammatory gene expression in adipose tissue according to diagnosis of anxiety and mood disorders in obese and non-obese subjects. Sci. Rep. 8 (1), 1–10.

Dahlström, E.H., Saksi, J., Forsblom, C., Uglebjerg, N., Mars, N., Thorn, L.M., et al., 2021. The low-expression variant of FABP4 is associated with cardiovascular disease in type 1 diabetes [Internet] Diabetes 70 (10), 2391–2401. https://doi.org/10.2337/db21-0056. Available from:

Ditzel, E.J., Nguyen, T., Parker, P., Camenisch, T.D., 2016. Effects of arsenite exposure during fetal development on energy metabolism and susceptibility to diet-induced fatty liver disease in male mice. *Environmental*. Health Perspectives 124 (2), 201–209. https://doi.org/10.1289/ehp.1409501.

Esser, N., Legrand-Poels, S., Piette, J., Scheen, A.J., Paquot, N., 2014. Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes.*Diabetes Research and Clinical Practice*. Elsevier Ireland Ltd.. https://doi.org/10.1016/j.diabres.2014.04.006

Falahi, E., Khalkhali Rad, A.H., Roosta, S., 2015. What is the best biomarker for metabolic syndrome diagnosis? Diabetes Metabol. Syndr. 9 (4), 366–372.

França, C.N., Mendes, C.C., Ferreira, C.E.S., 2018. Time collection and storage conditions of lipid profile. Braz. J. Med. Biol. Res. 51 (3).

Fry, R.C., Navasumrit, P., Valiathan, C., Svensson, J.P., Hogan, B.J., Luo, M., et al., 2007. Activation of inflammation/NF-κB signaling in infants born to arsenic-exposed mothers. PLoS Genet. 3 (11), 2180–2189. https://doi.org/10.1371/journal.pgen.0030207.

Fuentes, E., Fuentes, F., Vilahur, G., Badimon, L., Palomo, I., 2013. Mechanisms of chronic state of inflammation as mediators that link obese adipose tissue and metabolic syndrome. Mediat. Inflamm. https://doi.org/10.1155/2013/136584.

Galicia-Garcia, U., Benito-Vicente, A., Jebari, S., Larrea-Sebal, A., Siddiqi, H., Uribe, K.B., et al., 2020. Pathophysiology of type 2 diabetes mellitus. Int J Mol Sci. 21 (17), 6275. Available from: https://pubmed.ncbi.nlm.nih.gov/32872570.

Garciafigueroa, D.Y., Klei, L.R., Ambrosio, F., Barchowsky, A., 2013. Arsenic-stimulated lipolysis and adipose remodeling is mediated by G-protein-coupled receptors. Toxicol.Sci. 134 (2), 335–344. https://doi.org/10.1093/toxsci/kft108.

Gomez-Rubio, P., Roberge, J., Arendell, L., Harris, R.B., O'Rourke, M.K., Chen, Z., et al., 2011. Association between body mass index and arsenic methylation efficiency in adult women from southwest U.S. and northwest Mexico. Toxicol Appl Pharmacol [Internet] 252 (2), 176–182. https://doi.org/10.1016/j.taap.2011.02.007. Available from:

Grau-Perez, M., Kuo, C.C., Gribble, M.O., Balakrishnan, P., Jones Spratlen, M., Vaidya, D., et al., 2017. Association of low-moderate arsenic exposure and arsenic metabolism with incident diabetes and insulin resistance in the strong heart family study.

Hawkesworth, S., Wagatsuma, Y., Kippler, M., Fulford, A.J.C., Arifeen, S.E., Persson, L. A., et al., 2013. Early exposure to toxic metals has a limited effect on blood pressure or kidney function in later childhood, rural Bangladesh. Int. J. Epidemiol. 42 (1), 176–185.

He, J., Stryjecki, C., Reddon, H., Peralta-Romero, J., Karam-Araujo, R., Suarez, F., et al., 2019. Adiponectin is associated with cardio-metabolic traits in Mexican children. Sci. Rep. 9 (1).

Hinhumpatch, P., Navasumrit, P., Chaisatra, K., Promvijit, J., Mahidol, C., Ruchirawat, M., 2013. Oxidative DNA damage and repair in children exposed to low levels of arsenic in utero and during early childhood: application of salivary and urinary biomarkers. Toxicol. Appl. Pharmacol. 273 (3), 569–579. https://doi.org/10.1016/j.taap.2013.10.002.

Houde, A.A., Légaré, C., Biron, S., Lescelleur, O., Biertho, L., Marceau, S., et al., 2015. Leptin and adiponectin DNA methylation levels in adipose tissues and blood cells are associated with BMI, waist girth and LDL-cholesterol levels in severely obese men and women [Internet] BMC Med. Genet. 16 (1), 1–10 (Available from: ???).

Intarasunanont, P., Navasumrit, P., Waraprasit, S., Chaisatra, K., Suk, W.A., Mahidol, C., et al., 2012. Effects of arsenic exposure on DNA methylation in cord blood samples from newborn babies and in a human lymphoblast cell line.*Environmental Health: A Global Access*. Science Source 11 (1). https://doi.org/10.1186/1476-069X-11-31.

Kang, Y.E., Kim, J.M., Joung, K.H., Lee, J.H., You, B.R., Choi, M.J., et al., 2016. The roles of adipokines, proinflammatory cytokines, and adipose tissue macrophages in obesity-associated insulin resistance in modest obesity and early metabolic dysfunction. *PLoS ONE* 11 (4).

Klei, L.R., Yesica Garciafigueroa, D., Barchowsky, A., 2013. Arsenic activates endothelin-1 Gi protein-coupled receptor signaling to inhibit stem cell differentiation in adipogenesis.*Toxicol.* Sci. 131 (2), 512–520. https://doi.org/10.1093/toxsci/kfs323.

Leguisamo, N.M., Lehnen, A.M., Machado, U.F., Okamoto, M.M., Markoski, M.M., Pinto, G.H., et al., 2012. GLUT4 content decreases along with insulin resistance and high levels of inflammatory markers in rats with metabolic syndrome [Internet] Cardiovasc. Diabetol. 11 (1), 100. https://doi.org/10.1186/1475-2840-11-100. Available from:

Liss, K.H.H., Finck, B.N., 2017. PPARs and nonalcoholic fatty liver disease [Internet]. 2016/12/02 Biochimie 136, 65–74. Available from: https://pubmed.ncbi.nlm.nih.gov/27916647.

Liu, W., Zhou, X., Li, Y., Zhang, S., Cai, X., Zhang, R., et al., 2020. Serum leptin, resistin, and adiponectin levels in obese and non-obese patients with newly diagnosed type 2 diabetes mellitus: a population-based study [Internet] Medicine (Baltim.) 99 (6) [cited 2021 Nov 4], Available from:/pmc/articles/PMC7015632/.

Maull, E.A., Ahsan, H., Edwards, J., Longnecker, M.P., Navas-Acien, A., Pi, J., et al., 2012. Evaluation of the Association between Arsenic and Diabetes: A National Toxicology Program Workshop Review. Environmental Health Perspectives.

Mente, A., Meyre, D., Lanktree, M.B., Heydarpour, M., Darlene Davis, A., Miller, R., et al., 2013. Causal relationship between adiponectin and metabolic traits: a mendelian randomization study in a multiethnic population. PLoS One 8 (6).

Munir, H., Ward, L.S.C., Sheriff, L., Kemble, S., Nayar, S., Barone, F., et al., 2017. Adipogenic differentiation of mesenchymal stem cells alters their immunomodulatory properties in a tissue-specific manner.*Stem*. Cells 35 (6), 1636–1646. https://doi.org/10.1002/stem.2622.

Nilsson, E., Jansson, P.A., Perfilyev, A., Volkov, P., Pedersen, M., Svensson, M.K., et al., 2014. Altered DNA methylation and differential expression of genes influencing metabolism and inflammation in adipose tissue from subjects with type 2 diabetes. Diabetes 63 (9), 2962–2976.

Nohara, K., Tateishi, Y., Suzuki, T., Okamura, K., Murai, H., Takumi, S., et al., 2012. Late-onset increases in oxidative stress and other tumorigenic activities and tumors with a Ha-ras mutation in the liver of adult male C3H mice gestationally exposed to arsenic. Toxicol. Sci. 129 (2), 293–304.

Padmaja Divya, S., Pratheeshkumar, P., Son, Y.-O., Vinod Roy, R., Andrew Hitron, J., Kim, D., et al., 2015. Arsenic induces insulin resistance in mouse adipocytes and myotubes via oxidative stress-regulated mitochondrial sirt3-FOXO3a signaling pathway.*Toxicol.* Sciences 146 (2), 290–300. https://doi.org/10.1093/toxsci/kfv089.

Phookphan, P., Navasumrit, P., Waraprasit, S., Promvijit, J., Chaisatra, K., Ngaotepprutaram, T., et al., 2017. Hypomethylation of inflammatory genes (COX2, EGR1, and SOCS3) and increased urinary 8-nitroguanine in arsenic-exposed

newborns and children. Toxicol. Appl. Pharmacol. 316, 36–47. https://doi.org/10.1016/j.taap.2016.12.015.

Queipo-Ortuño, M.I., Escoté, X., Ceperuelo-Mallafré, V., Garrido-Sanchez, L., Miranda, M., Clemente-Postigo, M., et al., 2012. FABP4 dynamics in obesity: discrepancies in adipose tissue and liver expression regarding circulating plasma levels. PLoS One 7 (11).

Reddy, P., Lent-Schochet, D., Ramakrishnan, N., McLaughlin, M., Jialal, I., 2019. Metabolic syndrome is an inflammatory disorder: a conspiracy between adipose tissue and phagocytes. Clin. Chim. Acta 496, 35–44. June.

Rotter, V., Nagaev, I., Smith, U., 2003. Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-α, overexpressed in human fat cells from insulin-resistant subjects.J. Biol. Chem. 278 (46), 45777–45784. https://doi.org/10.1074/jbc.M301977200.

Shao, M., Hepler, C., Vishvanath, L., MacPherson, K.A., Busbuso, N.C., Gupta, R.K., 2017. Fetal development of subcutaneous white adipose tissue is dependent on Zfp423 [Internet] Mol. Metabol. 6 (1), 111–124. Available from: https://www.sciencedirect.com/science/article/pii/S221287781630206X.

Shearer, J.J., Neto, M.F., Umbaugh, S.S., Figueiredo, M.L., 2017. In vivo exposure to inorganic arsenic alters differentiation-specific gene expression of adipose- derived mesenchymal stem/stromal cells in C57BL/6J mouse model. *Toxicol* Sci. 157 (1), 172–182. https://doi.org/10.1093/toxsci/kfx026.

Sun, C., Mao, S., Chen, S., Zhang, W., Liu, C., 2021. PPARs-orchestrated metabolic homeostasis in the adipose tissue. Int J Mol Sci [Internet 22 (16), 8974. Available from: https://pubmed.ncbi.nlm.nih.gov/34445679.

Symonds, M.E., Mostyn, A., Pearce, S., Budge, H., Stephenson, T., 2003. Endocrine and nutritional regulation of fetal adipose tissue development. J. Endocrinol. 179 (3), 293–299.

Thanakun, S., Watanabe, H., Thaweboon, S., Izumi, Y., 2014. Comparison of salivary and plasma adiponectin and leptin in patients with metabolic syndrome. Diabetol. Metabol.Syndr. 6 (1). https://doi.org/10.1186/1758-5996-6-19.

Tokar, E.J., Qu, W., Waalkes, M.P., 2011. Arsenic, stem cells, and the developmental basis of adult cancer. Toxicol. Sci. 120 (Suppl. 1), 192–203.

ToxGuide, C.D.C., 2007. TM for arsenic as sources of exposure general populations [cited 2021 Nov 2]; Available from: www.atsdr.cdc.gov/toxpro2.html.

Trojnar, M., Patro-Małysza, J., , Kimber-Trojnar Ż, Leszczyńska-Gorzelak, B., Mosiewicz, J., 2019. Associations between fatty acid-binding protein 4–A proinflammatory adipokine and insulin resistance. Gestational and Type 2 Diabetes Mellitus 8. Cells.

Veljić, I., Polovina, M., Seferović, J.P., Seferović, P.M., 2018. Adipokine profile as a novel screening method for cardiometabolic disease: help or hindrance? [Internet] Eur J Prev Cardiol 25 (14), 1543–1547. https://doi.org/10.1177/2047487318795189. Available from:

Wang, G., Chen, Z., Bartell, T., Wang, X., 2014. Early life origins of metabolic syndrome: the role of environmental toxicants [Internet] Curr Environ Heal reports 1 (1), 78–89. Available from: https://pubmed.ncbi.nlm.nih.gov/24883264.

Yadav, S., Anbalagan, M., Shi, Y., Wang, F., Wang, H., 2013. Arsenic inhibits the adipogenic differentiation of mesenchymal stem cells by down-regulating peroxisome proliferator-activated receptor gamma and CCAAT enhancer-binding protein. Diabetol. Metabol. Syndr. 6 (1). https://doi.org/10.1186/1758-5996-6-19.

Yuan, Y., Marshall, G., Ferreccio, C., Steinmaus, C., Selvin, S., Liaw, J., et al., 2007. Acute myocardial infarction mortality in comparison with lung and bladder cancer mortality in arsenic-exposed region II of Chile from 1950 to 2000. Am. J. Epidemiol. 166 (12), 1381–1391.

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Assessing sustained uptake of latrine and child feces management interventions: Extended follow-up of a cluster-randomized controlled trial in rural Bangladesh 1–3.5 years after intervention initiation

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ABSTRACT

Background: Sanitation interventions typically result in modest increases in latrine access, and any gains in latrine access and use are often not sustained over time. Sanitation programs also rarely include child-focused interventions such as potties. We aimed to assess the sustained effect of a multi-component sanitation intervention on access to and use of latrines and child feces management tools in rural Bangladesh.

Methods: We conducted a longitudinal substudy nested within the WASH Benefits randomized controlled trial. The trial provided latrine upgrades, child potties and sani-scoops for feces removal, along with behavior change promotion to encourage use of the delivered hardware. Promotion visits to intervention recipients were frequent during the first 2 years after intervention initiation, decreased in frequency between years 2–3, and ceased after 3 years. We enrolled a random subset of 720 households from the sanitation and control arms of the trial in a substudy and visited them quarterly between 1 and 3.5 years after intervention initiation. At each visit, field staff recorded sanitation-related behaviors through spot-check observations and structured questionnaires. We assessed intervention effects on observed indicators of hygienic latrine access, potty use and sani-scoop use and investigated whether these effects were modified by duration of follow-up, ongoing behavior change promotion and household characteristics.

Results: The intervention increased hygienic latrine access from 37% among controls to 94% in the sanitation arm ($p < 0.001$). Access among intervention recipients remained high 3.5 years after intervention initiation, including periods with no active promotion. Gains in access were higher among households with less education, less wealth and larger number of residents. The intervention increased availability of child potties from 29% among controls to 98% in the sanitation arm ($p < 0.001$). However, fewer than 25% of intervention households reported exclusive child defecation in a potty or had observed indicators of potty and sani-scoop use, and gains in potty use declined over the follow-up period, even with ongoing promotion.

Conclusion: Our findings from an intervention that provided free products and intensive initial behavior change promotion suggest a sustained increase in hygienic latrine access up to 3.5 years after intervention initiation but infrequent use of child feces management tools. Studies should investigate strategies to ensure sustained adoption of safe child feces management practices.

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1. Introduction

Safely managed sanitation services are available to 62% of urban populations but only 44% of rural populations worldwide (WHO/UNICEF, 2021). If the current trends persist, 2.8 billion people will lack safely managed sanitation services by 2030 (WHO/UNICEF, 2021). Access to sanitation is considered a primary barrier against fecal-orally transmitted diseases. However, the effectiveness of sanitation improvements on interrupting disease transmission depends on their coverage and sustained adoption by users. Several sanitation trials have found either no impact or mixed impact of sanitation interventions on child diarrhea and growth (Arnold et al., 2010; Briceño et al., 2015; Clasen et al., 2014; Fenn et al., 2012; Patil et al., 2014; Pickering et al., 2015; Pickering et al., 2019). One possible reason could be that the sanitation interventions in these studies may not have increased latrine use sufficiently to reduce exposure to fecal pathogens (Clasen et al., 2014).

A systematic review and meta-analysis of sanitation intervention studies estimated that the interventions increased latrine coverage on average by 14% and latrine use by 13% (Garn et al., 2017). Even among households with latrine access, open defecation is often still practiced by household members (Barnard et al., 2013; Coffey et al., 2014). While approaches focused on behavior change such as the Community-Led Total Sanitation (CLTS) campaign can significantly increase latrine access and reduce open defecation (Harvey, 2011; Kar and Chambers, 2008; Mosler, 2012), problems persist with the long-term sustainability of latrines such as maintenance and repairs after initial installation (Mosler et al., 2018). Fecal sludge management remains an additional problem in the absence of mechanisms to hygienically transport fecal waste away from communities and treat it before being discharged into the environment. In low-income countries, >70% of fecal waste flows into the environment untreated (World Bank, 2011). Additionally, young children often continue to defecate directly in the living environment even when latrines are present (Islam et al., 2018). For example, in rural Bangladesh, more than 75% of young children defecate in the open despite widespread access to on-site latrines, and child feces are unhygienically disposed of within the domestic environment by the majority of households (Ercumen et al., 2018; Islam et al., 2018, 2020). Child-focused interventions such as potties are rarely included in conventional sanitation programs.

In this study, we present data from a substudy nested within the sanitation and control arms of a randomized controlled trial in rural Bangladesh (WASH Benefits) that provided latrine upgrades and child feces management tools to intervention households. We used longitudinal data collected between 1 and 3.5 years after the initiation of the interventions to assess their sustained impact on indicators of latrine access, use and maintenance, and use of child feces management tools. We also investigated effect modification of intervention impacts on these indicators by time since study onset, ongoing behavior change promotion and household factors.

2. Methods

2.1. Study design

WASH Benefits was a cluster randomized controlled trial of water, sanitation, hygiene and nutrition interventions with 5551 participating compounds in rural villages in the Gazipur, Kishoreganj, Mymensingh, and Tangail districts of central Bangladesh (ClinicalTrials.gov NCT01590095) (Arnold et al., 2013; Luby et al., 2018). The study areas were chosen to not have any ongoing government or NGO programs on water, sanitation, hygiene (WASH) or nutrition. The trial enrolled compounds with a pregnant woman in her first or second trimester. Six to eight adjacent enrolled compounds were grouped into a cluster, and eight adjacent clusters were grouped into a study block. Clusters within each block were randomized to one of six intervention arms or into a double-sized control arm resulting in geographically pair-matched clusters of intervention and control compounds. A 1-km buffer was enforced between clusters to minimize spillovers; analysis of trial data found no evidence of spillover between clusters in different arms of the trial (Luby et al., 2018). The trial followed the birth cohort born to the enrolled pregnant women (referred to as "index children" hereafter) for two years to measure diarrhea and child growth. Further details of the study design have been previously described (Arnold et al., 2013).

2.2. Sanitation interventions of the WASH Benefits trial

The sanitation intervention was implemented at the compound level (group of households shared by extended families). All households in the enrolled compound received upgrades to double-pit pour-flush latrines, with labor and minor financial contributions provided by households. If the index household (household where the index child lived) did not have a latrine, a latrine was constructed; for non-index households in the compound, existing latrines were upgraded. The latrines included two pits with five concrete rings per pit, a slab, water seal and a superstructure for privacy. The dual-pit design allows alternating use of pits so that the contents of the inactive pit can undergo pathogen inactivation and be safely emptied by household members and/or used as soil amendment. The slab and the water seal provide a barrier between latrine users and fecal material and also minimize flies. In the enrolled compounds, each household with children <5 years also received one child potty, and each household received one sani-scoop for removal and safe disposal of child and animal feces. Further details of the interventions have been described elsewhere (Arnold et al., 2013).

Hardware was delivered within a behavior change program, which consisted of periodic household visits from local community health promoters trained by the trial staff. The promotion visits primarily targeted the index household but other households in enrolled compounds were encouraged to attend. The visits included sharing messages using pictorial guides and conversations with the index child's caregivers. These activities were designed to encourage participants to correctly and consistently use the provided sanitation hardware and adopt safe sanitation practices in their daily lives (Dreibelbis et al., 2013). Messaging about latrine use focused on exclusive latrine use by all adults and older children in the compound and latrine training for young children. Households were informed to transfer the latrine superstructure (walls and roof) to the second pit when the first pit became full, but no messaging was given on pit emptying and maintenance. Messaging about child feces management included potty use for children aged 6 months and older until they stared to use the latrine, safe disposal of child feces into the latrine from the potty or from the courtyard using the sani-scoop immediately after defecation, and removal of animal feces from the courtyard using the sani-scoop.

Promoters visited intervention households intensively during the first two years after intervention initiation, with six visits per month on average. Promotion frequency was reduced to one visit per month at the end of the second year, gradually tapered down during the third year and ceased at the end of the third year. Promoters did not visit households in the control arm. Further details of intervention implementation have been described elsewhere (Unicomb et al., 2018). The parent trial measured intervention uptake 1–2 years after intervention initiation. In the sanitation arm, >95% of households had a latrine with a functional water seal, children in 54% of households were observed to defecate in a potty or hygienic latrine, 27% of households used the sani-scoop to handle human feces and 15% to handle animal feces (Luby et al., 2018, Parvez et al., 2018). These measurements only focused on a few key sanitation indicators as they covered all intervention arms and did not extend beyond 2 years after intervention initiation.

2.3. Longitudinal substudy

We conducted a longitudinal substudy among a randomly selected subset of households enrolled in the sanitation and control arms of the WASH Benefits study to leverage the design and infrastructure of the large-scale randomized controlled trial. Four households per cluster were randomly selected from each sanitation cluster and from one of two control clusters in the same block, maintaining the pair-matched design of the parent trial and resulting in a sample size of 720 households (360 per arm). Households were eligible for enrollment in the substudy if the index child was alive and available or if there was another child <24 months available in the household in the case of index child death.

We visited households enrolled in the substudy approximately quarterly for a total of 8 visits between 1 and 3.5 years after intervention initiation by the parent trial. Therefore, the substudy period captured approximately one year (substudy visits 1–3) with intensive promotion activities among intervention recipients, one year (substudy visits 4–6) with less intensive promotion and six months (substudy visits 7–8) with no promotion. At each visit, field staff observed indicators of access to and use of latrines, potties and sani-scoops through spot check observations and also recorded self-reported latrine use, maintenance, sharing and pit emptying practices, and self-reported use of potties and sani-scoops/similar tools through structured questionnaires. Latrine use was separately reported for adults and child age groups with different expected defecation practices (<3 years, 3–8 years, 8–15 years).

2.4. Composite sanitation indicators

We combined the spot-check observations for each individual data collection round to generate composite binary indicators for (1) hygienic latrine access, (2) potty use and (3) sani-scoop/similar tool use. A household was defined as having access to a hygienic latrine if the primary latrine was observed to have a functional water seal, feces were contained within a septic tank/pit and there were no visible feces on the slab or floor of the latrine. We defined a household as having indicators of potty use if a potty was observed to be present, accessible by caregivers and appeared recently used. We classified the potty as accessible if the caregiver could deliver it within 30 s without assistance to field staff standing where the child usually defecates; we chose 30 s as a reasonable estimate for how quickly a regularly used item can be retrieved. We classified the potty as recently used if it was observed to be either currently wet, or dry and clean (i.e., not covered with dust). We defined a household as having indicators of sani-scoop/similar tool use if a sani-scoop or other feces removal tool was observed to be present, accessible by adults and appeared recently used. We classified the tool as accessible if it was located in or near the courtyard and could be retrieved without assistance, and as recently used based on the same visual indicators as for the potties. We did not include reported behaviors in the composite indicators as they may be subject to biased reporting but analyzed them separately as individual outcomes.

2.5. Data analysis

To assess the impact of the sanitation intervention on latrine, child potty and sani-scoop/similar tool access and use, we compared the prevalence of each individual and composite spot-check indicator and reported practice between the sanitation and control arms, using pooled data from all eight data collection rounds. We used population-averaged generalized linear models (GLM) (Hubbard et al., 2010) with a Gaussian error distribution and identity link to estimate prevalence differences (PDs) (McNutt et al., 2003), using robust standard errors at the block level. The study block represents the highest level of outcome clustering in our trial, and robust standard errors by block account for outcome correlation across multiple levels in the study, including households within study blocks and multiple visits within households. We adjusted estimates for study block to account for the geographical pair-matching. We did not include any additional adjustment covariates in our models as the randomized study design led to well-balanced study groups. We conducted a Cochran-Armitage test for each composite indicator versus data collection round (separately within each study arm) to assess any linear time trends and also qualitatively compared the prevalence of each composite indicator at each individual data collection round between study arms. As a measure of consistency of access and use, we tabulated during how many of the eight data collection rounds a given household met the composite indicators for hygienic latrine access, potty use, and sani-scoop use.

We investigated effect modification on intervention impacts on hygienic latrine access, potty use and sani-scoop/similar tool use by the following factors: time since study onset, ongoing intervention promotion, age of index child and index child's primary caregiver, education of caregiver, education of father, household wealth, number of individuals in the compound, and number of children <5 years in the compound. For the time since study onset, we used a binary indicator variable for the first half (data collection rounds 1–4) vs. second half (rounds 5–8) of the follow-up period. We also compared periods of ongoing intervention promotion (rounds 1–6) vs. no promotion (rounds 7–8). We calculated a household wealth index using principal components analysis based on measured assets and housing materials (Howe et al., 2012; Vyas and Kumaranayake, 2006), including the following: presence of electricity; number of wardrobe, table/chair/bench, bed, television, refrigerator, motorcycle, sewing machine, mobile phones; materials of the wall, roof and floor of the house; and main fuel used for cooking. Based on this index, we defined a binary indicator variable for above-versus below--median wealth. For the continuous and categorical effect modification variables, we examined the empirical data distribution and determined a cut-off that corresponds to the median to generate binary indicators for the effect modification analysis. We included additive interaction terms between study arm and these binary variables in our linear models, and we interpreted p-values <0.2 for the interaction term as evidence for effect modification.

2.6. Ethical considerations

All households provided written informed consent. The protocol was reviewed and approved by human subjects review committees at the International Centre for Diarrhoeal Disease and Research, Bangladesh (icddr,b) (PR-11063), University of California, Berkeley (2011-09-3652), and Stanford University (25863).

3. Results

We enrolled 720 households starting in June 2014 and completed data collection in December 2016. Randomization balanced baseline characteristics between households in the sanitation and control arms (Table 1). Most households (80%) participated in all eight data collection rounds, while 10% of households completed seven rounds, 4% completed six rounds, and 6% completed between one and five rounds. The percent of households completing all rounds was slightly higher in the sanitation arm (83%) than in the control arm (76%). Households lost to follow-up vs. remaining in the study had similar baseline characteristics (Contreras et al., 2021).

3.1. Observed indicators of access and use

All indicators of latrine, child potty and sani-scoop/similar tool access and use were significantly higher in households in the sanitation intervention arm versus controls pooling observations from all eight data collection rounds over the 2.5-year follow-up period (Table 2). In the sanitation arm, 94% of households had access to a hygienic latrine compared to 37% of controls (prevalence difference [PD]: 57.6%, 95% CI: 52.0–63.3, $p < 0.001$) (Table 2). Hygienic latrine access was also

Table 1
Baseline characteristics among enrolled households in rural Bangladesh.

Household characteristics	Sanitation N = 360 %(n)	Control N = 360 %(n)
Respondent's age in years, mean (SD)	24 (5)	24 (5)
Mother's education		
No or primary	56 (201)	56 (200)
Secondary or above	44 (159)	44 (160)
Father's education		
No or primary	41 (148)	43 (154)
Secondary or above	59 (211)	57 (206)
Number of rooms in household, mean (SD)	1.9 (1.1)	2.0 (1.28)
Number of households in compound[a], mean (SD)	2.6 (1.65)	2.4 (1.38)
Number of children <3 years in household, mean (SD)	1.3 (0.46)	1.3 (0.54)
Number of children <3 years in compound, mean (SD)	2.0 (1.26)	2.0 (1.12)
Households with:		
Natural wall (made by jute/bamboo/mud)	28 (100)	34 (124)
Electricity	60 (216)	57 (205)
Cell phone	87 (313)	86 (309)
Television	33 (121)	31 (113)

SD: Standard deviation.
[a] **Compound** is a group of households around a central courtyard shared by extended families.

more consistent in the sanitation arm compared to controls; 65% of sanitation arm households had access to a hygienic latrine during all eight data collection visits compared to 13% of control households, and 2% of sanitation arm households did not have hygienic latrine access during any visit compared to 44% of control households (Fig. S1).

The intervention increased child potty presence from 29% among controls to 98% in the sanitation arm (PD: 68.8, 95% CI: 63.3–74.5, p < 0.001), while most households in both arms had a tool that can be used for feces removal (Table 2). Intervention households had higher levels of visual indicators for both child potty and sani-scoop/similar tool use compared with controls, but their use was ultimately low even in intervention households. In the sanitation arm, 15% of households had indicators of child potty use compared to 4% of controls (PD: 11.9, 95% CI: 8.8–15.0, p < 0.001). No households in either arm had indicators of child potty use during all visits, while 39% of sanitation households and 89% of control households did not have indicators of child potty use during any visit (Fig. S2). In the sanitation arm, 22% of households had indicators of sani-scoop/similar tool use while 13% of controls had indicators of a feces removal tool use (PD: 9.5, 95% CI: 2.2–16.7, p = 0.01) (Table 2). Only 2% of sanitation arm households and none of the control households had indicators of sani-scoop/similar tool use during all eight visits, and 46% of sanitation arm households did not have indicators of sani-scoop/similar tool use during any visit compared to 61% of control households (Fig. S3).

3.2. Reported use and maintenance

Reported exclusive latrine use for defecation by adults increased from 77% among controls to 86% in the sanitation arm (PD: 8.8, 95% CI: 4.1–13.6, p < 0.001) and latrine use by children 3–8 years old increased from 43% to 61% (PD: 17.4, 95% CI: 10.9–23.9, p < 0.001); the intervention did not significantly impact latrine use by children <3 years or 8–15 years old (Table 3). In the sanitation arm, 19% of households shared their primary latrine with other households compared to 52% of controls (PD: −33.5, 95% CI: −40.0–27.0, p < 0.001) (Table 3). Only 4% of sanitation arm households reported emptying the pit of their primary latrine in the time between two successive data collection visits vs. 23% of controls. In both arms, approximately two thirds of households reported burying pit contents, 30% reported discharging the waste into a water body and the rest reported releasing it into fields.

Table 2
Observed indicators of access and use for hygienic latrines and child feces management tools by study arm 1–3.5 years after intervention initiation.

	Sanitation N = 2735[a] % (n)	Control N = 2658[a] % (n)	PD[b] (95% CI)	p-value
Hygienic latrine access				
Functional water seal	96.6 (2644)	39.4 (1048)	57.4 (51.8, 63.0)	<0.001
Feces well-contained within a septic tank or pit	98.1 (2673)	21.0 (559)	77.2 (72.9, 81.6)	<0.001
No visible feces on the slab or floor	98.0 (2679)	89.4 (2341)	8.7 (5.6, 11.8)	<0.001
Composite indicator (all three above)	94.4 (2583)	36.9 (981)	57.6 (52.0, 63.3)	<0.001
Child potty use				
Potty present	97.7 (2672)	29.1 (771)	68.8 (63.3, 74.5)	<0.001
Accessible by mother/caregiver[c]	20.7 (566)	4.3 (115)	16.4 (12.6, 20.3)	<0.001
Appeared recently used[d]	58.6 (1603)	11.6 (316)	47.0 (42.2, 51.9)	<0.001
Composite indicator (all three above)	15.3 (418)	3.5 (93)	11.9 (8.8, 15.0)	<0.001
Sani-scoop use				
Sani-scoop or other feces removal tool present	99.6 (2723)	88.7 (2354)	10.9 (8.2, 13.5)	<0.001
Accessible by adult[e]	39.3 (1060)	23.2 (543)	16.1 (5.1, 27.1)	0.004
Appeared recently used[d]	56.3 (1520)	51.5 (1202)	4.9 (0.1, 9.5)	0.04
Composite indicator (all three above)	22.1 (605)	12.9 (342)	9.5 (2.2, 16.7)	0.01

PD: Prevalence difference; CI: Confidence interval.
[a] Pooled data from eight data collection rounds over 2.5 years.
[b] Estimated using robust standard errors for repeated measures and geographical clustering.
[c] Can be retrieved within 30 s without assistance when standing where child usually defecates.
[d] Currently wet, or dry and clean, i.e., not covered with dust.
[e] Located in or near the courtyard and can be retrieved without assistance.

In the sanitation arm, 41% of households reported last defecation by children <3 years in a potty compared to 9% of controls (PD: 31.9, 95% CI: 27.6–36.3, p < 0.001) and 19% reported exclusive potty use for defecation by children <3 years compared to 5% of controls (PD: 14.2, 10.3–17.8, p < 0.001) (Table 3). Among households in the sanitation arm, 59% reported disposing of child feces in the latrine compared to 11% of controls (PD: 47.4, 95% CI: 42.3–52.6, p < 0.001). Households in the sanitation arm were also significantly more likely to report using a sani-scoop or similar feces removal tool every day for some form of feces disposal (PD: 15.6, 95% CI: 10.1–20.6, p < 0.001); however, reported use to specifically handle child feces was lower in the sanitation arm compared to controls (PD: −18.9, 95% CI: −24.5, −13.3, p < 0.001) (Table 3).

3.3. Time trends

Observed indicators of hygienic latrine access were steady in the sanitation arm throughout the study period (Fig. 1), including periods with no ongoing promotion. The Cochran-Armitage test indicated no significant time trends in the sanitation arm (p = 0.10) while hygienic latrine access increased significantly among controls (p = 0.001). The difference between the sanitation and control arms in hygienic latrine access was somewhat smaller during the second half of the follow-up

Table 3
Reported use of latrines and child feces management tools by study arm 1–3.5 years after intervention initiation.

	Sanitation N = 2735[a] % (n)	Control N = 2658[a] % (n)	PD[b] (95% CI)	p-value
Exclusive latrine use for defecation				
Adults	86.2 (2357)	77.4 (2056)	8.8 (4.1, 13.6)	<0.001
Children <3 years	3.5 (80)	2.7 (58)	0.8 (−0.7, 2.3)	0.27
Children 3–8 years	60.6 (949)	43.3 (644)	17.4 (10.9, 23.9)	<0.001
Children 8–15 years	88.8 (1225)	84.8 (1055)	4.0 (−1.1, 9.1)	0.12
Latrine sharing				
Latrine shared with other households	18.7 (511)	52.1 (1384)	−33.5 (−40.0, −27.0)	<0.001
Latrine maintenance and pit emptying				
Latrine not maintained/ cleaned	0.1 (2)	6.2 (164)	−6.2 (−8.2, −4.2)	<0.001
Emptied pit since previous visit	3.5 (89)	23.2 (616)	−19.3 (−23.0, −15.7)	<0.001
Potty use (for children <3 years)				
Last child defecation in potty	41.1 (1125)	9.3 (247)	31.9 (27.6, 36.3)	<0.001
Child always uses potty for defecation	18.9 (518)	4.8 (128)	14.2 (10.3, 17.8)	<0.001
Child feces disposed of in latrine	58.7 (1607)	11.3 (301)	47.4 (42.3, 52.6)	<0.001
Sani-scoop/feces removal tool use				
Scoop/tool used every day for feces disposal	89.2 (2440)	73.7 (1961)	15.6 (10.1, 20.6)	<0.001
Scoop/tool used to handle child feces	52.2 (1435)	71.1 (1899)	−18.9 (−24.5, −13.3)	<0.001

PD: Prevalence difference; CI: Confidence interval.
[a] Pooled data from eight data collection rounds over 2.5 years.
[b] Estimated using robust standard errors for repeated measures and geographical clustering.

period (PD = 53.9, 95% CI: 47.9–60.0) than the first half (PD = 60.9, 95% CI: 55.2–66.8, p-value for interaction <0.001) and somewhat smaller during periods without promotion (PD = 53.2, 95% CI: 41.1, 62.7) than periods with promotion (PD = 59.8, 95% CI: 52.4–63.7, p-value for interaction = 0.21); however, this was driven by a modest improvement in hygienic latrine access in the control arm over time while access in the intervention arm remained steadily high around 94% throughout the study period (Table S1).

Observed indicators of child potty use declined in the sanitation arm after the first follow-up visit (Fig. 2). The Cochran-Armitage test indicated a significant decrease over time in both the sanitation arm (p = 0.0001) and control arm (p = 0.03). The difference between study arms in the prevalence of households with child potty use indicators was smaller during the second half of the follow-up period (PD = 9.3, 95% CI: 5.9–12.7) than the first half (PD = 14.3, 95% CI: 10.8–17.9, p-value for interaction = 0.002) and smaller during periods without promotion (PD = 9.0, 95% CI: 5.7, 12.3) than periods with promotion (PD = 14.5, 95% CI: 11.3–17.6, p-value for interaction <0.001) (Table S2). Indicators of sani-scoop/similar tool use remained steady over time (Fig. 3) with no evidence of time trends from the Cochran-Armitage test in either the sanitation arm (p = 0.80) and control arm (p = 0.43). The difference between study arms in the prevalence of households with indicators of sani-scoop/similar tool use was similar between the two halves of the study and between periods with and without promotion (Table S3).

3.4. Household factors associated with latrine, child potty and sani-scoop access and use

Compared to controls, the sanitation intervention increased hygienic latrine access more substantially among households where index children were below the median age (28 months), households where the caregiver or the father had no/only primary education vs. secondary/higher education, households with below-median vs. above-median wealth, and compounds with ≥10 residents and ≥2 children under 5 years (all interaction p-values <0.05, Table S1). These differences were driven by lower hygienic latrine access in the control arm among households with less education, less wealth and larger number of compound residents and children; hygienic latrine access was similar across these subgroups in the sanitation arm. For example, among households where the caregiver had no or primary education, hygienic latrine access increased by 73% (from 23% among controls to 96% in the sanitation arm) while among households where the caregiver had secondary or higher education, hygienic latrine access increased by 45% (from 48% to 93%). Similarly, hygienic latrine access increased by 75% (from 21% to 95%) among households with below-median wealth and by 41% (from 52% to 93%) among those with above-median wealth. Compared to controls, the intervention increased child potty use to a greater degree among households where children were <28 months old, and households located in compounds with ≥2 children under 5 years (interaction p-values <0.2, Table S2). There were no significant effect modifiers of intervention impacts on observed sani-scoop use (Table S3).

4. Discussion

In our substudy nested within a randomized controlled trial that provided sanitation hardware along with initial intensive behavior change promotion, the intervention led to a sustained increase in access to a hygienic latrine with a functioning water seal, feces well-contained in a septic tank or pit, and no feces on the slab or floor of the latrine 1–3.5 years after intervention initiation. Access remained high during periods with less intensive or no promotion. The gains in hygienic latrine access were more pronounced among households with less education, less wealth and larger number of residents and children. While the intervention significantly increased reported exclusive latrine use by adults and children 3–8 years old, reported exclusive latrine use by adults was high (77%) among controls, indicating existing sanitation norms and habits. The intervention increased availability of child feces management tools but their use remained low among intervention recipients, and any gains in potty use in the intervention arm declined over time.

The parent WASH Benefits study was an efficacy trial where latrine upgrades were provided to households free of charge and accompanied by initial intensive behavior change promotion. As such, the increase in hygienic latrine access among intervention recipients was substantially higher than what has typically been achieved by programmatic sanitation improvements. A systematic review on sustained adoption of sanitation interventions (defined as latrine ownership, presence, quality, functionality or use after the end of the intervention period) found that the most influential program factors associated with sustainability included frequent, personal contact with a health promoter and accountability over a period of time (K. Hulland et al., 2015). A study in Bangladesh, focused on areas that were previously declared “open defecation free” because all residents had installed latrines in response to a government program, found that 4.5 years later, households were more likely to have an improved or shared latrine if they received follow-up visits by a community health promoter about latrine use (Hanchett et al., 2011). Our study implemented intensive in-person promotion (6 visits/month) for the first two years after intervention initiation and less frequent promotion during the third year. No apparent decrease in hygienic latrine access after promotion activities tapered down or ceased suggests that functional hygienic latrines, once

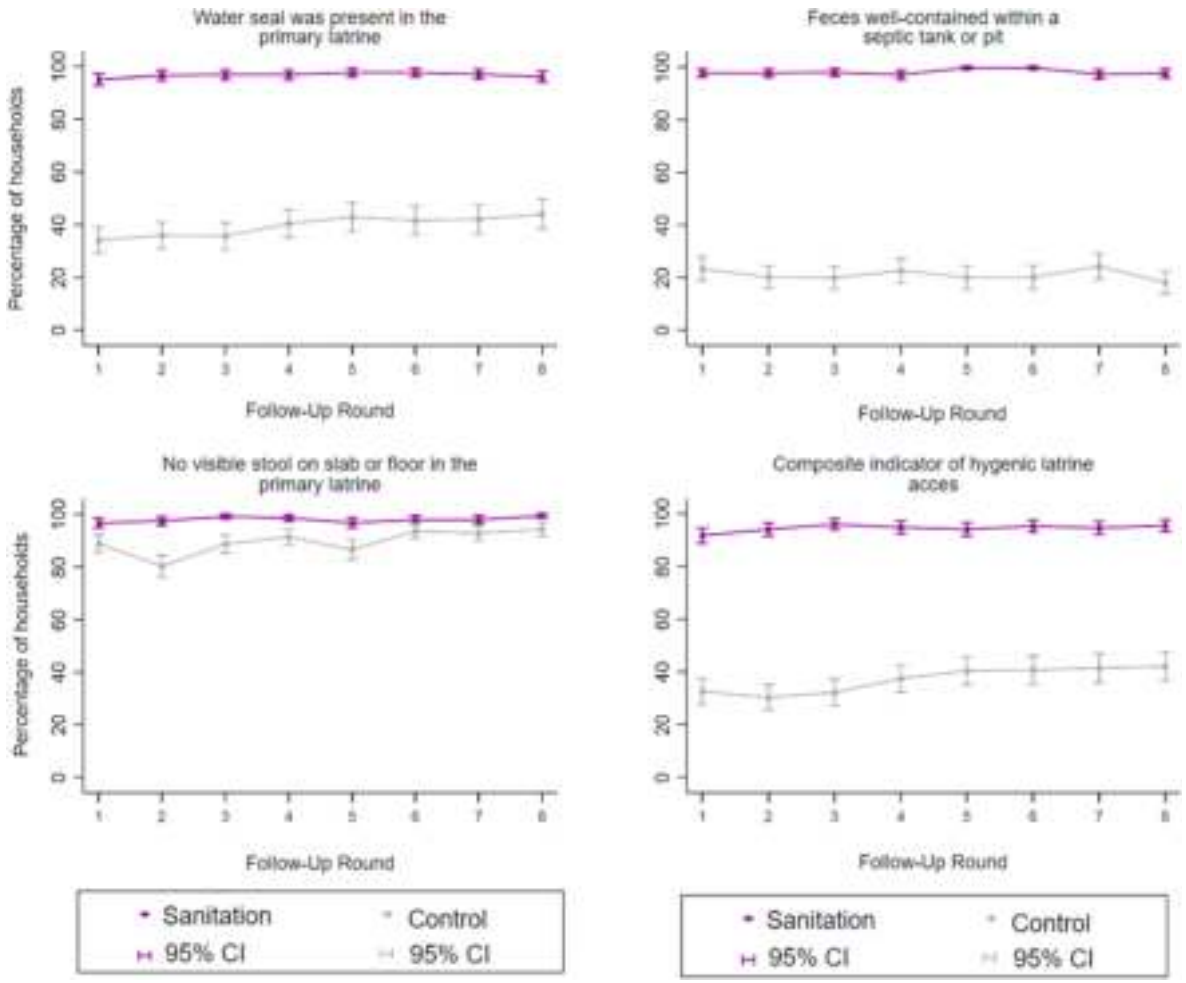

Fig. 1. Observed indicators of hygienic latrine access by study arm and round of data collection. Composite indicator defined as the primary latrine observed to have a functional water seal, feces contained within a septic tank/pit and no visible feces on the slab or floor of the latrine. Data were collected between June 2014–December 2016, with each round completed over approximately three months. Intensity of intervention promotion was high during rounds 1–3 and low during rounds 4–6; there was no promotion during rounds 7–8. Each data collection round included approximately 720 households (360 per arm).

constructed, can be maintained without ongoing behavior change promotion. However, we note that our study was conducted in a setting with existing sanitation habits, as evidenced by high reported latrine use and secular increases in observed hygienic latrine access over time among controls. These existing norms may have supported ongoing maintenance of hygienic latrines among intervention recipients regardless of promotion activities.

Households in the sanitation arm also emptied the latrine pit less frequently, which may have been due to the recent construction of the pit latrine by the study, the dual-pit design which requires less frequent desludging, and slower pit fill-up due to reduced latrine sharing. Our findings are consistent with studies in Mozambique which found less frequent emptying of septic tanks at intervention compounds which received sanitation upgrades, including construction of sanitation infrastructure or subsidized provision of pour-flush toilets draining to a septic tank (Bauerl et al., 2016; Capone et al., 2020). In the latter of these two studies, the intervention was also significantly associated with increased hygienic emptying of septic tanks (Capone et al., 2020). In our study, unhygienic pit emptying practices (e.g., release into waterbodies) persisted among approximately a third of intervention recipients. We note that the delivered sanitation intervention did not include any hardware, facilities or behavioral messaging for safe emptying of pits. However, the double-pit design was intended to allow households to switch the super structure to the second pit when the first one filled up such that the contents of the first pit could decompose before being emptied. Many households in rural Bangladesh are located close to a pond, and intervention households may have continued to empty pits into water bodies out of convenience and habit. Future work should identify barriers against safe emptying of latrine pits to move up the sanitation ladder toward safely managed sanitation services.

Interventions substantially increased availability of child potties while the majority of households in both groups had a scoop or similar tool that can be used for removal of child feces. However, the use of these child feces management tools was low and inconsistent among intervention recipients. Similarly, in a recent trial in India that promoted safe child feces management along with broader latrine use, the intervention increased the percentage of households where the caregiver safely disposed of child feces by approximately 20 percentage points but only a third of intervention recipients practiced safe disposal (Caruso et al., 2022). Previous studies have identified latrine access and use by adults, and availability of child feces management tools as important determinants of safe child feces disposal (Ellis et al., 2020; Miller-Petrie et al., 2016). In our study, child feces management products as well as latrines were provided for free, and latrine use among adults was high among intervention recipients, suggesting additional barriers to child feces management. Such barriers may include lack of perceived risk from child feces, time needed for child feces management and competing demands on caregivers' time for household tasks or income-generating activities, and social norms (Ellis et al., 2020; K. R. Hulland et al., 2015; Mbuya and Humphrey, 2016; Miller-Petrie et al.,

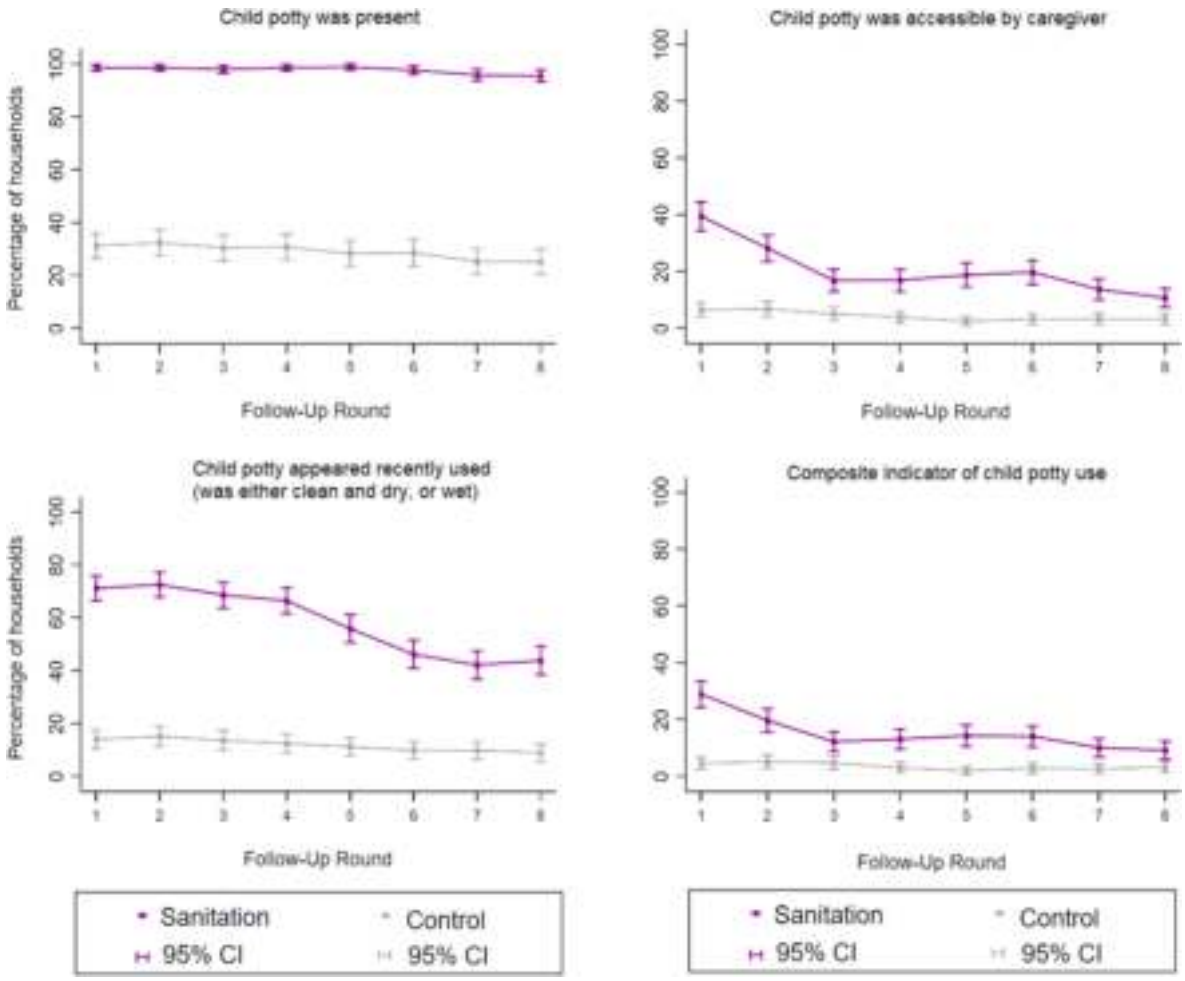

Fig. 2. Observed indicators of child potty use by study arm and round of data collection. Composite indicator defined as a potty that was observed to be present, accessible by caregivers and appeared recently used. Data were collected between June 2014–December 2016, with each round completed over approximately three months. Intensity of intervention promotion was high during rounds 1–3 and low during rounds 4–6; there was no promotion during rounds 7–8. Each data collection round included approximately 720 households (360 per arm).

2016).

In rural Bangladesh, potties are uncommon and not widely available for purchase (Sultana et al., 2013), and rural parents are typically not aware about the benefits of using potties or may not know how to train their children to use the potty (Hussain et al., 2017). Stool of young children is typically considered harmless or less harmful and less disgusting than adult feces in South Asia (Brown et al., 2013), and therefore, consistently using a child potty for child defecation and scoop for child feces disposal may be less prioritized. The potties and scoops provided as part of the WASH Benefits interventions were iteratively piloted and tailored with community input, using the integrated behavioral model for water, sanitation and hygiene (IBM-WASH) (Dreibelbis et al., 2013). During the piloting stage, semi-structured interviews, group discussions, and observations among 26 households in the study area indicated that caregivers found the potties acceptable and reported time savings from using a potty for child feces management (Hussain et al., 2017). Some children refused to defecate in the potty, and younger children (<1 year old) were too small to sit on it and had to be held (Hussain et al., 2017). The behavior change program provided along with the WASH Benefit interventions included discussions and activities on potty familiarization, potty training, problems children encountered while defecating in the potty, potty cleaning and maintenance, benefits and barriers of using potty, and feces disposal location (Hussain et al., 2017). Future studies should investigate additional strategies to promote safe child feces management practices in low-income settings.

Our previous work in rural Bangladesh suggested that unsafe child feces disposal is associated with increased *E. coli* contamination of child hands and stored drinking water but showed no clear associations with child gastrointestinal illness (Islam et al., 2018, 2020). A study in rural India found higher levels of *E. coli* on the floor or ground after a child defecated on these surfaces and the feces were removed, and on tools used to dispose of the feces even after cleaning (Bauza et al., 2020). In the same study, unsafe child feces disposal was associated with increased levels of *E. coli* in stored drinking water and on caregiver hands (Bauza et al., 2020). A microbial source tracking study in urban slums in Kenya found that young children's feces were the main source of fecal contamination detected inside the home environment, while contamination detected outside the home was more commonly from feces of adults and older children (Bauza et al., 2019). Out of five studies reviewed in a recent meta-analysis, two found that safe child defecation and safe feces disposal was associated with a reduction in diarrhea while the others did not find an association (Morita et al., 2016). In a study in rural Bangladesh, children in households where caregivers reported unsafe child feces disposal had higher environmental enteropathy scores and odds of being wasted (George et al., 2016). A study in Mozambique suggested that deposition of a small amount of child feces onto soil can support ongoing transmission of *Ascaris* infections (Capone et al., 2022). These findings suggest that child feces are a potential source of fecal pathogens in the environment that could contribute to adverse effects on

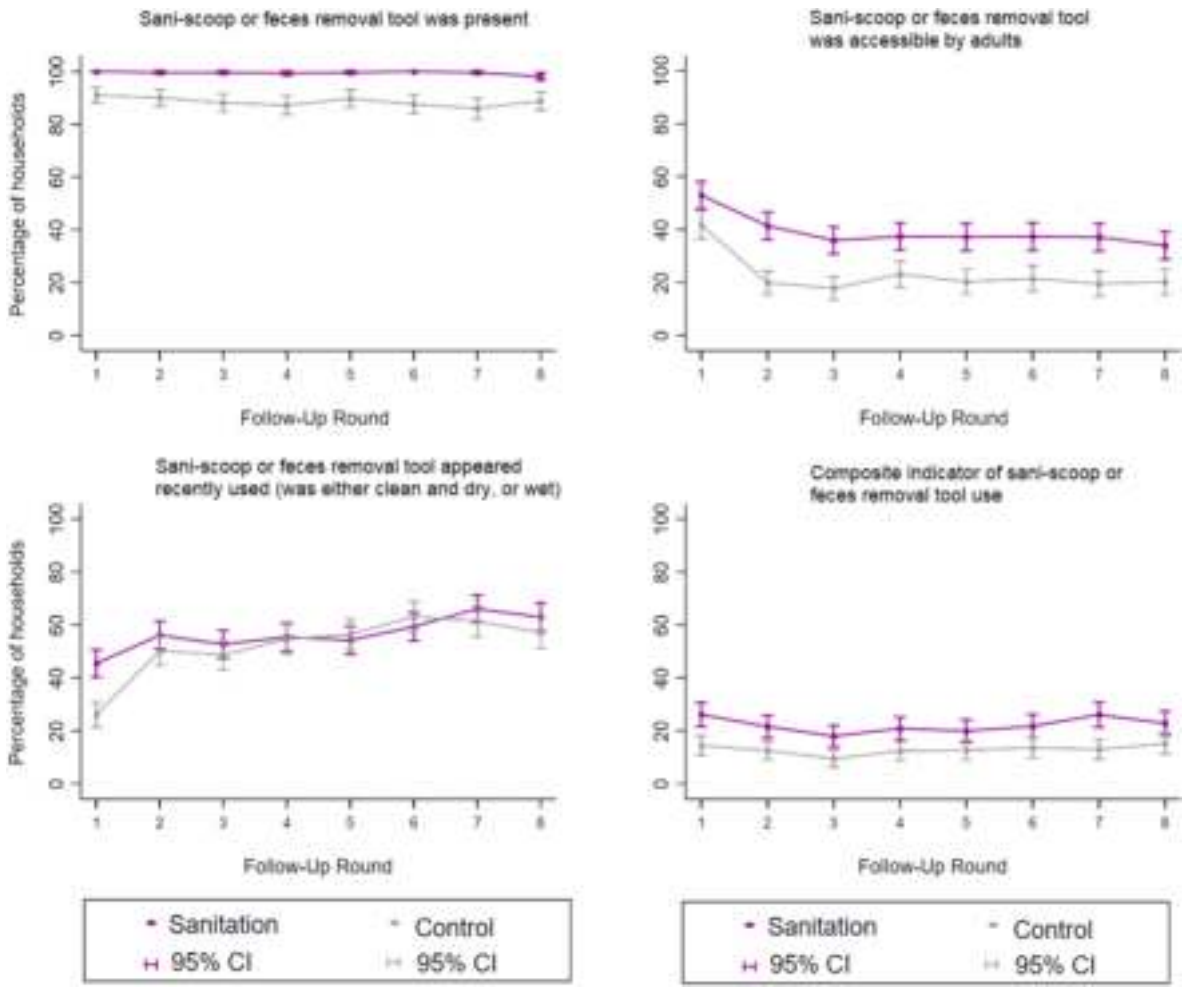

Fig. 3. Observed indicators of sani-scoop or feces removal tools use by study arm and round of data collection. Composite indicator defined as a sani-scoop or other feces removal tool that was observed to be present, accessible by adults and appeared recently used. Data were collected between June 2014–December 2016, with each round completed over approximately three months. Intensity of intervention promotion was high during rounds 1–3 and low during rounds 4–6; there was no promotion during rounds 7–8. Each data collection round included approximately 720 households (360 per arm).

child health. Sanitation programs that solely focus on the feces of adults and older children are unlikely to reduce fecal contamination in the home environment without measures for hygienic defecation and feces disposal for young children. Interventions aiming to reduce fecal exposure should continue to develop and test approaches to reduce child feces in the environment, alone or combined with broader sanitation improvements (Caruso et al., 2022; Sclar et al., 2022).

Our study had some limitations. While the observable uptake indicators objectively reflected the availability and condition of infrastructure and supplies they may not accurately represent actual use as availability does not ensure use. However, our observed indicators included visual signs of likely recent use, such as the tools being wet or free of dust. Additionally, our study had a longitudinal design where we visited the same households several times. Therefore, it is possible that anticipation of a data collection visit might alert participants to improve their practices during the data collection period and, therefore, overestimate uptake (Arnold et al., 2015). We attempted to reduce this limitation by arriving unannounced to minimize reactivity (Cousens et al., 1996). Another limitation is that the intervention was delivered under optimal conditions during an efficacy trial, and so these findings do not readily generalize to routine programs with more limited resources.

5. Conclusion

In a randomized controlled trial that provided latrine upgrades along with initial intensive behavior change promotion, high access to hygienic latrines with a functional water seal and well-contained fecal waste was sustained among intervention recipients up to 3.5 years after intervention initiation. Access was high even after behavior change promotion stopped. Our findings from a setting with existing sanitation norms indicate that latrine quality, once established, can be maintained without ongoing promotion activities. Future studies should investigate strategies to achieve and sustain access to and use of latrines that effectively isolate feces from the environment in settings with different baseline sanitation norms. Despite free provision of potties and sani-scoop tools and messages on safe child feces management included in the behavior change promotion program, adoption of these tools remained low among intervention recipients. Studies should investigate barriers to safe child feces management practices and strategies to ensure their sustained adoption, as well as test the effectiveness of child feces management interventions, alone or combined with broader sanitation improvements, in reducing fecal exposures.

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Data statement

De-identified data used for this analysis will be made freely available on OSF upon publication (https://osf.io/6u7cn/).

Declaration of competing interest

We declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114149.

References

Arnold, B.F., Khush, R.S., Ramaswamy, P., London, A.G., Rajkumar, P., Ramaprabha, P., Colford, J.M., 2010. Causal inference methods to study nonrandomized, preexisting development interventions. Proc. Natl. Acad. Sci. USA 107 (52), 22605–22610.

Arnold, B.F., Khush, R.S., Ramaswamy, P., Rajkumar, P., Durairaj, N., Ramaprabha, P., Colford Jr., J.M., 2015. Reactivity in rapidly collected hygiene and toilet spot check measurements: a cautionary note for longitudinal studies. Am. J. Trop. Med. Hyg. 92 (1), 159–162.

Arnold, B.F., Null, C., Luby, S.P., Unicomb, L., Stewart, C.P., Dewey, K.G., Clasen, T., 2013. Cluster-randomised controlled trials of individual and combined water, sanitation, hygiene and nutritional interventions in rural Bangladesh and Kenya: the WASH Benefits study design and rationale. BMJ Open 3 (8), e003476.

Barnard, S., Routray, P., Majorin, F., Peletz, R., Boisson, S., Sinha, A., Clasen, T., 2013. Impact of Indian Total Sanitation Campaign on latrine coverage and use: a cross-sectional study in Orissa three years following programme implementation. PLoS One 8 (8).

Bauerl, M., Arsénio, A., Muximpua, O., Hawkins, P., 2016. Emptying of Sanitation Facilities in Maputo, Mozambique. Assessment of Attitudes and Practices at a Household Level.

Bauza, V., Madadi, V., Ocharo, R.M., Nguyen, T.H., Guest, J.S., 2019. Microbial source tracking using 16S rRNA amplicon sequencing identifies evidence of widespread contamination from young children's feces in an urban slum of Nairobi, Kenya. Environ. Sci. Technol. 53 (14), 8271–8281.

Bauza, V., Majorin, F., Routray, P., Sclar, G.D., Caruso, B.A., Clasen, T., 2020. Child feces management practices and fecal contamination: a cross-sectional study in rural Odisha, India. Sci. Total Environ. 709, 136169.

Briceño, B., Coville, A., Martinez, S., 2015. Promoting Handwashing and Sanitation: Evidence from a Large-Scale Randomized Trial in Rural Tanzania. The World Bank.

Brown, J., Cairncross, S., Ensink, J.H., 2013. Water, sanitation, hygiene and enteric infections in children. Arch. Dis. Child. 98 (8), 629–634.

Capone, D, Barker, T, Cumming, O, Flemister, A, Geason, R, Kim, E, Knee, J, Linden, Y, Manga, M, Meldrum, M, Nala, R, 2022. Persistent Ascaris Transmission Is Possible in Urban Areas Even Where Sanitation Coverage Is High. Environ. Sci. Technol. 56 (22), 15969–15980. Oct 26.

Capone, D., Buxton, H., Cumming, O., Dreibelbis, R., Knee, J., Nalá, R., Brown, J., 2020. Impact of an intervention to improve pit latrine emptying practices in low income urban neighborhoods of Maputo, Mozambique. Int. J. Hyg Environ. Health 226, 113480.

Caruso, B.A., Sclar, G.D., Routray, P., Nagel, C.L., Majorin, F., Sola, S., Clasen, T., 2022. Effect of a Low-Cost Behaviour Change Intervention on Latrine Use and Safe Child Faeces Disposal in Rural Odisha, India: A Cluster-Randomized Controlled Trial. India: A Cluster-Randomized Controlled Trial.

Clasen, T., Boisson, S., Routray, P., Torondel, B., Bell, M., Cumming, O., Odagiri, M., 2014. Effectiveness of a rural sanitation programme on diarrhoea, soil-transmitted helminth infection, and child malnutrition in Odisha, India: a cluster-randomised trial. Lancet Global Health 2 (11), e645–e653.

Coffey, D., Gupta, A., Hathi, P., Khurana, N., Spears, D., Srivastav, N., Vyas, S., 2014. Revealed preference for open defecation. Econ. Polit. Wkly. 49 (38), 43.

Contreras, J.D., Islam, M., Mertens, A., Pickering, A.J., Kwong, L.H., Arnold, B.F., Sen, D., 2021. Longitudinal effects of a sanitation intervention on environmental fecal contamination in a cluster-randomized controlled trial in rural Bangladesh. Environ. Sci. Technol. 55 (12), 8169–8179.

Cousens, S., Kanki, B., Toure, S., Diallo, I., Curtis, V., 1996. Reactivity and repeatability of hygiene behaviour: structured observations from Burkina Faso. Soc. Sci. Med. 43 (9), 1299–1308.

Dreibelbis, R., Winch, P.J., Leontsini, E., Hulland, K.R., Ram, P.K., Unicomb, L., Luby, S. P., 2013. The integrated behavioural model for water, sanitation, and hygiene: a systematic review of behavioural models and a framework for designing and evaluating behaviour change interventions in infrastructure-restricted settings. BMC Publ. Health 13 (1), 1015.

Ellis, A., McClintic, E.E., Awino, E.O., Caruso, B.A., Arriola, K.R., Ventura, S.G., Webb-Girard, A., 2020. Practices and perspectives on Latrine use, child feces disposal, and clean play environments in Western Kenya. Am. J. Trop. Med. Hyg. 102 (5), 1094.

Ercumen, A., Pickering, A.J., Kwong, L.H., Mertens, A., Arnold, B.F., Benjamin-Chung, J., Islam, S., 2018. Do sanitation improvements reduce fecal contamination of water, hands, food, soil, and flies? Evidence from a cluster-randomized controlled trial in rural Bangladesh. Environ. Sci. Technol. 52 (21), 12089–12097.

Fenn, B., Bulti, A.T., Nduna, T., Duffield, A., Watson, F., 2012. An evaluation of an operations research project to reduce childhood stunting in a food-insecure area in Ethiopia. Publ. Health Nutr. 15 (9), 1746–1754.

Garn, J.V., Sclar, G.D., Freeman, M.C., Penakalapati, G., Alexander, K.T., Brooks, P., Clasen, T.F., 2017. The impact of sanitation interventions on latrine coverage and latrine use: a systematic review and meta-analysis. Int. J. Hyg Environ. Health 220 (2), 329–340.

George, C.M., Oldja, L., Biswas, S., Perin, J., Sack, R.B., Ahmed, S., Azmi, I.J., 2016. Unsafe child feces disposal is associated with environmental enteropathy and impaired growth. J. Pediatr. 176, 43–49.

Hanchett, S., Krieger, L., Kahn, M.H., Kullmann, C., Ahmed, R., 2011. Long-term Sustainability of Improved Sanitation in Rural Bangladesh.

Harvey, P., 2011. Community-led total sanitation, Zambia: stick, carrot or balloon? Waterlines 95–105.

Howe, L.D., Galobardes, B., Matijasevich, A., Gordon, D., Johnston, D., Onwujekwe, O., Hargreaves, J.R., 2012. Measuring socio-economic position for epidemiological studies in low-and middle-income countries: a methods of measurement in epidemiology paper. Int. J. Epidemiol. 41 (3), 871–886.

Hubbard, A.E., Ahern, J., Fleischer, N.L., Van der Laan, M., Satariano, S.A., Jewell, N., Satariano, W.A., 2010. To GEE or not to GEE: comparing population average and mixed models for estimating the associations between neighborhood risk factors and health. Epidemiology 467–474.

Hulland, K., Martin, N., Dreibelbis, R., Valliant, J., Winch, P., 2015. What Factors Affect Sustained Adoption of Safe Water, Hygiene and Sanitation Technologies? A Systematic Review of Literature. EPPI-Centre, Social Science Research Unit, UCL Institute of Education, University College London, London.

Hulland, K.R., Chase, R.P., Caruso, B.A., Swain, R., Biswal, B., Sahoo, K.C., Dreibelbis, R., 2015. Sanitation, stress, and life stage: a systematic data collection study among women in Odisha, India. PLoS One 10 (11), e0141883.

Hussain, F., Luby, S.P., Unicomb, L., Leontsini, E., Naushin, T., Buckland, A.J., Winch, P. J., 2017. Assessment of the acceptability and feasibility of child potties for safe child feces disposal in rural Bangladesh. Am. J. Trop. Med. Hyg. 97 (2), 469–476.

Islam, M., Ercumen, A., Ashraf, S., Rahman, M., Shoab, A.K., Luby, S.P., Unicomb, L., 2018. Unsafe disposal of feces of children< 3 years among households with latrine access in rural Bangladesh: association with household characteristics, fly presence and child diarrhea. PLoS One 13 (4), e0195218.

Islam, M., Rahman, M., Unicomb, L., Kafi, M.A.H., Rahman, M., Alam, M., Hubbard, A.E., 2020. Child defecation and feces management practices in rural Bangladesh: associations with fecal contamination, observed hand cleanliness and child diarrhea. PLoS One 15 (7), e0236163.

Kar, K., Chambers, R., 2008. Handbook on Community-Led Total Sanitation.

Luby, S.P., Rahman, M., Arnold, B.F., Unicomb, L., Ashraf, S., Winch, P.J., Benjamin-Chung, J., 2018. Effects of water quality, sanitation, handwashing, and nutritional interventions on diarrhoea and child growth in rural Bangladesh: a cluster randomised controlled trial. Lancet Global Health 6 (3), e302–e315.

Mbuya, M.N., Humphrey, J.H., 2016. Preventing environmental enteric dysfunction through improved water, sanitation and hygiene: an opportunity for stunting reduction in developing countries. Matern. Child Nutr. 12, 106–120.

McNutt, L.-A., Wu, C., Xue, X., Hafner, J.P., 2003. Estimating the relative risk in cohort studies and clinical trials of common outcomes. Am. J. Epidemiol. 157 (10), 940–943.

Miller-Petrie, M.K., Voigt, L., McLennan, L., Cairncross, S., Jenkins, M.W., 2016. Infant and young child feces management and enabling products for their hygienic collection, transport, and disposal in Cambodia. Am. J. Trop. Med. Hyg. 94 (2), 456.

Morita, T., Godfrey, S., George, C.M., 2016. Systematic review of evidence on the effectiveness of safe child faeces disposal interventions. Trop. Med. Int. Health 21 (11), 1403–1419.

Mosler, H.-J., 2012. A systematic approach to behavior change interventions for the water and sanitation sector in developing countries: a conceptual model, a review, and a guideline. Int. J. Environ. Health Res. 22 (5), 431–449.

Mosler, H.-J., Mosch, S., Harter, M., 2018. Is Community-Led Total Sanitation connected to the rebuilding of latrines? Quantitative evidence from Mozambique. PLoS One 13 (5).

Parvez, SM, Azad, R, Rahman, M, Unicomb, L, Ram, PK, Naser, AM, Stewart, CP, Jannat, K, Rahman, MJ, Leontsini, E, Winch, PJ, 2018 Dec. Achieving optimal technology and behavioral uptake of single and combined interventions of water, sanitation hygiene and nutrition, in an efficacy trial (WASH benefits) in rural Bangladesh. Trials 19, 1–6.

Patil, S.R., Arnold, B.F., Salvatore, A.L., Briceno, B., Ganguly, S., Colford Jr., J.M., Gertler, P.J., 2014. The effect of India's total sanitation campaign on defecation

behaviors and child health in rural Madhya Pradesh: a cluster randomized controlled trial. PLoS Med. 11 (8), e1001709.
Pickering, A.J., Djebbari, H., Lopez, C., Coulibaly, M., Alzua, M.L., 2015. Effect of a community-led sanitation intervention on child diarrhoea and child growth in rural Mali: a cluster-randomised controlled trial. Lancet Global Health 3 (11), e701–e711.
Pickering, A.J., Null, C., Winch, P.J., Mangwadu, G., Arnold, B.F., Prendergast, A.J., Benjamin-Chung, J., 2019. The WASH Benefits and SHINE trials: interpretation of WASH intervention effects on linear growth and diarrhoea. Lancet Global Health 7 (8), e1139–e1146.
Sclar, G.D., Bauza, V., Mosler, H.-J., Bisoyi, A., Chang, H.H., Clasen, T.F., 2022. Study design and rationale for a cluster randomized trial of a safe child feces management intervention in rural Odisha, India. BMC Publ. Health 22 (1), 1–12.
Sultana, R., Mondal, U.K., Rimi, N.A., Unicomb, L., Winch, P.J., Nahar, N., Luby, S.P., 2013. An improved tool for household faeces management in rural Bangladeshi communities. Trop. Med. Int. Health 18 (7), 854–860.
Unicomb, L., Begum, F., Leontsini, E., Rahman, M., Ashraf, S., Naser, A.M., Parvez, S.M., 2018. WASH Benefits Bangladesh trial: management structure for achieving high coverage in an efficacy trial. Trials 19 (1), 1–11.
Vyas, S., Kumaranayake, L., 2006. Constructing socio-economic status indices: how to use principal components analysis. Health Pol. Plann. 21 (6), 459–468.
WHO, UNICEF, July, 2021. Billions of people will lack access to safe water, sanitation and hygiene in 2030 unless progress quadruples – warn WHO. UNICEF. https://www.unicef.org/bangladesh/en/press-releases/billions-people-will-lack-access-safe-water-sanitation-and-hygiene-2030-unless.
2011 World Bank, 2011 Jan. Economic impacts of inadequate sanitation in India. The World Bank, pp. 1–128. Report No.: 68159. Available: http://documents.worldbank.org/curated/en/820131468041640929/Economic-impacts-of-inadequate-sanitation-in-India.

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Associations of per- and polyfluoroalkyl substances and alternatives with reproductive hormones in women of childbearing age

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ABSTRACT

Background: Experimental studies suggested that per- and polyfluoroalkyl substances (PFAS) may have endocrine-disrupting effects. However, the epidemiological evidence on the associations of PFAS with female reproductive hormones is sparse and limited to perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA).

Objective: To evaluate effects of legacy and emerging PFAS alternatives on female reproductive hormones.

Methods: A total of 433 reproductive-aged females were recruited from 2014 to 2016. Information on age, age at menarche, gravity, menstrual cycle, BMI, education, and income was obtained from medical records and questionnaires. Serum samples were collected for reproductive hormones, and plasma samples for PFAS measurement by ultraperformance liquid chromatography - tandem mass spectrometer (UPLC-MS/MS). Multiple linear regression and quantile g-computation (q-gcomp) were used to examine the associations of individual PFAS and their mixture with reproductive hormones.

Results: Multiple linear regression analysis showed significant effects of certain PFAS on total testosterone (TT): adjusted estimate (β) for perfluoroheptanoic acid (PFHpA) was 0.57 (95% CI: 0.18, 0.97). Moreover, a positive association was detected between PFAS mixture and TT in the q-gcomp model. Higher concentrations of 6:2 chlorinated polyfluorinated ether sulfonic acid (6:2 Cl-PFESA) were associated with significantly lower prolactin level ($\beta = -0.07$, 95% CI: −0.14, −0.001).

Conclusion: Our study found that exposure to PFAS alternatives was associated with altered levels of reproductive hormones in women of childbearing age.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) are a class of persistent environmental organic chemicals and widely used in consumer and commercial products, including textiles, nonstick cookware, food packaging, and firefighting foams (Sunderland et al., 2019). Humans are ubiquitously exposed to PFAS through diet, drinking water and indoor/outdoor environment (De Silva et al., 2021). Perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) are the two most well-known PFAS. Numerous animal and human studies have demonstrated that these chemicals have developmental toxicity, reproductive toxicity, neurotoxicity, carcinogenicity (Steenland et al., 2010; Tsuda, 2016). Both PFOS and PFOA have, therefore, been banned in Europe and North America in recent years (Stockholm Convention, 2009; Official Journal of the European Union, 2017). Consequently, PFAS alternatives have been rapidly developed and came into market in a large quantity.

Short-chain PFAS analogues, defined as $C < 8$ for perfluorocarboxylic acid (PFCAs) and $C < 6$ for perfluoroalkyl sulfonic acids (PFSAs), are one class of PFAS alternatives (ITRC, 2020). Perfluorobutanoic acid (PFBA) and perfluorobutane sulfonate (PFBS) are the most common representatives. Structurally more complex

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alternatives such as 6:2 chlorinated polyfluorinated ether sulfonic acid (6:2 Cl-PFESA) and hexafluoropropylene oxide dimer acid (HFPO-DA, also called GenX) have also emerged (Brase et al., 2021). From 2016 to 2021, the concentrations of PFBA (median concentration ranging from 6.25 to 28.4 ng/L), PFBS (from 2.15 to 19.9 ng/L) and HFPO-DA (from 0.77 to 3.10 ng/L), have been increasing in Chinese rivers and lakes (Yao et al., 2022). Studies showed that emerging PFAS alternatives have similar multi-system toxicity as legacy PFAS do (Jane et al., 2022). Nonetheless, little is known regarding the potential effects of these emerging PFAS alternatives on reproductive hormones.

PFAS may interfere with the endocrine homeostasis, including reproductive hormones (Barrett et al., 2015; Harlow et al., 2021; Ding et al., 2022). Reproductive hormones in reproductive-aged women, play a vital role in the follicle growth, endometrial maturation, and regulation of metabolism (Barbieri, 2014; Taraborrelli, 2015; Macotela et al., 2020). Furthermore, normal homeostasis of reproductive hormones is critical in maintaining regular menstruation and successful conception (Jabbour et al., 2006; Maybin and Critchley, 2012; Ben-Nagi and Panay, 2014).

A few studies have investigated the relationship between PFAS exposure and reproductive hormones in women of child-bearing age. The results were inconsistent. Some studies found no significant association between PFAS exposure and reproductive hormone levels in healthy adult women (Knox et al., 2011; Tsai et al., 2015; Heffernan et al., 2018; Zhang et al., 2018), while other studies noted that PFAS had endocrine-disruption effects (Barrett et al., 2015; Harlow et al., 2021; Ding et al., 2022).

So far, no study has explored the association of emerging PFAS alternatives with reproductive hormones. Additionally, most previous reports adopted conventional multiple regression models to estimate the PFAS toxicities without accounting for the joint exposure of individual PFAS with strong correlations. Thus, we conducted a cross-sectional analysis to explore the association between legacy and emerging PFAS exposure and reproductive hormones with advanced statistical analyses. As emerging PFAS alternatives have been widely used in China (Wang et al., 2013; Pan et al., 2018), and their levels in Chinese rivers and lakes (0.17–6.59 ng/L) are much higher than those in other countries (0.01–0.05 ng/L) (Pan et al., 2018), it is urgent to evaluate reproductive toxicities of PFAS alternatives.

2. Methods

2.1. Study population

We used data from a multi-center, case-control study on the association between environmental endocrine disruptors and unexplained recurrent miscarriage. The study design, eligibility, recruitment, and investigation have been described in detail previously (Nian et al., 2022a). The current analysis used data from 560 controls only. They were healthy, fertile women who underwent a physical examination, had no chronic diseases (cancer, diabetes, cardiovascular disorders, lung, kidney, and liver diseases), endocrine disorders (endocrine neoplasia, thyroid disease, and reproductive disease), autoimmune diseases (autoimmune thyroid disease, systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis), or active infection (toxoplasmosis, rubella virus, cytomegalovirus, herpes simplex virus, hepatitis viruses, and human immunodeficiency virus). We excluded 120 individuals who had inadequate amount of plasma to measure PFAS and 7 individuals whose data on reproductive hormone were missing, leaving 433 women for this analysis. Table S1 compares the characteristics of included and excluded participants. A written informed consent was obtained from all the participants before investigation. All participants were interviewed by a research assistant using a standard questionnaire on demographic characteristics, socioeconomic status, lifestyle, and medical history. Women went through a complete workup, including the measurements of reproductive hormones, e.g., follicle stimulating hormone (FSH), luteinizing Hormone (LH), prolactin (PRL), estradiol (E_2), progesterone (P), and total testosterone (TT) in the morning of Days 2–4 of a menstrual cycle. This study was approved by the Ethics Committee of Xinhua Hospital affiliated to Shanghai Jiao Tong University School of Medicine (XHEC-C-2015-046).

2.2. Measurements of plasma PFAS

Detailed information regarding PFAS measurement was provided previously (Nian et al., 2022a, 2022b). Briefly, we measured the concentration of 35 PFAS using 0.1 mL plasma with ultra-performance liquid chromatography (UPLC, Agilent 1290) connected to an Agilent 6495C triple quadrupole tandem mass spectrometry (Agilent Technologies, Palo Alto and Santa Clara, CA USA). Our current analysis only used individual PFAS with a detection rate >85% in the population, including three emerging PFAS alternatives [(6:2 Cl-PFESA, 8:2 chlorinated perfluoroalkyl ether sulfonic acid (8:2 Cl-PFESA), and HFPO-DA)], four short-chain PFAS [perfluorobutanesulfonic acid (PFBS), perfluorobutanoic acid (PFBA), perfluoroheptanoic acid (PFHpA), perfluorohexanoic acid (PFHxA)], and eight legacy PFAS [PFOA, PFOS, perfluorohexanesulfonic acid (PFHxS), perfluoroheptanesulfonic acid (PFHpS), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), and perfluorododecanoic acid (PFDoA)]. We defined the limit of detection (LOD) as the peak analyte level with a signal-to-noise (S/N) ratio of 3. And the values below the LOD were replaced with the LOD divided by the square root of 2 (Hornung RW, 1990).

2.3. Outcome assessment

Blood samples were obtained by venipuncture between days 2 and 4 of women's menstrual cycle and centrifuged at 4 °C for 3000 rpm for 10 min. The serum samples were used directly for reproductive hormones measurements. FSH, LH, E_2, PRL, P, and TT were measured with chemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany) (Jiao et al., 2021). For the concentrations below LOD, we replaced with a value of LOD/$\sqrt{2}$.

2.4. Confounders

Potential confounders were selected based on previous literature (Tsai et al., 2018; Park et al., 2019; Chang et al., 2021; Li et al., 2022), including age (year, continuous), age at menarche (year, continuous), body mass index (BMI, kg/m^2, continuous), gravidity (nulliparous, 1–2, and ≥3), menstrual cycle (regularity, and irregularity), education level (primary school, middle school, and higher than middle school), annual family income (<100,000 CNY, ≥100,000 CNY, and missing), and study sites.

2.5. Statistical analysis

Mean ± standard deviation (SD), median with interquartile range (IQR), and numbers and percentage were used to describe the characteristics of variables with normal, skewed distributions and categorical data, respectively. PFAS concentrations were expressed as median (IQRs), and were natural log transformed to reduce skewness in further analyses. Considering reproductive hormones were measured in two different hospitals, we calculated the z-score to improve the comparability and interpretation. We also adopted Box-Cox transformation to correct deviations from sample normality of reproductive hormones (Box and Cox, 1964). Spearman correlation coefficients among PFAS concentrations were calculated.

Multiple linear regression models were employed to analyze the association of individual PFAS exposure with reproductive hormones. Also, plasma PFAS concentrations were converted to quartiles and the lowest was used as the reference to estimate β coefficients (95% CI) of

reproductive hormones. All regression analyses were adjusted for general characteristics including age, age at menarche, BMI, gravidity, menstrual cycle, income, education, and study site.

We used quantile-based g-computation (qgcomp) to assess the PFAS mixtures effects on reproductive hormones. This method estimated the reproductive hormones changes of a quantile while all PFAS increased in a specific mixture. It also allows all PFAS to be related to the hormones in different directions. Meanwhile, the contribution of individual PFAS to the overall effect was then assessed by the relative strength of weights in each direction and visualized in the graph.

The statistical significance level was set at 0.05. Statistical analyses were performed using the R software (version 4.0.3; R foundation for Statistical Computing, Vienna, Austria) for qgcomp analysis ("qgcomp" package). We also used STATA (version 17; Stata Corp LP, College Station, USA) for Box-Cox regression and SAS (version 9.4; SAS Institute Inc., Cary, NC, USA) for other analyses.

3. Results

3.1. Participant characteristics

The demographic characteristics of participants are shown in Table 1. The mean age was 29.6 years old and average years at menarche was 14.1 years old. Comparing to women who were excluded from the present analyses, those who were included were younger (mean age: 29.6 vs 31.6 years), more likely to be nulliparous (58.9% vs 21.3%) and from Shandong province (65.4% vs 20.5%) (Table S1).

Table 2 presents the median concentrations of FSH, LH, E_2, P, TT, and PRL were 6.32 IU/L, 5.13 IU/L, 38.8 pg/mL, 0.64 ng/mL, 0.31 ng/mL, and 15.05 mIU/L, respectively.

Table 3 shows that the 15 measured PFAS were detected in the vast majority of women. PFOA had the highest median concentration (6.16 ng/mL), followed by PFOS (4.11 ng/mL) and 6:2 Cl-PFESA (2.27 ng/mL). PFAS were variably correlated, ranging from 0.01 to 0.91 (see Fig. S1).

3.2. Results of multiple linear regressions

Table 4 presents linear regression between individual PFAS and reproductive hormones. After adjusting for the confounders, ln-PFHpA [$\beta = 0.57$, (95% CI: 0.18, 0.97)] was positively associated with TT level. Similarly, natural log-transformed PFOA was positively associated with TT. Conversely, ln-6:2 Cl-PFESA concentration was significantly associated with a decreased level of PRL [$\beta = -0.07$, (95% CI: −0.14, −0.001) in the covariate-adjusted models. Other PFAS were not associated with reproductive hormones. We further examined the potential effect modification by parity using an interaction term (PFAS*parity). Tables S3 and S4 show that there was no clear or consistent effect modification by parity in our study.

Additionally, Fig. 1 and Tables S5–S6 show the linear trends of the associations between reproductive hormones and PFAS quartiles. Statistically significant positive trends were found for 6:2 Cl-PFESA, PFHpA, and PFBA with TT after controlling for potential confounders (Fig. 1). Further, similar trends were found for PFOA, PFNA, PFDA, and PFUnDA with TT (Tables S5–S6).

Table 1
Characteristics of participants.

Variable	Median (IQR), mean ± SD, or n (%)
Age (year)	29.6 ± 4.7
BMI (kg/m^2)	22.7 ± 3.4
Age at menarche (year)	14.1 ± 1.4
Gravidity (%)	
Nulliparous	255 (58.9%)
1-2	152 (35.1%)
≥3	26 (6%)
Menstrual cycle irregularity (%)	70 (16.2%)
Education	
Primary school	60 (13.9%)
Middle school	245 (56.5%)
Above middle school	128 (29.6%)
Income	
<100,000 CNY	276 (63.7%)
≥100,000 CNY	99 (22.9%)
Missing	58 (13.4%)
Study site	
Shandong	283 (65.36%)
Zhejiang	150 (34.64%)

Abbreviations: SD: standard deviation; IQR: interquartile range; CNY: Chinese Yuan; BMI: body mass index.

3.3. Qgcomp analysis

Fig. 2 shows the overall association of the PFAS mixture with TT in qgcomp analysis. Similar to the results from multiple linear regressions, TT was statistically significant with per natural-log unit (ng/mL) increase of PFAS mixture [1.08 (95% CI: 0.15, 2.02)].

4. Discussion

This study evaluated the relationship of plasma PFAS with reproductive hormones among women of child-bearing age. We found that PFAS alternatives and legacy PFAS were positively associated with TT levels.

Emerging PFAS alternatives such as 6:2 Cl-PFESA and 8:2 Cl-PFESA are mainly reported in China. Similar to our study, Jin et al. (2020) and Pan et al. (2017) found that 6:2 Cl-PFESA (3.7 ng/mL, 4.16 ng/mL) and 8:2 Cl-PFESA (0.01 ng/mL, 0.06 ng/mL) concentrations were comparable to our research (6:2 Cl-PFESA: 2.27 ng/mL, 8:2 Cl-PFESA: 0.07 ng/mL). However, due to the relatively high LOD (0.80 ng/mL and 0.14 ng/mL), HFPO-DA was not detected in two other studies (Pan et al., 2017; Liu et al., 2021). Besides, legacy PFAS levels in this study differed from those in other countries (Fig. S2).

We observed a significant increase in TT in association with PFAS from both multiple linear regression analysis and qgcomp. TT plays an important role in female reproductive system as the precursor for E_2 (Walters and Handelsman, 2018). Elevated TT is a marker for hyperandrogenism in hirsute amenorrheic women and one of the criteria to diagnose polycystic ovary syndrome (PCOS), a most common endocrine disorder in reproductive age women (Rosenfield and Ehrmann, 2016).

Our findings were partially consistent with previous population-based studies that explored the associations between PFAS and reproductive hormones in women of childbearing age. Similar to our research, Heffernan et al. (2018) observed that PFOA, PFHxS, and PFNA had positive associations with TT and no significant relationship with E_2 in 29 healthy women (mean age: 32.9 years). Zhang et al. (2018) found no signification association of PFOA, PFOS, and PFHxS with FSH, LH, E_2, PRL, and T in 120 healthy women (mean age: 29.7 years). But they found that PFOS and PFHxS exposure in patients with primary ovarian insufficiency (POI) were positively associated with FSH concentration and negatively associated with the E_2 level. And PFOA and PFOS exposure had a positive association between PRL in patients with POI. In addition, a study suggested no significant association between PFOA and estradiol from the C8 Health Project in women aged 18–42 years. They also found that PFOS was negatively associated with estradiol in the same population but the association did not reach statistical significance (Knox et al., 2011). Similarly, PFOA, PFOS, PFNA, and PFUnDA had no significant relationship with FSH, LH, E_2 and TT in 445 healthy British adult women (age range: 18–30 years) (Tsai et al., 2015). However, in contrast to our findings, several studies observed endocrine disruption of PFAS in adult women. One research from Norway indicated that PFOS may be associated with decreased production of E_2 and P in 178 healthy and naturally cycling women (aged 25–35 years) (Barrett et al., 2015). The discrepancy between Barrett's and our studies might be partly due

Table 2
Distribution of reproductive hormones (n = 433).

Reproductive hormones	LOD	Detection rate %	P25	P50	P75	min	max
FSH (IU/L) (P_{25}, P_{75})	0.1	100	5.37	6.32	7.42	1.86	15.2
LH (IU/L) (P_{25}, P_{75})	0.1	100	4.02	5.13	6.92	0.94	48.9
E_2 (pg/mL) (P_{25}, P_{75})	5	98.6	27	38.8	96.3	3.03	915.9
P (ng/mL) (P_{25}, P_{75})	0.03	77.6	0.28	0.64	1.46	0.02	61.9
TT (ng/mL) (P_{25}, P_{75})	0.025	94.7	0.17	0.31	0.7	0.017	70
PRL (mIU/L) (P_{25}, P_{75})	1	95.4	10.86	15.05	20.7	0.71	69.3

Abbreviations: LOD: limit of detection; FSH: follicle-stimulating hormone; LH: luteinizing hormone; E_2: estradiol; P: progesterone; TT: total testosterone; PRL: prolactin.

Table 3
Distribution of plasma PFAS (n = 433).

PFAS (ng/mL)	LOD (ng/mL)	Detection rate %	P_{25} (ng/mL)	P_{50} (ng/mL)	P_{75} (ng/mL)	Min (ng/mL)	Max (ng/mL)
6:2 Cl-PFESA	0.0027	100	1.00	2.27	5.2	0.03	50.2
8:2 Cl-PFESA	0.0054	99.8	0.04	0.07	0.11	0.004	9.73
HFPO-DA	0.0064	99.9	0.01	0.03	0.04	0.005	0.41
PFOA	0.0083	100	2.94	6.16	12	0.35	106.7
PFOS	0.0005	100	2.37	4.11	6.55	0.46	98.2
PFNA	0.0037	100	0.37	0.61	0.97	0.08	4.51
PFDA	0.0017	100	0.26	0.51	1.02	0.03	8.06
PFHxS	0.0025	100	0.13	0.21	0.38	0.03	4.07
PFHpS	0.0051	99.7	0.06	0.08	0.11	0.007	1.48
PFDoA	0.0038	99.8	0.08	0.11	0.16	0.003	14.35
PFUnDA	0.0072	100	0.25	0.46	0.80	0.01	3.36
PFBS	0.003	87.1	0.02	0.04	0.07	0.002	2.43
PFHpA	0.002	97.9	0.03	0.07	0.11	0.001	1.98
PFBA	0.006	97.4	0.06	0.09	0.12	0.004	8.93
PFHxA	0.002	82.0	0.01	0.02	0.04	0.001	1.4

Abbreviations: See Supplemental Table S2 for PFAS and PFAS alternatives definition.

to the difference in PFOS levels (14.78 ng/mL in nulliparas and 12.65 ng/mL in multiparas in Barrett's study vs 4.11 ng/mL in ours). Behr et al. (2018) did not find endocrine disrupting properties of PFAS in vitro at concentrations relevant to human exposure. In addition, two studies based on the Study of Women's Health Across the Nation (SWAN) investigated mid-aged women (aged 42–52 years) and found positive associations of PFOA and PFOS with FSH but inverse associations of PFNA and PFOA with E_2 (Harlow et al., 2021; Ding et al., 2022). But our study did not show any significant association between PFAS and E_2. This inconsistency might be attributable to the large age difference as women in our study were much younger (mean age of 29.6 years). Since reproductive hormone levels decline with age, the association may be more obvious in older women.

Our study also discovered that some short-chain PFAS was associated with reproductive hormone levels in women. Short-chain PFAS have much shorter half-lives in human blood, leading to a much lower blood level than long-chain PFAS (Xu et al., 2020). However, their

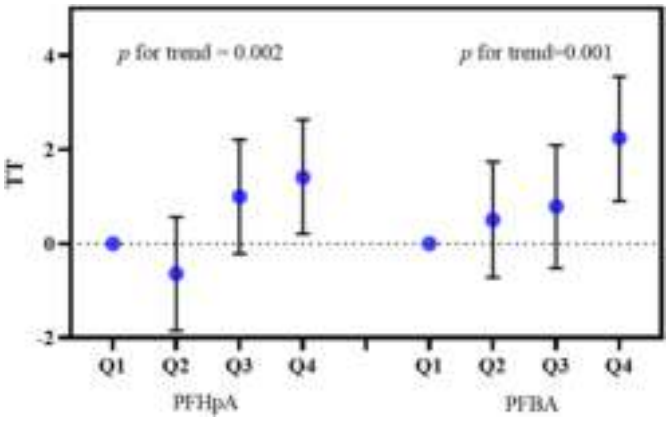

Fig. 1. Associations between quartiles of PFAS alternatives (Q1 = reference) and female reproductive hormones. Adjust for age, BMI, age at menarche, gravity, menstrual cycle, study site, income and education. See Supplemental Table S2 for PFAS alternatives definition; TT: total testosterone. *p*-values for trend were calculated using categories representing the median value of corresponding quartile (Q1: quartile 1; Q2: quartile 2; Q3: quartile 3; Q4: quartile 4).

Table 4
Associations between single ln-transformed PFAS and reproductive hormones in multivariable linear regression (n = 433)[a].

	FSH β (95 %CI)	LH β (95 %CI)	E_2 β (95 %CI)	P β (95 %CI)	TT β (95 %CI)	PRL β (95 %CI)
6:2 Cl-PFESA	0.01 (−0.05, 0.06)	−0.01 (−0.04, 0.01)	0.01 (−0.03, 0.06)	0.00 (−0.08, 0.08)	0.07 (−0.37, 0.51)	**−0.07 (-0.14, -0.001)**
8:2 Cl-PFESA	0.00 (−0.09, 0.09)	0.00 (−0.04, 0.03)	0.01 (−0.05, 0.08)	−0.09 (−0.22, 0.04)	−0.44 (−1.12, 0.23)	−0.07 (−0.17, 0.04)
HFPO-DA	0.01 (−0.05, 0.07)	0.01 (−0.01, 0.03)	0.03 (−0.01, 0.07)	0.03 (−0.05, 0.11)	0.15 (−0.29, 0.59)	0.01 (−0.06, 0.08)
PFBS	0.03 (−0.02, 0.08)	0.00 (−0.02, 0.02)	0.02 (−0.02, 0.06)	0.01 (−0.07, 0.08)	0.26 (−0.14, 0.67)	−0.04 (−0.11, 0.02)
PFHpA	0.01 (−0.04, 0.06)	0.01 (−0.01, 0.03)	0.04 (0.00, 0.07)	**0.09 (0.02, 0.17)**	**0.57 (0.18, 0.97)**	−0.01 (−0.07, 0.05)
PFBA	−0.01 (−0.07, 0.05)	0.00 (−0.03, 0.02)	0.02 (−0.02, 0.07)	**0.14 (0.05, 0.23)**	0.44 (−0.03, 0.91)	0.01 (−0.07, 0.08)
PFHxA	0.01 (−0.02, 0.05)	0.01 (−0.01, 0.02)	0.01 (−0.02, 0.04)	0.01 (−0.04, 0.07)	−0.10 (−0.39, 0.19)	−0.02 (−0.07, 0.02)
PFOA	−0.02 (−0.08, 0.04)	−0.01 (−0.03, 0.02)	−0.03 (−0.07, 0.02)	0.08 (−0.01, 0.17)	**0.64 (0.15, 1.12)**	0.03 (−0.05, 0.11)
PFOS	0.01 (−0.07, 0.09)	−0.01 (−0.04, 0.02)	0.00 (−0.06, 0.06)	0.00 (−0.11, 0.11)	0.01 (−0.59, 0.61)	−0.05 (−0.15, 0.04)
PFNA	0.01 (−0.07, 0.09)	−0.01 (−0.03, 0.02)	0.02 (−0.04, 0.09)	0.05 (−0.07, 0.16)	0.59 (−0.03, 1.21)	−0.02 (−0.12, 0.08)
PFDA	0.01 (−0.06, 0.08)	−0.01 (−0.04, 0.01)	0.01 (−0.04, 0.06)	0.01 (−0.10, 0.11)	0.22 (−0.32, 0.76)	−0.06 (−0.14, 0.03)
PFHxS	0.00 (−0.08, 0.08)	0.01 (−0.02, 0.04)	0.00 (−0.06, 0.06)	0.10 (−0.01, 0.22)	0.61 (−0.01, 1.23)	−0.02 (−0.12, 0.08)
PFHpS	0.02 (−0.09, 0.12)	0.02 (−0.02, 0.06)	−0.01 (−0.09, 0.07)	0.14 (−0.01, 0.28)	0.05 (−0.74, 0.84)	−0.02 (−0.15, 0.10)
PFDoA	−0.05 (−0.11, 0.01)	0.00 (−0.03, 0.02)	0.03 (−0.02, 0.07)	0.03 (−0.05, 0.12)	0.00 (−0.47, 0.48)	−0.02 (−0.09, 0.06)
PFUnDA	0.00 (−0.08, 0.08)	−0.01 (−0.04, 0.02)	0.01 (−0.05, 0.07)	0.07 (−0.05, 0.18)	0.17 (−0.46, 0.80)	−0.05 (−0.15, 0.05)

Abbreviations: See Supplemental Table S2 for PFASs and PFAS alternatives definition; FSH: follicle-stimulating hormone; LH: luteinizing hormone; E_2: estradiol; P: progesterone; TT: total testosterone; PRL: prolactin.
Bold characters indicate significance, $p < 0.05$.
[a] Adjusted for age, BMI, age at menarche, gravity, menstrual cycle, study site, income and education.

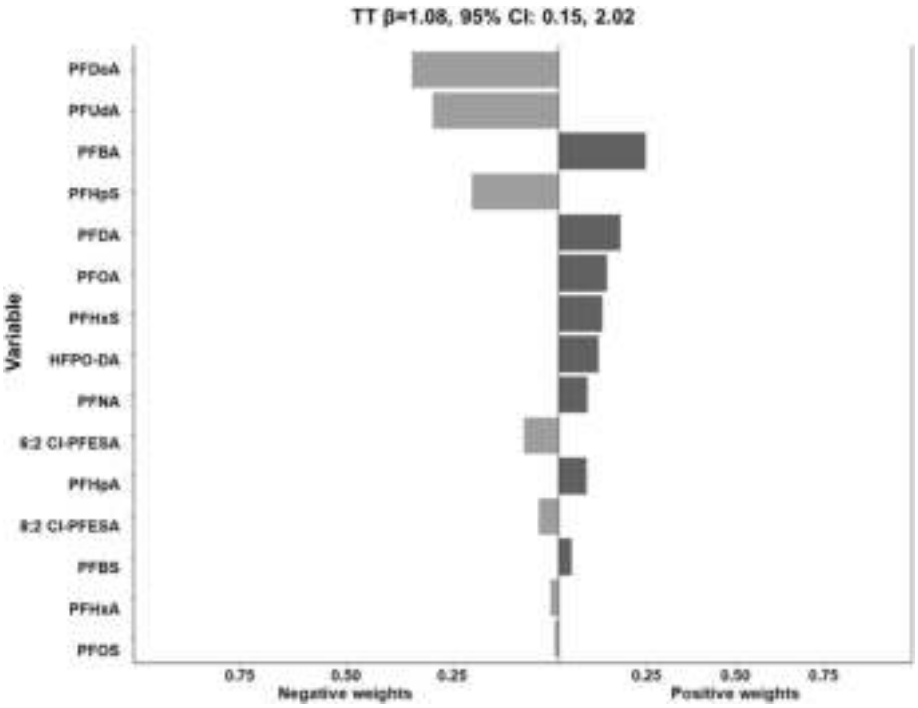

Fig. 2. Weights representing the proportion of the positive or negative partial effect for TT with the PFAS in the quantile g-computation model. All PFAS were ln-transformed. Models were adjusted for age, BMI, age at menarche, gravity, menstrual cycle, study site, income and education. See Supplemental Table S2 for PFAS alternatives definition; TT: total testosterone.

concentrations in the water system and human urine can be quite high (Peng et al., 2021), suggesting that humans may still be exposed to high levels of short-chain PFAS. Thus, the toxicity of the short-chain PFAS cannot be ignored. Previous findings demonstrated that short-chain PFAS had reproductive toxicity on *C. elegans* by increasing production of reactive oxygen species (Chen et al., 2018).

6:2 Cl-PFESA is a PFAS alternative and has a long half-life. *In vivo* experiments in fish (Shi et al., 2018) suggested that 6:2 Cl-PFESA could cause reproductive toxicity by altering the histological structure of the gonads, decreasing gonadosomatic index and female egg production, and elevating blood testosterone levels. Our observation supports the toxicological findings, suggesting that 6:2 Cl-PFESA may be a potential endocrine disruptor (He et al., 2022). But more research is needed to confirm its effects in future studies.

The mechanisms of endocrine-disruption of PFAS are still unclear. Female *silurana tropicalis* exposed to PFOS (0.29, 0.62, and 1.1 mg/L) had significant higher plasma testosterone concentrations compared to the control and exhibited a concentration-related increase in the testosterone (Fort et al., 2019). Peroxisome proliferator-activated receptors, particularly the PPARγ isoforms, have been confirmed to be the key targets in the PFAS-related endocrine disruption (Vanden et al., 2006). Activation of PPARγ could inhibit the expression of aromatase. Aromatase is a critical enzyme to convert androgens to oestrogens through suppressing nuclear factor-kappa B (NF-κB) expression (Fan et al., 2005). Besides, Chaparro-Ortega et al. (2018) found that PFAS inhibited progesterone and androstenedione secretion in stimulated theca cells, and restrained progesterone and estradiol secretion in stimulated granulose cells. Chronic exposure of adult female rats to PFOS (0.1 mg/kg/day) suppresses biosynthesis of E_2 possibly through reduced mRNA expression of *Star* mediated by reduced histone acetylation (Feng et al., 2015). Furthermore, PFOS reduced cell viability and induced apoptosis in human placental syncytiotrophoblasts by increasing pro-apoptosis proteins such as Bax and cleaved-caspase3, and decreasing pro-caspase3 and anti-apoptosis protein Bcl-2, and thus, disrupting sex steroid hormone levels in women (Zhang et al., 2015). But some toxicological findings displayed that PFAS had no endocrine properties in vitro at human relevant dose (Sunderland et al., 2019).

The present study has several strengths. Most of all, we evaluated the joint effects of 15 PFAS compounds on reproductive hormones with more advanced statistical models. These methods provide a more realistic and comprehensive way to evaluate reproductive toxicities of PFAS than the conventional single regression analysis. We further assessed a variety of PFAS, including emerging PFAS alternatives, short-chain PFAS, and legacy PFAS.

Our study also has some limitations that should not be ignored. First, reproductive hormones are known to fluctuate substantially by time. Even within a narrow window during the menstrual cycle, individual variations could be large (Baerwald et al., 2012). The large random errors in these hormones reduce the statistical power to identify a significant difference. Second, the present study was a cross-sectional study, and the determination of the causal relationship between PFAS exposure and sex hormones was not possible. For example, PRL is recognized as a metabolic hormone to promote metabolism (Ben-Jonathan and Hugo, 2015; Lopez-Vicchi et al., 2020). Low PRL values may be associated with metabolic disease (Macotela et al., 2020). Low metabolism might result in PFAS accumulation in the body. Besides, lifestyles such as eating habits may have a certain impact on both PFAS exposure and outcomes (Sanchez-Zamorano et al., 2016; Zhou et al., 2019). Thus, we were unable to rule out the possibility of false-positive or false-negative results. Therefore, the correlations we found require further investigation.

5. Conclusion

We found that exposure to PFAS was associated with increased TT in women of childbearing age. This study provided human evidence for the association of PFAS alternatives with reproductive hormones. However, further studies are warranted to confirm our findings and to elucidate the mechanisms of PFAS alternatives.

Author contributions

CY.L. and M.N. contributed to the conceptualization of the study and manuscript draft; M.N. and W.Q performed data analysis; MH.X. and LY. Z. validated analysis; J.Z. and H.Y. contributed to investigation and data curation; H.Y. revised the manuscript; J.Z. acquired funding. All authors substantially revised the manuscript and have approved the submitted version.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114158.

References

Barbieri, R.L., 2014. The endocrinology of the menstrual cycle. Methods Mol. Biol. 1154, 145–169. https://doi.org/10.1007/978-1-4939-0659-8_7.

Barrett, E.S., Chen, C., Thurston, S.W., Haug, L.S., Sabaredzovic, A., Fjeldheim, F.N., et al., 2015. Perfluoroalkyl substances and ovarian hormone concentrations in naturally cycling women. Fertil. Steril. 103, 1261–1270. https://doi.org/10.1016/j.fertnstert.2015.02.001.

Ben-Jonathan, N., Hugo, E., 2015. Prolactin (PRL) in adipose tissue: regulation and functions. Adv. Exp. Med. Biol. 846, 1–35. https://doi.org/10.1007/978-3-319-12114-7_1.

Ben-Nagi, J., Panay, N., 2014. Premature ovarian insufficiency: how to improve reproductive outcome? Climacteric 17, 242–246. https://doi.org/10.3109/13697137.2013.860115.

Box, G., Cox, D.R., 1964. An analysis of transformations. J R Stat Soc Series B Stat Methodol 26, 211–252. https://doi.org/10.1111/j.2517-6161.1964.tb00553.x.

Brase, R.A., Mullin, E.J., Spink, D.C., 2021. Legacy and emerging per- and polyfluoroalkyl substances: analytical techniques, environmental fate, and health effects. Int. J. Mol. Sci. 22 https://doi.org/10.3390/ijms22030995.

Chang, C.J., Ryan, P.B., Smarr, M.M., Kannan, K., Panuwet, P., Dunlop, A.L., et al., 2021. Serum per- and polyfluoroalkyl substance (PFAS) concentrations and predictors of exposure among pregnant African American women in the Atlanta area, Georgia. Environ. Res. 198, 110445 https://doi.org/10.1016/j.envres.2020.110445.

Chaparro-Ortega, A., Betancourt, M., Rosas, P., Vazquez-Cuevas, F.G., Chavira, R., Bonilla, E., et al., 2018. Endocrine disruptor effect of perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) on porcine ovarian cell steroidogenesis. Toxicol. Vitro 46, 86–93. https://doi.org/10.1016/j.tiv.2017.09.030.

Chen, F., Wei, C., Chen, Q., Zhang, J., Wang, L., Zhou, Z., et al., 2018. Internal concentrations of perfluorobutane sulfonate (PFBS) comparable to those of perfluorooctane sulfonate (PFOS) induce reproductive toxicity in Caenorhabditis elegans. Ecotoxicol. Environ. Saf. 158, 223–229. https://doi.org/10.1016/j.ecoenv.2018.04.032.

Stockholm Convention, 2009. Stockholm Convention Online.

De Silva, A.O., Armitage, J.M., Bruton, T.A., Dassuncao, C., Heiger-Bernays, W., Hu, X.C., et al., 2021. PFAS exposure pathways for humans and wildlife: a synthesis of current knowledge and key gaps in understanding. Environ. Toxicol. Chem. 40, 631–657. https://doi.org/10.1002/etc.4935.

Ding, N., Harlow, S.D., Randolph, J.F., Mukherjee, B., Batterman, S., Gold, E.B., Park, S. K., 2022. Perfluoroalkyl substances and incident natural menopause in midlife women: the mediating role of sex hormones. Am. J. Epidemiol. 191, 1212–1223. https://doi.org/10.1093/aje/kwac052.

Fan, W., Yanase, T., Morinaga, H., Mu, Y.M., Nomura, M., Okabe, T., et al., 2005. Activation of peroxisome proliferator-activated receptor-gamma and retinoid X receptor inhibits aromatase transcription via nuclear factor-kappaB. Endocrinology 146, 85–92. https://doi.org/10.1210/en.2004-1046.

Feng, X.J., Wang, X.L., Cao, X.Y., Xia, Y.K., Zhou, R., Chen, L., 2015. Chronic exposure of female mice to an environmental level of perfluorooctane sulfonate suppresses estrogen synthesis through reduced histone H3K14 acetylation of the StAR promoter leading to deficits in follicular development and ovulation. Toxicol. Sci. 148, 368–379. https://doi.org/10.1093/toxsci/kfv197.

Fort, D.J., Mathis, M.B., Fort, C.E., Fort, H.M., Fort, T.D., Guiney, P.D., Weeks, J.A., 2019. Effect of perfluorooctanesulfonate exposure on steroid hormone levels and steroidogenic enzyme activities in juvenile Silurana tropicalis. J. Appl. Toxicol. 39, 1066–1078. https://doi.org/10.1002/jat.3794.

Harlow, S.D., Hood, M.M., Ding, N., Mukherjee, B., Calafat, A.M., Randolph, J.F., et al., 2021. Per- and polyfluoroalkyl substances and hormone levels during the menopausal transition. J. Clin. Endocrinol. Metab. 106, e4427–e4437. https://doi.org/10.1210/clinem/dgab476.

He, Y., Lv, D., Li, C., Liu, X., Liu, W., Han, W., 2022. Human exposure to F-53B in China and the evaluation of its potential toxicity: an overview. Environ. Int. 161, 107108 https://doi.org/10.1016/j.envint.2022.107108.

Heffernan, A.L., Cunningham, T.K., Drage, D.S., Aylward, L.L., Thompson, K., Vijayasarathy, S., et al., 2018. Perfluorinated alkyl acids in the serum and follicular fluid of UK women with and without polycystic ovarian syndrome undergoing fertility treatment and associations with hormonal and metabolic parameters. Int. J. Hyg Environ. Health 221, 1068–1075. https://doi.org/10.1016/j.ijheh.2018.07.009.

Hornung Rw, R.L., 1990. Estimation of average concentration in the presence of Nondetectable values. Appl. Occup. Environ. Hyg 5, 46–51.

ITRC, 2020. PFAS — Per- and Polyfluoroalkyl Substances.

Jabbour, H.N., Kelly, R.W., Fraser, H.M., Critchley, H.O., 2006. Endocrine regulation of menstruation. Endocr. Rev. 27, 17–46. https://doi.org/10.1210/er.2004-0021.

Jane, L.E.L., Yamada, M., Ford, J., Owens, G., Prow, T., Juhasz, A., 2022. Health-related toxicity of emerging per- and polyfluoroalkyl substances: comparison to legacy PFOS and PFOA. Environ. Res. 212, 113431 https://doi.org/10.1016/j.envres.2022.113431.

Jiao, X., Meng, T., Zhai, Y., Zhao, L., Luo, W., Liu, P., Qin, Y., 2021. Ovarian reserve markers in premature ovarian insufficiency: within different clinical stages and different etiologies. Front. Endocrinol. 12, 601752 https://doi.org/10.3389/fendo.2021.601752.

Jin, H., Lin, S., Dai, W., Feng, L., Li, T., Lou, J., Zhang, Q., 2020. Exposure sources of perfluoroalkyl acids and influence of age and gender on concentrations of chlorinated polyfluorinated ether sulfonates in human serum from China. Environ. Int. 138, 105651 https://doi.org/10.1016/j.envint.2020.105651.

Knox, S.S., Jackson, T., Javins, B., Frisbee, S.J., Shankar, A., Ducatman, A.M., 2011. Implications of early menopause in women exposed to perfluorocarbons. J. Clin. Endocrinol. Metab. 96, 1747–1753. https://doi.org/10.1210/jc.2010-2401.

Li, J., Luo, K., Liu, X., Tang, S., Zhang, J., Chen, D., 2022. Chemical-specific determinants for pre-conceptional exposure to emerging and legacy per- and polyfluoroalkyl substances. Sci. Total Environ. 819, 152501 https://doi.org/10.1016/j.scitotenv.2021.152501.

Liu, J., Gao, X., Wang, Y., Leng, J., Li, J., Zhao, Y., Wu, Y., 2021. Profiling of emerging and legacy per-/polyfluoroalkyl substances in serum among pregnant women in China. Environ. Pollut. 271, 116376 https://doi.org/10.1016/j.envpol.2020.116376.

Lopez-Vicchi, F., De Winne, C., Brie, B., Sorianello, E., Ladyman, S.R., Becu-Villalobos, D., 2020. Metabolic functions of prolactin: physiological and pathological aspects. J. Neuroendocrinol. 32, e12888 https://doi.org/10.1111/jne.12888.

Macotela, Y., Triebel, J., Clapp, C., 2020. Time for a New perspective on prolactin in metabolism. Trends Endocrinol. Metabol. 31, 276–286. https://doi.org/10.1016/j.tem.2020.01.004.

Maybin, J.A., Critchley, H.O., 2012. Steroid regulation of menstrual bleeding and endometrial repair. Rev. Endocr. Metab. Disord. 13, 253–263. https://doi.org/10.1007/s11154-012-9228-2.

Nian, M., Huo, X., Zhang, J., Mao, Y., Jin, F., Shi, Y., Zhang, J., 2022a. Association of emerging and legacy per- and polyfluoroalkyl substances with unexplained recurrent spontaneous abortion. Ecotoxicol. Environ. Saf. 239, 113691 https://doi.org/10.1016/j.ecoenv.2022.113691.

Nian, M., Zhou, W., Feng, Y., Wang, Y., Chen, Q., Zhang, J., 2022b. Emerging and legacy PFAS and cytokine homeostasis in women of childbearing age. Sci. Rep. 12, 6517. https://doi.org/10.1038/s41598-022-10501-8.

Official Journal of the European Union, 2017. Commission Regulation (EU) 2017/1000 of 13 June 2017 Amending Annex XVII to Regulation (EC) No 1907/2006 of the European Parliament and of the Council Concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as Regards Perfluorooctanoic Acid (PFOA), its Salts and PFOA-Related Substances (Text with EEA Relevance).

Pan, Y., Zhang, H., Cui, Q., Sheng, N., Yeung, L., Guo, Y., et al., 2017. First report on the occurrence and bioaccumulation of hexafluoropropylene oxide trimer acid: an emerging concern. Environ. Sci. Technol. 51, 9553–9560. https://doi.org/10.1021/acs.est.7b02259.

Pan, Y., Zhang, H., Cui, Q., Sheng, N., Yeung, L., Sun, Y., et al., 2018. Worldwide distribution of novel perfluoroether carboxylic and sulfonic acids in surface water. Environ. Sci. Technol. 52, 7621–7629. https://doi.org/10.1021/acs.est.8b00829.

Park, S.K., Peng, Q., Ding, N., Mukherjee, B., Harlow, S.D., 2019. Determinants of per- and polyfluoroalkyl substances (PFAS) in midlife women: evidence of racial/ethnic and geographic differences in PFAS exposure. Environ. Res. 175, 186–199. https://doi.org/10.1016/j.envres.2019.05.028.

Peng, L., Xu, W., Zeng, Q., Cheng, Y., Zhang, Y., Guo, Y., et al., 2021. Distribution characteristics of per- and polyfluoroalkyl substances (PFASs) in human urines of acrylic fiber plant and chemical plant. Environ. Sci. Pollut. Res. Int. 28, 69181–69189. https://doi.org/10.1007/s11356-021-15355-7.

Rosenfield, R.L., Ehrmann, D.A., 2016. The pathogenesis of polycystic ovary syndrome (PCOS): the hypothesis of PCOS as functional ovarian hyperandrogenism revisited. Endocr. Rev. 37, 467–520. https://doi.org/10.1210/er.2015-1104.

Sanchez-Zamorano, L.M., Flores-Luna, L., Angeles-Llerenas, A., Ortega-Olvera, C., Lazcano-Ponce, E., Romieu, I., et al., 2016. The Western dietary pattern is associated with increased serum concentrations of free estradiol in postmenopausal women: implications for breast cancer prevention. Nutr. Res. 36, 845–854. https://doi.org/10.1016/j.nutres.2016.04.008.

Shi, G., Guo, H., Sheng, N., Cui, Q., Pan, Y., Wang, J., et al., 2018. Two-generational reproductive toxicity assessment of 6:2 chlorinated polyfluorinated ether sulfonate (F-53B, a novel alternative to perfluorooctane sulfonate) in zebrafish. Environ. Pollut. 243, 1517–1527. https://doi.org/10.1016/j.envpol.2018.09.120.

Steenland, K., Fletcher, T., Savitz, D.A., 2010. Epidemiologic evidence on the health effects of perfluorooctanoic acid (PFOA). Environ. Health Perspect. 118, 1100–1108. https://doi.org/10.1289/ehp.0901827.

Sunderland, E.M., Hu, X.C., Dassuncao, C., Tokranov, A.K., Wagner, C.C., Allen, J.G., 2019. A review of the pathways of human exposure to poly- and perfluoroalkyl substances (PFASs) and present understanding of health effects. J. Expo. Sci. Environ. Epidemiol. 29, 131–147. https://doi.org/10.1038/s41370-018-0094-1.

Taraborrelli, S., 2015. Physiology, production and action of progesterone. Acta Obstet. Gynecol. Scand. 94 (Suppl. 161), 8–16. https://doi.org/10.1111/aogs.12771.

Tsai, M.S., Lin, C.Y., Lin, C.C., Chen, M.H., Hsu, S.H., Chien, K.L., et al., 2015. Association between perfluoroalkyl substances and reproductive hormones in adolescents and young adults. Int. J. Hyg Environ. Health 218, 437–443. https://doi.org/10.1016/j.ijheh.2015.03.008.

Tsai, M.S., Miyashita, C., Araki, A., Itoh, S., Bamai, Y.A., Goudarzi, H., et al., 2018. Determinants and temporal trends of perfluoroalkyl substances in pregnant women: the Hokkaido study on environment and children's health. Int. J. Environ. Res. Publ. Health 15. https://doi.org/10.3390/ijerph15050989.

Tsuda, S., 2016. Differential toxicity between perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA). J. Toxicol. Sci. 41, P27–P36. https://doi.org/10.2131/jts.41.SP27.

Vanden, H.J., Thompson, J.T., Frame, S.R., Gillies, P.J., 2006. Differential activation of nuclear receptors by perfluorinated fatty acid analogs and natural fatty acids: a comparison of human, mouse, and rat peroxisome proliferator-activated receptor-alpha, -beta, and -gamma, liver X receptor-beta, and retinoid X receptor-alpha. Toxicol. Sci. 92, 476–489. https://doi.org/10.1093/toxsci/kfl014.

Walters, K.A., Handelsman, D.J., 2018. Role of androgens in the ovary. Mol. Cell. Endocrinol. 465, 36–47. https://doi.org/10.1016/j.mce.2017.06.026.

Wang, S., Huang, J., Yang, Y., Hui, Y., Ge, Y., Larssen, T., et al., 2013. First report of a Chinese PFOS alternative overlooked for 30 years: its toxicity, persistence, and presence in the environment. Environ. Sci. Technol. 47, 10163–10170. https://doi.org/10.1021/es401525n.

Xu, Y., Fletcher, T., Pineda, D., Lindh, C.H., Nilsson, C., Glynn, A., et al., 2020. Serum half-lives for short- and long-chain perfluoroalkyl acids after ceasing exposure from drinking water contaminated by firefighting foam. Environ. Health Perspect. 128, 77004 https://doi.org/10.1289/EHP6785.

Yao, J.Z., Sheng, N., Guo, Y., Yeung, L., Dai, J.Y., Pan, Y.T., 2022. Nontargeted identification and temporal trends of per- and polyfluoroalkyl substances in a fluorochemical industrial zone and adjacent taihu lake. Environ. Sci. Technol. 56, 7986–7996. https://doi.org/10.1021/acs.est.2c00891.

Zhang, N., Wang, W.S., Li, W.J., Liu, C., Wang, Y., Sun, K., 2015. Reduction of progesterone, estradiol and hCG secretion by perfluorooctane sulfonate via induction of apoptosis in human placental syncytiotrophoblasts. Placenta 36, 575–580. https://doi.org/10.1016/j.placenta.2015.02.008.

Zhang, S., Tan, R., Pan, R., Xiong, J., Tian, Y., Wu, J., Chen, L., 2018. Association of perfluoroalkyl and polyfluoroalkyl substances with premature ovarian insufficiency in Chinese women. J. Clin. Endocrinol. Metab. 103, 2543–2551. https://doi.org/10.1210/jc.2017-02783.

Zhou, W., Zhao, S., Tong, C., Chen, L., Yu, X., Yuan, T., et al., 2019. Dietary intake, drinking water ingestion and plasma perfluoroalkyl substances concentration in reproductive aged Chinese women. Environ. Int. 127, 487–494. https://doi.org/10.1016/j.envint.2019.03.075.

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Early-life exposure to a mixture of organophosphate esters and child behavior

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ABSTRACT

Organophosphate esters (OPEs), widely used as flame retardants and plasticizers for commercial and residential purposes, are suspected of being neurotoxic. We aimed to assess exposure to an OPE mixture in early life and its relationship to parent-reported child behavior. We measured urinary concentrations of three OPE metabolites, bis-2-chloroethyl phosphate (BCEP), bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), and diphenyl phosphate (DPHP), at pregnancy (16 and 26 weeks of gestation and delivery) and postnatal time points (ages 1, 2, 3, and 5 years) in the Health Outcomes and Measures of the Environment Study, a longitudinal pregnancy and birth cohort in Cincinnati, Ohio, USA (enrolled 2003–2006, n = 219). We used latent variable analysis in structural equations models and quantile g-computation to investigate associations of a mixture of the three OPE metabolites with parent-reported child behaviors at 3 and 8 years, measured using the Behavioral Assessment System for Children, Second Edition. Higher log-transformed urinary OPE latent variable values at 16 weeks were associated with fewer externalizing problem behaviors (ß = −5.74; 95% CI = −11.24, −0.24) and fewer overall behavioral problems at age 3 years (ß = −5.26; 95% CI = −10.33, −0.19), whereas having higher OPEs at delivery was associated with poorer overall behavioral problems at age 3 years (ß = 2.87; 95% CI = 0.13, 5.61). OPE latent variable values at 16 weeks, 26 weeks, and delivery were not associated with child behavior at 8 years. However, higher OPE latent variable values at 3 years were associated with fewer externalizing behaviors at 8 years (ß = −2.62; 95% CI = −5.13, −0.12). The quantile g-computation estimates had directions largely consistent with the latent variable analysis results. Pregnancy and postnatal urinary OPE metabolite mixtures were associated with child internalizing, externalizing, and overall negative behaviors at 3 and 8 years, but we did not identify a consistent pattern in terms of the direction of the effects or a particularly sensitive time point.

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1. Introduction

In the first 5 years of life, the brain undergoes about 90% of its total development (Brown and Jernigan, 2012). Poor early-life developmental trajectories in the brain can result in poorer overall functioning in adulthood and can significantly impact one's risk of psychiatric disorders and lifetime earning potential (Caspi et al., 1996; Loth et al., 2014; Vergunst et al., 2019). Brain function is highly complex, with various regions controlling different processes, and stimuli such as hormones, stress, and lived experiences able to affect neural circuitry. However, some observable metrics that are derived from higher-level processes and the integration of multiple brain domains can be used to measure brain development broadly. Behavior is one of these constructs that can give clinicians and researchers information about overall brain maturity and function in children.

Environmental toxicants, including endocrine-disrupting chemicals, can negatively alter brain development and behavior (Grandjean and Landrigan, 2015). Organophosphate esters (OPEs) are suspected of disrupting thyroid hormones, the stress response system, and sex hormones, all of which are necessary for proper brain development (Luo et al., 2020; Ren et al., 2015; Tao et al., 2021; Liu et al., 2016; Percy et al., 2021). OPE exposure may cause alterations in neurotransmitters, gene expression, inflammation, synaptogenesis, and neural network activity (Patisaul et al., 2021). OPEs have been added to consumer products as flame retardant and plasticizing chemicals since the 1970s, but a large increase in OPE production volume in recent years has prompted a closer investigation into these chemicals to determine the extent of their potential effects on human health (Doherty et al., 2019a).

OPEs can be released from consumer products and settle into homes, offices, and cars (Bergh et al., 2011; Wei et al., 2015; van der Veen and de Boer, 2012). Once released into the environment, humans are exposed to OPEs through various pathways, including accidental ingestion and inhalation of dust, dermal sorption, and food and water contamination (He et al., 2018; Han et al., 2019; Li et al., 2019; Kim and Kannan, 2018; Kim et al., 2019). In recent years, work has been done to characterize the toxicity profile of OPEs to determine adverse exposure-related health outcomes in humans. Endocrine disruption may occur via altered gene regulation and hormone receptor activity in the presence of OPEs, which can induce neurotoxicity through changes in neuronal growth and differentiation and inflammation (Yan et al., 2021). In epidemiologic studies, exposure to OPEs has been associated with alterations in child neurobehavior. Still, the evidence is inconsistent concerning which developmental windows are most sensitive, which OPEs are associated with adverse effects, and which neurobehavioral domains are affected.

Only one study of OPEs and child behavior so far has examined postnatal exposures; however, it had a cross-sectional design and no internal biomarkers of exposure (Lipscomb et al., 2017). Other studies have focused only on OPE exposures during pregnancy, but all assessed exposure at a single time point, which increases measurement error and eliminates the ability to assess potential sensitive windows to exposure during pregnancy, especially with short-half life chemicals that are measured in urine (Castorina et al., 2017; Choi et al., 2021; Liu et al., 2021; Doherty et al., 2019b). Further, although OPEs are applied in chemical mixtures, and humans experience exposure to a combination of chemicals in their daily lives, much of the current literature on the health effects of OPEs has studied associations as single chemicals. This approach, however, does not adequately capture the total and combined effects of OPE mixtures in the environment. Further, studying chemical mixtures allows researchers to model how reducing overall OPE exposure might affect health, which approximates how a theoretical public health intervention of restricting or eliminating OPEs from consumer goods might impact the population.

This study aims to assess exposure to an OPE mixture in early life and its relationship to parent-reported child behavior. We used data from a well-established pregnancy and birth cohort to estimate the association of pregnancy and postnatal exposure to OPEs with child behavior at ages 3 and 8 years.

2. Materials and methods

2.1. Study population

All participants in the present study were recruited from March 2003 to February 2006 for the Health Outcomes and Measures of the Environment (HOME) Study, a pregnancy and birth cohort in Cincinnati, Ohio (Braun et al., 2017, 2020). The HOME Study was established to investigate the role of common environmental exposures on child health and development in a population targeted to mimic the demographics of the Cincinnati area. Pregnant women were eligible to participate if they were at least 18 years of age; at 16 ± 3 weeks' gestation; living in a home built before 1978 (relating to the original study goal of examining lead exposure); fluent in English; not diagnosed with bipolar disorder, schizophrenia, diabetes, or cancer that required radiation or chemotherapy; not taking medication for thyroid disorders or seizures; HIV negative; and not planning to move outside of the Greater Cincinnati Area.

For the present study, we included pregnant women and their singleton children if they had at least one measurement of urinary OPE metabolites during pregnancy (16 weeks, 26 weeks, or delivery) or early childhood (ages 1, 2, 3, or 5 years), and a parent completed the Behavioral Assessment System for Children, 2nd Edition (BASC-2) at either the 3- or 8-year study visits. The 3- and 8-year time points for behavioral assessments were chosen to maximize available sample sizes and avoid cross-sectionality with urinary OPE metabolite measurements. All participants provided written informed consent for themselves and their children, and the Institutional Review Board (IRB) at Cincinnati Children's Hospital Medical Center (CCHMC) approved the study protocol. The Centers for Disease Control and Prevention (CDC) deferred to the CCHMC IRB as the IRB of record.

2.2. Urine collection and analysis

Pregnant study participants provided urine samples in polypropylene specimen cups at approximately 16 and 26 weeks of pregnancy during prenatal care appointments and during the hospital visit for delivery (may have taken place before or after delivery). The time of day for urine collection was not standardized. The samples were aliquoted and stored at −20 °C until overnight shipment to the Centers for Disease Control and Prevention (CDC) laboratory. Child study participants who were not yet toilet-trained provided urine samples via a surgical diaper insert provided by the study team during a study visit. Child study participants who were in the process of being toilet-trained provided urine samples using a training potty lined with surgical inserts during a study visit, and toilet-trained children provided samples in polypropylene specimen cups during a study visit. Surgical inserts were expressed by laboratory technicians in vials and aliquoted. Child urine samples were also aliquoted; all urine aliquots were stored at −20 °C until overnight shipment to the laboratory. Trained laboratory technicians measured the specific gravity in the urine samples using the ATAGO PAL-10S pocket refractometer (ATAGO CO., Tokyo, Japan).

The CDC's National Center for Environmental Health Laboratory received all study urine samples and quantified concentrations of three OPE metabolites: bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), a metabolite of tris(1,3-dichloro-2-propyl) phosphate (TDCIPP); bis-2-

chloroethyl phosphate (BCEP), a metabolite of tris(2-chloroethyl) phosphate (TCEP); and diphenyl phosphate (DPHP), a metabolite of triphenyl phosphate (TPHP) and 2-ethylhexyl diphenyl phosphate (EHDPP), among other OPEs, in 200 μL of urine. Following enzymatic hydrolysis, technicians used automated off-line solid-phase extraction, reversed-phase high-performance liquid chromatography, and isotope dilution-electrospray ionization tandem mass spectrometry to quantify total (free and conjugated) concentrations of OPE metabolites (Jayatilaka et al., 2017, 2019). Please see elsewhere for quality control and other analytic details (Percy et al., 2020).

The limit of detection (LOD) was 0.1 μg/L for all three urinary OPE metabolites. The percent of samples with detectable OPE concentrations ranged from 83.9 to 95.5% for BCEP, 88.6–100% for BDCIPP, and 97.6–100% for DPHP (Supplemental Table 1). For concentrations below the LOD, we substituted a value of LOD/$\sqrt{2}$ (Hornung and Reed, 1990). Then, OPE metabolite concentration values were standardized by urinary specific gravity to account for dilution using the following formula (MacPherson et al., 2018; Duty et al., 2005):

$$OPE_{SG\_Adj} = OPE_i \frac{(SG_m - 1)}{(SG_i - 1)}$$

where OPE_i is the observed OPE metabolite concentration, SG_m is the median specific gravity for the cohort at the appropriate time point, and SG_i is the specific gravity of the urine sample.

2.3. Child behavior measurements

The BASC-2 is a 160-item parent-report assessment of a child's behavior in public and home settings (Reynolds and Kamphaus, 2004). BASC-2 yields four composite scales: Internalizing Problems, Externalizing Problems, Behavioral Symptom Index (BSI), and Adaptive Skills (not examined in this study). The Internalizing Problems scale measures harmful behaviors directed toward oneself, including anxiety, depression, and somatization. The Externalizing Problems scale measures harmful behaviors directed toward others, including aggression and hyperactivity. The BSI composite is an overall measurement of maladaptive behaviors, including aggression, atypicality, attention problems, depression, hyperactivity, and withdrawal. BASC-2 scores are age normalized to a population mean of 50 and a standard deviation of 10, with higher scores indicating more problem behavior. The internal consistency of the BASC-2 ranges from r = 0.87-0.95 for the subscales across ages 3 and 8 years, and correlations with other measures of child behavior range from r = 0.65-0.84 depending on the subscale (Reynolds and Kamphaus, 2004). Caregivers of study participants, usually mothers (98%), completed the BASC-2 at the 3- and 8-year study visits after receiving instructions from trained study staff.

2.4. Covariate measurements

We considered the following as potential covariates for adjusted models: breastfeeding status (ever vs. never), maternal race, household income at baseline, maternal depression at the time of BASC-2 measurement, maternal education at baseline, maternal age at baseline, marital status at baseline, and the caregiving environment. We then used a Directed Acyclic Graph to determine the final covariate set: maternal depression, breastfeeding status, maternal race (white or non-white), household income, and caregiving environment (Supplemental Fig. 1). All covariate information was collected via questionnaire at study visits except for caregiving environment. This variable was assessed using the Home Observation and Measurement of the Environment, a semi-structured questionnaire and interview that captures the quality and quantity of caregiving in the child's home environment based on observations by trained research assistants in study participants' homes at age 12 months (Caldwell and Bradley, 1984). The internal consistency of the HOME score is r = 0.89, and the test-retest reliability from 12 to 24 months is r = 0.77. In addition, maternal depression was measured by the Beck's Depression Inventory (Beck et al., 1996). We did not have any missing covariate data.

2.5. Statistical analysis

We completed descriptive analyses and then natural-log transformed the specific-gravity standardized urinary OPE metabolite concentrations before statistical analyses to reduce the influence of outliers. We calculated the intraclass correlation coefficients (ICCs) using a two-way mixed effects model of log-transformed OPE metabolites at pregnancy and postnatal time points to determine the correlation of repeated measurements. ICC values reflect the strength of the correlations: ≤0.4 (poor), 0.4–0.75 (fair to good), and ≥0.75 (excellent) (Rosner, 2011).

To explore the associations between urinary OPE metabolites and child behavior, we used two statistical methods chosen to provide a summary of how multiple OPEs are jointly associated with our outcomes of interest, as this conceptualization reflects real-world human exposures and can provide insight into the potential effects of a public health policy to reduce OPEs. First, we used structural equations modeling (SEM) to create and model latent variables of total OPE exposure at each time point based on the three urinary OPE metabolites BCEP, BDCIPP, and DPHP. Then we used quantile g-computation to complement our latent variable SEMs, as both methods estimate the incremental effects of exposure to multiple chemicals on an outcome.

We used Mplus for SEM (Muthen and Muthen, 2017). The outcome variables were the Internalizing Problems, Externalizing Problems, and BSI composite scores from the BASC-2 at ages 3 and 8 years, which are analyzed separately. We constructed latent variables to represent OPE exposure at a single time point, including the observed BCEP, BDCIPP, and DPHP urinary concentrations (Supplemental Figs. 2–4). Latent variable analysis allows the measurement of latent or unobserved variables (i.e., joint OPE exposure) based on measured variables (i.e., multiple urinary OPE metabolite concentrations). We first used confirmatory factor analysis (CFA) to ensure that our latent variable measurement models fit appropriately. The fit was considered excellent if the comparative fit index (CFI) was above 0.95, the Tucker Lewis Index (TLI) was above 0.95, and the root mean square error of approximation (RMSEA) was below 0.07 (Hooper et al., 2008). Next, we used SEM to model associations of latent OPE exposure variables with BASC-2 composite scores after adjustment for covariates (Fig. 1) (Supplemental Figs. 2–4). As our outcome variables were all continuous, we used the maximum likelihood estimator for our models. Parameter estimates can be interpreted as the average change in BASC-2 composite score for a one-unit increase in total log-OPE metabolite concentration at the given time point.

We used R version 4.1.2 and the *qgcomp* package for quantile g-computation (R Core Team, 2019; Keil et al., 2020). This method models how a simultaneous one-quantile increase of all chemicals in a defined mixture affects the specified outcome. It is based on a joint marginal structural model and allows individual chemicals in the mixture to have either positive or negative weights (Keil et al., 2020). We used four quantiles in our analysis, and effect estimates can be interpreted as the difference in BASC-2 scores for a simultaneous one-quartile increase in BCEP, BDCIPP, and DPHP urinary concentrations. We created two different models: 1) an estimate of a linear dose-response parameter for a single time point (16 weeks, 26 weeks, delivery, 1 year, 2 years, 3 years, and 5 years) of combined OPE exposure biomarkers (i.e., combined BCEP, BDCIPP, and DPHP at 16 weeks), and 2) an estimate of the linear dose-response parameter for longitudinal combined pregnancy (16 weeks, 26 weeks, and delivery) and combined postnatal (1 year, 2 years, 3 years, and 5 years) OPE exposure biomarkers (i.e., combined BCEP, BDCIPP, and DPHP across pregnancy or across the postnatal period) using cluster weighting and 5000 bootstrapped estimates for standard errors.

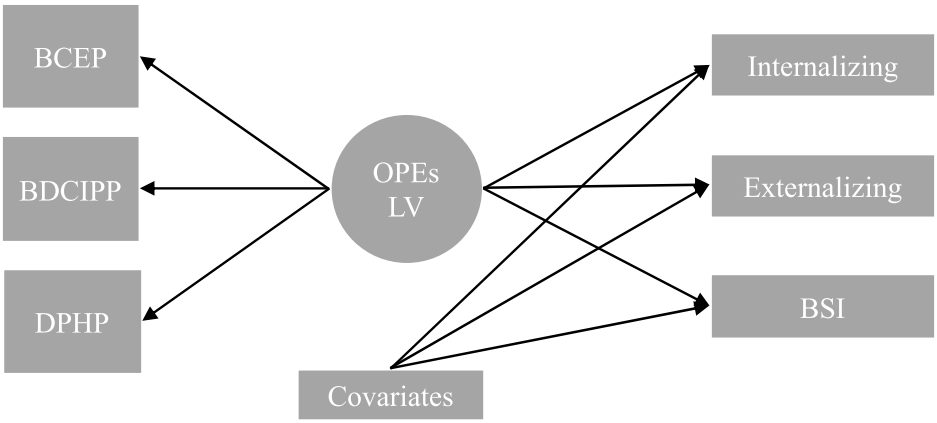

Fig. 1. Simplified SEM diagram for modeling strategy.
Abbreviations: BCEP, bis-2-chloroethyl phosphate; BDCIPP, bis(1,3-dichloro-2-propyl) phosphate; DPHP, diphenyl phosphate; OPEs, organophosphate esters; LV, latent variable; BSI, Behavioral Symptom Index.

3. Results

Of the 389 women who gave birth to singleton infants and participated in the HOME Study, 170 were excluded because they either did not have at least one measurement of OPEs for themselves or their child or had not completed a BASC-2 questionnaire at either ages 3 or 8 years. Our final analytical sample was 219 mother-child dyads. On average, the pregnant study participants were aged 29.2 years at delivery, 61.2% were non-Hispanic white persons, and the majority had some college education. Children were 56.2% female, and 80.8% were ever breastfed (Table 1).

Specific gravity-standardized BCEP concentrations were the lowest at all time points, ranging from a median of 0.50 μg/L at 26 weeks of gestation to 1.21 μg/L at 3 years of age. DPHP concentrations, which were the highest during pregnancy and ages 1 and 2 years, ranged from a median of 1.32 μg/L at 26 weeks of gestation to 2.70 μg/L at 2 years of age. BDCIPP concentrations were highest at ages 3 and 5 years (medians of 3.65 and 3.36 μg/L, respectively, see Table 1). ICC values were <0.4 for all OPE metabolites during pregnancy and childhood, indicating poor correlation of the urinary OPE concentrations over time (Supplemental Table 1).

The fit statistics for all SEMs were considered very good, with CFIs ≥0.917, TLIs ≥0.933, and RMSEA ≤0.054 (Supplemental Table 2). The sample size for analyses of prenatal OPEs and behavior at age 3 years was n = 206; the sample size for analyses of prenatal OPEs and behavior at age 8 years was n = 210; the sample size for analyses of postnatal OPEs and behavior at age 8 years was n = 196. For all latent variables except the 2-year variable, the OPEs loaded as BDCIPP > BCEP > DPHP. In the 2-year variable, the loadings were BCEP > BDCIPP > DPHP (Supplemental Table 3).

In SEMs, higher urinary OPE latent variable values at 16 weeks of gestation were associated with fewer Externalizing Problems (ß = −5.74; 95% CI = −11.24, −0.24) and better BSI scores (ß = −5.26; 95% CI = −10.33, −0.19) at age 3 years. However, higher urinary OPE latent variable values at delivery were associated with more Internalizing Problems (ß = 2.93; 95% CI = −0.07, 5.94) and poorer BSI scores (ß = 2.87; 95% CI = 0.13, 5.61) at age 3 years. In addition, the urinary OPE latent variable at age 3 years was associated with fewer Externalizing Problems at age 8 (ß = −2.62; 95% CI = −5.13, −0.12). Confidence intervals for estimates of pregnancy urinary OPE latent variables and child behavior at age 8 years included the null, as did all latent variable models at ages 26 weeks, 1 year, 2 years, and 5 years (Fig. 2, Supplemental Table 4).

In quantile g-computation models for individual exposure time points, the relative magnitudes and directions of estimates were similar

Table 1
Maternal and child characteristics and specific gravity-corrected urinary OPE metabolite concentrations (n = 219), the HOME Study (2003–2006).

Maternal Characteristic	N	%
Race		
Non-Hispanic White	134	61.2
Other race	85	38.8
Education		
High school or less	55	25.1
Any college or trade school	125	57.1
Graduate school	39	17.8
Age at delivery [median, IQR]	29.3	24.9, 33.2
Child Characteristic	**N**	**%**
Sex		
Male	96	43.8
Female	123	56.2
Ever breastfed		
Yes	177	80.8
No	42	19.2
OPE Metabolite Concentrations (μg/L)[a]	**Median**	**IQR**
BCEP		
16 weeks	0.60	0.34, 1.07
26 weeks	0.50	0.23, 1.09
Delivery	0.53	0.28, 1.10
1 year	1.13	0.47, 2.73
2 years	0.95	0.49, 2.54
3 years	1.21	0.50, 3.04
5 years	0.80	0.39, 1.87
BDCIPP		
16 weeks	0.81	0.49, 1.49
26 weeks	0.61	0.29, 1.13
Delivery	0.64	0.62, 1.32
1 year	1.60	0.79, 3.40
2 years	2.04	1.03, 4.30
3 years	3.65	1.50, 7.12
5 years	3.36	1.59, 8.15
DPHP		
16 weeks	1.63	0.94, 3.13
26 weeks	1.32	0.79, 2.33
Delivery	1.68	0.93, 3.35
1 year	2.28	1.59, 4.00
2 years	2.70	1.65, 4.57
3 years	2.68	1.70, 4.81
5 years	2.69	1.60, 6.42

Abbreviations: HOME Study, Health Outcomes and Measures of the Environment Study; IQR, interquartile range; OPE, organophosphate ester; BCEP, bis-2-chloroethyl phosphate; BDCIPP, bis(1,3-dichloro-2-propyl) phosphate; DPHP, diphenyl phosphate.
[a] The limit of detection for all OPE metabolites was 0.1 μg/L.

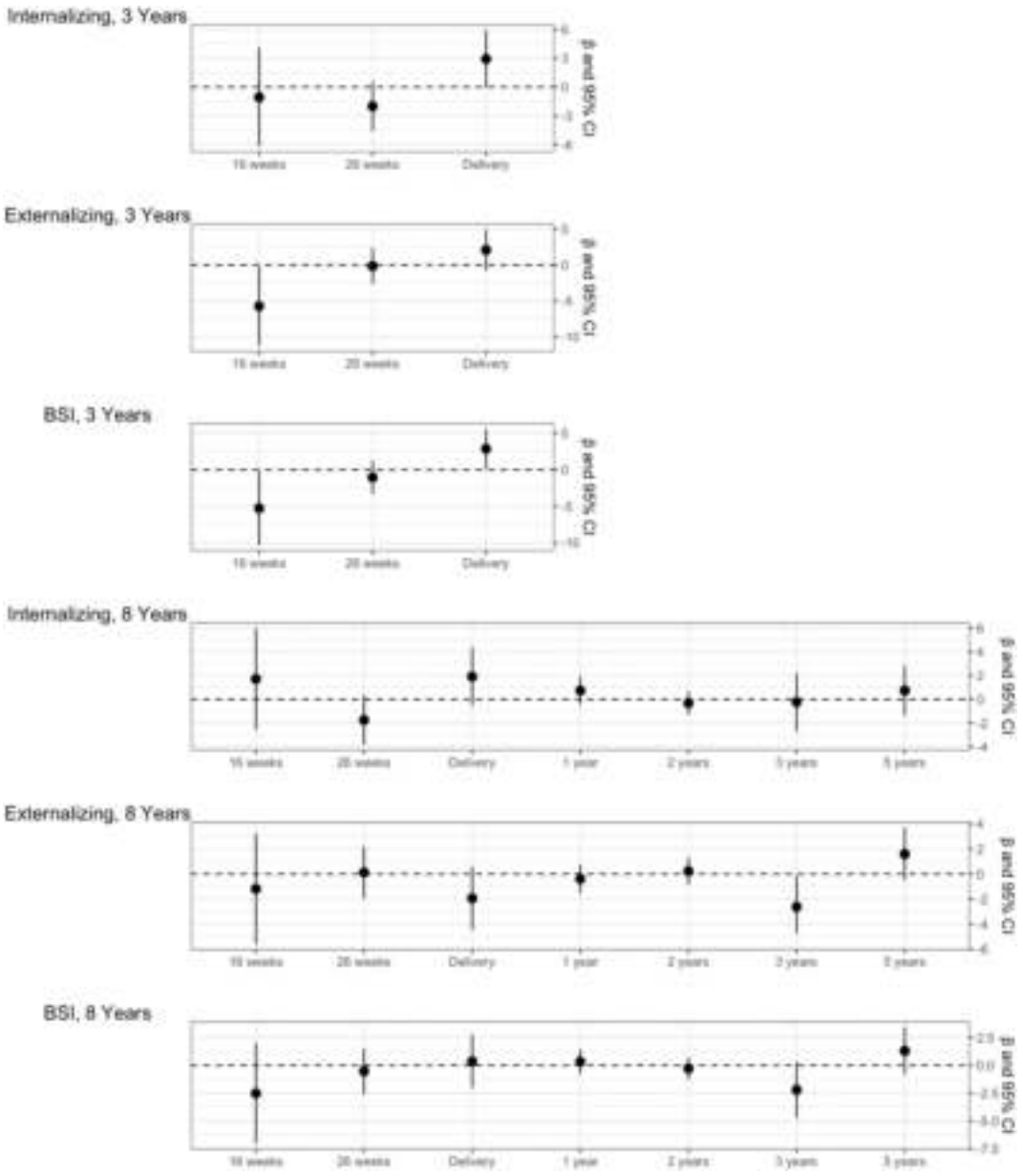

Fig. 2. SEM models for natural-log transformed OPE latent variables and BASC-2 outcome t-scores (n = 219). All models are adjusted for maternal depression, breastfeeding status, maternal race, income, and HOME score.
Abbreviations: CI, confidence interval; BSI, Behavioral Symptom Index.

to SEM results, but all confidence intervals included the null (Table 2). However, in the longitudinal quantile g-computation models, pregnancy concentrations of OPEs were associated with fewer Externalizing Problems at age 8 years (ß = −0.96, 95% CI = −1.93, 0.01), a result not observed with the other model types (Table 3).

4. Discussion

In this longitudinal cohort study of OPE exposures during early brain development and child behavior at ages 3 and 8 years, we found that *in utero* exposures were more frequently and significantly associated with internalizing problems, externalizing problems, and overall maladaptive behavior at age 3 years compared with childhood exposures in latent variable analyses. However, the direction of associations was inconsistent. OPE exposure at delivery was associated with more behavior problems, while OPE exposure at 16 weeks of gestation and age 3 years was associated with fewer behavior problems. In single-timepoint quantile g-computation models, the magnitude and direction of the null associations were similar to the latent variable results. We did observe a small and significant decrease in externalizing behavior problems at age 8 years with *in utero* exposure longitudinal quantile g-computation models.

The associations we observed showing decreased behavioral issues at age 8 years with higher OPEs earlier in life could be partly due to remedial actions taken by parents and schools that would not yet have been accomplished at age 3 years. Externalizing behaviors in particular are likely to be intervened upon in school-age children. Although we do not have the data to test this hypothesis, it could be an area of future study for other researchers.

Others have also reported associations between OPE exposures and child behavior measured by the BASC-2. Doherty et al. measured urinary OPE metabolites once during pregnancy, around 27 weeks, in a cohort of North Carolina women and reported associations of BDCIPP and DPHP concentrations with higher scores on the Externalizing Problems and BSI

Table 2
Quantile g-computation models for OPEs and BASC-2 outcomes, estimates for increasing urine exposure biomarkers by one quartile (n = 219). All models are adjusted for maternal depression, breastfeeding status, maternal race, income, and HOME score.

Exposure assessment	Outcome	Estimate	95% CI
16 Weeks	Internalizing, 3 Years	−0.36	−2.39, 1.67
	Externalizing, 3 Years	−1.02	−2.93, 0.88
	BSI, 3 Years	−0.64	−2.36, 1.08
26 Weeks	Internalizing, 3 Years	−0.61	−2.35, 1.13
	Externalizing, 3 Years	−0.49	−2.13, 1.14
	BSI, 3 Years	−0.72	−2.24, 0.80
Delivery	Internalizing, 3 Years	0.34	−1.66, 2.34
	Externalizing, 3 Years	1.08	−0.79, 2.95
	BSI, 3 Years	0.58	−1.10, 2.26
16 Weeks	Internalizing, 8 Years	0.52	−1.07, 2.11
	Externalizing, 8 Years	−1.14	−2.68, 0.41
	BSI, 8 Years	−1.01	−2.53, 0.52
26 Weeks	Internalizing, 8 Years	−0.07	−1.43, 1.30
	Externalizing, 8 Years	−0.36	−1.73, 1.01
	BSI, 8 Years	−0.37	−1.72, 0.98
Delivery	Internalizing, 8 Years	0.41	−1.16, 1.98
	Externalizing, 8 Years	−0.64	−2.15, 0.87
	BSI, 8 Years	−0.27	−1.82, 1.27
1 Year	Internalizing, 8 Years	0.21	−1.36, 1.77
	Externalizing, 8 Years	−0.65	−2.33, 1.04
	BSI, 8 Years	−0.43	−2.05, 1.20
2 Years	Internalizing, 8 Years	−0.59	−2.32, 1.13
	Externalizing, 8 Years	−0.67	−2.24, 0.90
	BSI, 8 Years	−1.14	−2.83, 0.54
3 Years	Internalizing, 8 Years	0.18	−1.41, 1.77
	Externalizing, 8 Years	−1.17	−2.70, 0.37
	BSI, 8 Years	−1.30	−2.78, 0.18
5 Years	Internalizing, 8 Years	0.56	−0.91, 2.03
	Externalizing, 8 Years	0.37	−1.18, 1.91
	BSI, 8 Years	0.19	−1.28, 1.67

Abbreviations: CI, confidence interval; BSI, Behavioral Symptom Index.

Table 3
Longitudinal OPE exposure and BASC-2 outcome t-scores quantile g-computation models, estimates for increasing exposure biomarker concentrations by one quartile (n = 187). All models are adjusted for maternal depression, breastfeeding status, maternal race, income, and HOME score.

Exposure assessment	Outcome	Estimate	95% CI
Pregnancy	Internalizing, 3 years	−0.22	−1.34, 0.89
	Externalizing, 3 years	−0.12	−1.34, 1.10
	BSI, 3 years	−0.27	−1.38, 0.84
Pregnancy	Internalizing, 8 years	0.21	−0.67, 1.10
	Externalizing, 8 years	−0.96	−1.93, 0.01
	BSI, 8 years	−0.60	−1.55, 0.36
Postnatal	Internalizing, 8 years	0.30	−0.54, 1.14
	Externalizing, 8 years	−0.22	−1.10, 0.65
	BSI, 8 years	−0.37	−1.25, 0.51

Abbreviations: CI, confidence interval; BSI, Behavioral Symptom Index.

composite scores at age 3 years (Doherty et al., 2019b). However, urinary concentrations of isopropyl-phenyl phenyl phosphate (ip-PPP), an OPE not measured in our study, were associated with lower Internalizing Problems and BSI composite scores. The observation of mixed results concerning the direction of the effects is consistent with our study. Castorina et al. also measured urinary OPE metabolites once during pregnancy, around 26 weeks, in a cohort of California women (mainly Hispanic, living in farm-working communities) and assessed child behavior at age 7 years with the BASC-2. They did not report associations with composite scales but did observe that BDCIPP and ip-PPP concentrations were associated with higher scores on the attention and hyperactivity subscales, respectively, which contribute to the Externalizing and BSI composite scores (Castorina et al., 2017). We observed some evidence of an association between pregnancy OPEs and externalizing behaviors at 8 years in our quantile g-computation analysis, but in the opposite direction to what Castorina et al. reported. Neither of these cited studies reported multiple time points of OPE exposure or associations with repeated measurements of child behavior, which makes them more susceptible to measurement error.

We are only aware of one study that reported associations between childhood OPE exposure and behavior. In 3- to 5-year-old children, Lipscomb et al. measured levels of OPEs parent compounds in silicone wristbands worn for 7 days and observed that higher OPEs were associated with less responsible behavior and more externalizing problems based on a teacher-reported rating scale of social skills (Lipscomb et al., 2017). They did not observe any associations with internalizing behaviors. However, this study was limited by its cross-sectional design and lack of internal biomarkers of exposure.

While our mixture-based analysis—which more closely approximated real-world exposures—was a strength of the study, our results should be interpreted cautiously. Our findings were inconsistent with respect to which exposure periods during brain development were sensitive to OPEs and the effects on behavioral domains. This variability could result from spurious findings, uncontrolled confounding, or our inability to account for the effects of other chemical exposures from different classes in our mixtures analysis. A limitation to our mixture approach is that we were unable to include more than three OPEs in our models. We previously attempted to quantify the concentrations of additional OPE metabolites in our participants' urine, but the number of samples that were below the limit of detection made the inclusion of additional metabolites unfeasible for our statistical methods (Percy et al., 2020). This is likely due to the time period in which our samples were collected, during the phase-out of polybrominated diphenyl ethers from use as flame retardants, when environmental concentrations of OPEs were lower than they are today. Other cohorts might be better able to include a broader group of OPEs in future mixtures analyses.

A limitation of our study is that we relied on a parent-report questionnaire to assess child behavior instead of utilizing a direct assessment or a self-report method. We also did not specifically exclude intellectually disabled mothers from the study, which may have impacted the reliability of a parent-report questionnaire. However, the BASC-2 is useful for this study in that it is logistically convenient and repeatable at ages 3 and 8 years. Other assessment options, such as the Bayley Scales of Infant and Child Development (Bayley, 1993) or a child-reported questionnaire, would have only been appropriate for one age group and would have limited our ability to directly compare our results between ages. It cannot be entirely ruled out that higher exposed women might have completed the BASC-2 with a systematic bias, but we attempted to control for this type of confounding by adjusting for maternal depression. However, we cannot rule out residual confounding in our analyses.

However, the study had several strengths. OPEs have a short biological half-life and poor reliability between measurements (Percy et al., 2020; Wang et al., 2020), so frequent quantification of OPE metabolite concentrations is critical to reducing exposure misclassification as the chemicals do not accumulate for long in the body. We had three urinary OPE metabolite measurements during pregnancy and four during early childhood; this is more than any other study currently in the literature. We also assessed child behavior at two different time points, at age 3 years during early childhood and at age 8 years, when child behavioral measures tend to be more reliable. Finally, we utilized two methods to quantify associations between OPE mixtures and child behavior, and the results were largely consistent.

This study is the first that utilized a chemical mixtures approach to study pregnancy and postnatal OPE exposure and behavior in children. Therefore, additional research will provide helpful information to determine the direction and magnitude of the associations and the public health relevancy of the results. Given our findings and the findings of others, we would urge decision-makers to inform the public about ways to reduce exposure to OPEs. Chlorinated OPEs, like the

parent compounds of BCEP and BDCIPP, are typically used as flame retardants, while the parent compound of DPHP is used as a plasticizing chemical and as a component of commercial flame retardant mixtures, including FM550 (van der Veen and de Boer, 2012). Therefore, recommendations that include frequent cleaning in rooms containing electronics or furniture that may have been treated with OPEs as flame retardant chemicals, the use of indoor air purification to keep down dust levels, and reducing dependance on plastic products may be appropriate.

5. Conclusions

Pregnancy and early childhood mixtures of urinary OPE metabolites were associated with some measures of child behavior in this pregnancy and birth cohort. We did not observe consistent patterns concerning the direction of the association, vulnerable time points, or the type of behaviors affected. However, the results were similar across two different statistical methods. The heterogeneity may be actual or due to measurement error, and further research on other populations can help inform public health decisions.

Declaration of competing interest

Dr. Braun was financially compensated for serving as an expert witness in litigation related to perfluorooctanonic acid contamination in drinking water.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114162.

References

Bayley, N., 1993. Bayley Scales of Infant Devlopment: Manual, second ed. The Psychological Corporation, San Antonio.
Beck, A.T., Steer, R.A., Brown, G.K., 1996. Manual for the Beck Depression Inventory-II.
Bergh, C., Torgrip, R., Emenius, G., et al., 2011. Organophosphate and phthalate esters in air and settled dust - a multi-location indoor study. Indoor Air 21 (1), 67–76.
Braun, J.M., Kalloo, G., Chen, A., et al., 2017. Cohort profile: the health outcomes and measures of the environment (HOME) study. Int. J. Epidemiol. 46 (1), 24-24i.
Braun, J.M., Buckley, J.P., Cecil, K.M., et al., 2020. Adolescent follow-up in the health outcomes and measures of the environment (HOME) study: cohort profile. BMJ Open 10 (5), 1–11.
Brown, T.T., Jernigan, T.L., 2012. Brain development during the preschool years. Neuropsychol. Rev. 22 (4), 313–333.
Caldwell, B., Bradley, R., 1984. Home Observation for Measurement of the Environment.
Caspi, A., Moffitt, T.E., Newman, D.L., et al., 1996. Behavioral observations at age 3 Years predict adult psychiatric disorders. Arch. Gen. Psychiatr. 53, 1033–1039.
Castorina, R., Bradman, A., Stapleton, H.M., et al., 2017. Current-use flame retardants: maternal exposure and neurodevelopment in children of the CHAMACOS cohort. Chemosphere 189, 574–580. https://doi.org/10.1016/j.chemosphere.2017.09.037 [electronic article].
Choi, G., Keil, A.P., Richardson, D.B., et al., 2021. Pregnancy exposure to organophosphate esters and the risk of attention-deficit hyperactivity disorder in the Norwegian mother, father and child cohort study. Environ. Int. 154, 106549 https://doi.org/10.1016/j.envint.2021.106549 [electronic article].
Doherty, B.T., Hammel, S.C., Daniels, J.L., et al., 2019a. Organophosphate esters: are these flame retardants and plasticizers affecting children's health? Curr Environ Health Rep 6 (4), 201–213.
Doherty, B.T., Hoffman, K., Keil, A.P., et al., 2019b. Prenatal exposure to organophosphate esters and behavioral development in young children in the Pregnancy, Infection, and Nutrition Study. Neurotoxicology 73 (March), 150–160.
Duty, S.M., Ackerman, R.M., Calafat, A.M., et al., 2005. Personal care product use predicts urinary concentrations of some phthalate monoesters. Environ. Health Perspect. 113 (11), 1530–1535.
Grandjean, P., Landrigan, P.J., 2015. Neurobehavioural effects of developmental toxicity. Lancet Neurol. 13 (3), 330–338.
Han, L., Sapozhnikova, Y., Nuñez, A., 2019. Analysis and occurrence of organophosphate esters in meats and fish consumed in the United States. J. Agric. Food Chem. 67 (46), 12652–12662.
He, C., Wang, X., Tang, S., et al., 2018. Concentrations of organophosphate esters and their specific metabolites in food in Southeast Queensland, Australia: is dietary exposure an important pathway of organophosphate esters and their metabolites? Environ. Sci. Technol. 52 (21), 12765–12773.
Hooper, D., Coughlan, J., Mullen, M.R., 2008. Structural equation modeling: guidelines for determining model fit. Electron. J. Bus. Res. Methods 6 (1), 53–60.
Hornung, R., Reed, L., 1990. Estimation of average concentration in the presence of nondetectable values. Appl. Occup. Environ. Hyg 5, 46–51.
Jayatilaka, N.K., Restrepo, P., Williams, L., et al., 2017. Quantification of three chlorinated dialkyl phosphates, diphenyl phosphate, 2,3,4,5-tetrabromobenzoic acid, and four other organophosphates in human urine by solid phase extraction-high performance liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 409 (5), 1323–1332.
Jayatilaka, N.K., Restrepo, P., Davis, Z., et al., 2019. Quantification of 16 urinary biomarkers of exposure to flame retardants, plasticizers, and organophosphate insecticides for biomonitoring studies. Chemosphere 235, 481–491.
Keil, A.P., Buckley, J.P., O'Brien, K.M., et al., 2020. A quantile-based g-computation approach to addressing the effects of exposure mixtures. Environ. Health Perspect. 128 (4), 1–10.
Kim, U.J., Kannan, K., 2018. Occurrence and distribution of organophosphate flame retardants/plasticizers in surface waters, tap water, and rainwater: implications for human exposure. Environ. Sci. Technol. 52 (10), 5625–5633.
Kim, U.-J., Wang, Y., Li, W., et al., 2019. Occurrence of and human exposure to organophosphate flame retardants/plasticizers in indoor air and dust from various microenvironments in the United States. Environ. Int. 125 (January), 342–349 [electronic article]. https://linkinghub.elsevier.com/retrieve/pii/S0160412018327296.
Li, J., Zhao, L., Letcher, R.J., et al., 2019. A review on organophosphate Ester (OPE) flame retardants and plasticizers in foodstuffs: levels, distribution, human dietary exposure, and future directions. Environ. Int. 127 (March), 35–51. https://doi.org/10.1016/j.envint.2019.03.009 [electronic article].
Lipscomb, S.T., McClelland, M.M., MacDonald, M., et al., 2017. Cross-sectional study of social behaviors in preschool children and exposure to flame retardants. Environ. Health 16 (1), 1–10.
Liu, X., Jung, D., Jo, A., et al., 2016. Long-term exposure to triphenylphosphate alters hormone balance and HPG, HPI, and HPT gene expression in zebrafish (Danio rerio). Environ. Toxicol. Chem. 35 (9), 2288–2296.
Liu, W., Luo, D., Xia, W., et al., 2021. Prenatal exposure to halogenated, aryl, and alkyl organophosphate esters and child neurodevelopment at two years of age. J. Hazard Mater. 408 (December 2020), 124856 https://doi.org/10.1016/j.jhazmat.2020.124856 [electronic article].
Loth, A.K., Drabick, D.A.G., Leibenluft, E., et al., 2014. Do childhood externalizing disorders predict adult depression? A meta-analysis. J. Abnorm. Child Psychol. 42 (7), 1103–1113.
Luo, K., Liu, J., Wang, Y., et al., 2020. Associations between organophosphate esters and sex hormones among 6–19-year old children and adolescents in NHANES 2013–2014. Environ. Int. 136 (January), 105461.
MacPherson, S., Arbuckle, T.E., Fisher, M., 2018. Adjusting urinary chemical biomarkers for hydration status during pregnancy. J. Expo. Sci. Environ. Epidemiol. 28 (5), 481–493.
Muthen, L., Muthen, B., 2017. Mplus User's Guide, eighth ed.
Patisaul, H.B., Behl, M., Birnbaum, L.S., et al., 2021. Beyond cholinesterase inhibition: developmental neurotoxicity of organophosphate ester flame retardants and plasticizers. Environ. Health Perspect. 129 (10), 1–12.
Percy, Z., Vuong, A.M., Ospina, M., et al., 2020. Organophosphate esters in a cohort of pregnant women : variability and predictors of exposure. Environ. Res. 184 (February), 109255 https://doi.org/10.1016/j.envres.2020.109255 [electronic article].
Percy, Z., Vuong, A.M., Xu, Y., et al., 2021. Maternal urinary organophosphate esters and alterations in maternal and neonatal thyroid hormones. Am. J. Epidemiol. 190 (9), 1793–1802.
R Core Team, 2019. R: A Language and Environment for Statistical Computing.
Ren, X., Cao, L., Yang, Y., et al., 2015. In vitro assessment of thyroid hormone receptor activity of four organophosphate esters. J. Environ. Sci. (China) [electronic article] 45, 185–190. https://doi.org/10.1016/j.jes.2015.12.021.
Reynolds, C., Kamphaus, R., 2004. Behavior Assessment System for Children, second ed. American Guidance Services Publishing, Circle Pines, MN.
Rosner, B., 2011. Fundamentals of Biostatistics. Cengage Learning, Boston, MA.
Tao, Y., Hu, L., Liu, L., et al., 2021. Prenatal exposure to organophosphate esters and neonatal thyroid-stimulating hormone levels: a birth cohort study in Wuhan, China. Environ. Int. 156, 106640 https://doi.org/10.1016/j.envint.2021.106640 [electronic article].
van der Veen, I., de Boer, J., 2012. Phosphorus flame retardants: properties, production, environmental occurrence, toxicity and analysis. Chemosphere 88 (10), 1119–1153. https://doi.org/10.1016/j.chemosphere.2012.03.067 [electronic article].
Vergunst, F., Tremblay, R.E., Nagin, D., et al., 2019. Association of behavior in boys from low socioeconomic neighborhoods with employment earnings in adulthood. JAMA Pediatr. 173 (4), 334–341.
Wang, X., Liu, Q., Zhong, W., et al., 2020. Estimating renal and hepatic clearance rates of organophosphate esters in humans: impacts of intrinsic metabolism and binding

affinity with plasma proteins. Environ. Int. 134 (November 2019), 105321 https://doi.org/10.1016/j.envint.2019.105321 [electronic article].
Wei, G.L., Li, D.Q., Zhuo, M.N., et al., 2015. Organophosphorus flame retardants and plasticizers: sources, occurrence, toxicity and human exposure. Environ. Pollut. 196, 29–46. https://doi.org/10.1016/j.envpol.2014.09.012 [electronic article].
Yan, Z., Jin, X., Liu, D., et al., 2021. The potential connections of adverse outcome pathways with the hazard identifications of typical organophosphate esters based on toxicity mechanisms. Chemosphere 266, 128989.

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Effect of a novel hygiene intervention on older children's handwashing in a humanitarian setting in Kahda district, Somalia: A cluster-randomised controlled equivalence trial

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ABSTRACT

Introduction: Improving handwashing with soap (HWWS) among children in humanitarian emergencies has the potential to reduce the transmission of several important infectious diseases. However, there is limited evidence on which approaches are effective in increasing HWWS among children in humanitarian settings. One recent innovation – the "Surprise Soap" intervention – was shown to be successful in a small-scale efficacy trial in a humanitarian setting in Iraq. This intervention includes soap with embedded toys delivered through a short household session comprising a glitter game, instruction of how and when to wash hands, and HWWS practice. Whilst promising, this approach has not been evaluated at programmatic scale in a complex humanitarian setting.

Methods: We conducted a cluster-randomised controlled equivalence trial of the Surprise Soap intervention in IDP camps in Kahda district, Somalia. Proportionate stratified random sampling was employed to recruit 200 households, with at least one child aged 5–12, across the camps. Eligible households were randomly allocated to receive the Surprise Soap intervention (n = 100) or an active comparator handwashing intervention in which plain soap was delivered in a short household session comprising standard health-based messaging and instruction of how and when to wash hands (n = 100). The primary outcome was the proportion of pre-specified occasions when HWWS was practiced by children aged 5–12 years, measured at baseline, 4-weeks, 12 weeks, and 16 weeks post invention delivery.

Results: HWWS increased in both groups (by 48 percentage points in the intervention group and 51 percentage points in the control group, at the 4-week follow up), however, there was no evidence of a difference in HWWS between the groups at the 4-week (adjusted RR (aRR) = 1.0, 95% CI 0.9–1.1), 12-week (aRR = 1.1, 95% CI 0.9–1.3), or 16-week (aRR = 1.0, 95% CI 0.9–1.2) follow-up.

Conclusions: In this complex humanitarian setting, where soap availability and past exposure to handwashing promotion was low, it appears that well-designed, household-level targeted handwashing interventions that include soap provision can increase child HWWS and potentially reduce disease risk, but the Surprise Soap intervention offers no marginal benefit over a standard intervention that would justify the additional costs.

1. Introduction

In humanitarian emergencies, conditions such as overcrowding, unclean water and sanitation facilities, limited access to healthcare, and environmental contamination leave people at high risk of disease (UNHCR, 2015, Toole and Waldman, 1997; Connolly et al., 2004; Kouadio et al., 2012). Faecal-oral diseases such as diarrhoea, for example, are responsible for up to 40% of all deaths in the acute phase of

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an emergency (Connolly et al., 2004).

In these high-risk environments, handwashing with soap (HWWS) can be an effective means of preventing disease transmission. Systematic reviews consistently show that HWWS is effective in reducing diarrhoeal disease by up to 48% (Curtis and Cairncross, 2003; Freeman et al., 2014; Fewtrell et al., 2005; Cairncross et al., 2010; Waddington and Snilstveit, 2009; Ejemot-Nwadiaro et al., 2015; Wolf et al., 2018) and it is ranked as one of the most cost effective of all public health interventions (Horton and Levin, 2016; Jamison et al., 2006; Walker et al., 2010). HWWS can also reduce the risk of acute respiratory infections (ARIs) by 21–23% (Aiello et al., 2008; Rabie and Curtis, 2006) and has been linked to the reduction of certain neglected tropical diseases, such as trachoma (Stocks et al., 2014) and certain soil-transmitted helminth infections (STHs) (Strunz et al., 2014).

Children can account for more than half of the population in humanitarian settings (UNHCR, 2015). Diarrhoeal diseases and ARIs are responsible for most deaths among children, and the burden of trachoma and STHs are also concentrated in this age group (Vos et al., 2020). Increasing HWWS among children in humanitarian settings has the potential to achieve a large public health impact, not only via direct improvements in health outcomes but also via extended benefits such as improvements in school attendance (Willmott et al., 2015; Nandrup-Bus, 2009; Talaat et al., 2011; Azor-Martinez et al., 2016; Mohamed et al., 2020) which may lead to improved academic attainment (Lamdin, 1996; Morrissey et al., 2014) and associated economic and health benefits later in life (Gakidou et al., 2010).

HWWS interventions that aim to reduce the infectious disease burden among young children are predominately targeted at their caregivers – a logical approach considering they assume responsibility for much of the child's behaviour. Older children (classified as children between the age of 5–14 by the Global Burden of Disease studies (Vos et al., 2020)), however, spend more time outside the household, for example at school, and are often expected to have responsibility for washing their own hands. Increasing HWWS among older children is of public health importance, not only to reduce disease transmission risk among this group but also as they may act as effective agents of change for behavioural practices in the community and can take an active role in their handwashing practices as well as that of other family members (Bresee et al., 2016; Onyango-Ouma et al., 2005, Winter et al., 2021; Blanton et al., 2010; Tidwell et al., 2020). Currently though, there is limited evidence around which approaches are effective in increasing HWWS among older children in humanitarian settings (Watson et al., 2017). Even in stable settings, few rigorous studies of HWWS promotion interventions targeting older children have been published and the effects of these have been mixed (Watson et al., 2017, 2021).

One recent intervention that has shown promise in a humanitarian setting is a novel motive-based intervention, referred to as the 'Surprise Soap' intervention (Watson et al., 2019). This intervention aims to encourage older children's HWWS by appealing to their innate motives of play and curiosity. It involves the delivery of bars of Surprise Soap – transparent soaps with a toy embedded inside – in a short, fun household session that does not rely on traditional health-based messaging, which research has shown to be a poor motivator of behaviour change, particularly among children (White et al., 2020; Biran et al., 2009; Curtis et al., 2009; Rheinländer et al., 2015). The theorised mechanism of change for the Surprise Soap intervention is simply that children are more motivated to wash their hands with soap when there is a toy inside.

In 2018 this intervention was evaluated in an internally displaced persons (IDP) camp in Iraq (Watson et al., 2019) in a small proof-of-concept trial. At the 4-week follow-up, children in the intervention group were observed to practice HWWS almost 4 times more often compared to the counterfactual, a standard health-based household-level handwashing intervention (adjusted RR = 3.94, 95% CI 1.59–9.79) (Alexander et al., 2013). These findings are promising and indicate that this rapidly deployable intervention might be an effective means to increase children's HWWS in humanitarian emergencies and thereby reduce the risk of infectious disease. However, this pilot study was conducted in just one IDP camp with a homogeneous population (100% Yezidi), in which children already had a high exposure to hygiene promotion and good access to soap and water, we do not know if it can be effective in more complex humanitarian settings. The study follow-up was also limited to only 4 weeks whereas the acute phase of emergencies may last significantly longer, requiring interventions that can sustain HWWS over longer periods.

To address this information gap, we conducted a cluster-randomised controlled equivalence trial over 16 weeks to compare the effectiveness of the Surprise Soap intervention and a household-level intervention comprising of standard messages and plain soap, in a complex humanitarian setting where the population is mixed, access to handwashing facilities is limited, and past exposure to extensive handwashing promotion is low. Findings will both contribute to the limited evidence base for HWWS interventions targeting children and guide humanitarian agencies' decisions on the deployment of the Surprise Soap intervention at scale.

2. Materials and methods

2.1. Study design and participants/eligibility

This study was a cluster-randomised controlled equivalence trial with an intervention arm and an active control arm. Households were eligible to participate in the study if they included at least one child between the age of 5 and 12 and had no plans to travel away for more than one week over the ensuing six months. Individual households were then randomly assigned (1:1) to an intervention arm receiving the Surprise Soap intervention or the active control arm receiving a standard handwashing promotion intervention.

2.2. Study setting

The study took place across three IDP camps – Banaaney Two, Samadeq, and Alkowsar - in the Kahda district of the Banadir region of Somalia. The Kahda district is one of 17 districts in the Banadir region with a population of almost half a million, comprising host and IDP communities (Camp Coordination and Camp Management Cluster (CCCM). 2022). It is one of the districts with the largest IDP populations in the Banadir region with a growing population of displaced persons due to ongoing and protracted humanitarian emergencies (Camp Coordination and Camp Management Cluster (CCCM). 2022). We included three camps where the humanitarian agency, Action Against Hunger is engaged and where access to education, food security and livelihoods, health, housing, land and property, protection, shelter and non-food items, and water, sanitation and hygiene are all classed as 'extreme' or 'severe' by the Camp Coordination and Camp Management Cluster (CCCM) (Camp Coordination and Camp Management Cluster (CCCM). 2022). Most of the population in these camps live in self-made shelters known as 'buul'. They access drinking water from paid communal water points, water kiosks, or from vendors and shops. Very few households report having access to a functioning handwashing station with soap and water and children in these settings have had little previous exposure to hygiene promotion.

2.3. Intervention content and delivery

Households assigned to the intervention group received the Surprise Soap intervention, and households assigned to the active control group received a standard handwashing promotion intervention. Each intervention was delivered to children at their house the day after baseline observation was carried out. Hygiene promoters, already active under Action Against Hunger were trained to deliver both interventions. The main features of the two interventions are presented in Table 1 and further details given below.

Table 1
Overview of intervention activities in each study arm.

	Intervention group	Active control group
Intervention	Surprise Soap Intervention	Standard Intervention
Setting	Household	Household
Intensity	One-off session	One-off session
Session length	10 minutes	10 minutes
Approach	Motive-based	Education-based
Products	Surprise Soap x 5 bars, plus later replenishments	Plain soap identical to Surprise Soap but minus the toy x 5 bars, plus later replenishments
Activities	Glitter game to demonstrate germs spreading Demonstration of handwashing technique Information on key times to wash hands Children practicing handwashing with Surprise Soap	Handwashing-related health-messaging using F-diagram Demonstration of handwashing technique Information on key times to wash hands
Delivery agent	Action Against Hunger hygiene promoters	Action Against Hunger hygiene promoters

2.4. Surprise Soap intervention

The Surprise soap intervention consisted of distribution of Surprise Soap bars within a short (approximately 10-min) household session. Surprise Soaps are round transparent glycerine soaps with toy animals embedded inside (Fig. 1). All soaps were manufactured by the company, KIMA, in Jordan. Brief formative work which involved showing photos of potential toy options and soliciting feedback from IDP camp leaders, hygiene promoters, and adult residents of the camps, was undertaken by Action Against Hunger to ensure the toys were culturally appropriate. On arriving at their designated household, hygiene promoters gathered the children of the household together and initiated a "glitter game" to demonstrate how germs spread: petroleum jelly and glitter were applied to one child's hands who then 'high fived' the other children, transferring the 'glitter germs' between hands. The hygiene promoter then revealed the Surprise Soap bars to the children, explaining that the more often they wash their hands with the soap, the faster they will reach the toy inside, and listing five key handwashing times (before eating, before preparing food, before serving food to another person, after using the toilet, and before cleaning another person's faeces). The hygiene promoter then gave a demonstration of ideal handwashing technique and invited the children to practice washing the glitter from their hands using the Surprise Soap and then left a parcel of five Surprise Soaps with the children in the household. At least one adult of the household, usually a caregiver, was present during intervention delivery but they were not instructed in any way about the use of these toy soaps. Directly after the 4-week, 12-week, and 16-week follow-up household observations, the hygiene promoters visited the households again to distribute further packages of Surprise Soap but did not repeat the household session. No handwashing messages were delivered during these follow-up visits.

Fig. 1. Surprise Soap image.

2.5. Standard intervention

The standard intervention consisted of the distribution of plain soap, identical to the Surprise Soap in colour, size, shape, volume, and quality but without a toy inside, delivered within a short household session (approximately 10 minutes – comparable to the length of the Surprise Soap household session) to control for the effects of soap provision and household-level delivery. The household session focused on standard health-based messages using some of Action Against Hunger's existing handwashing promotion material. Hygiene promoters gathered the children and showed them the F-diagram, explaining how the spread of germs from faeces to mouths via hands can lead to diseases such as diarrhoea. They explained that HWWS can prevent these diseases, listed five key times to practice HWWS (as above), and demonstrated ideal handwashing technique. A parcel of five plain soaps were left with the children. Plain soap was also replenished directly after the 4-week, 12-week and 16-week follow-up household observations, in the same quantities as Surprise Soap, without repeating the household session.

2.6. Outcomes

The primary outcome for the trial was the proportion of five key potential handwashing occasions that were accompanied by HWWS (both hands) for children aged 5–12 years. The five 'key potential handwashing occasions' were: (i) after defecation or using the toilet, (ii) before eating, (iii) before preparing food, (iv) before serving food to another person and, (v) after cleaning another child's faeces. This outcome was measured at all follow-up visits. The two secondary outcomes were: the proportion of all observed handwashing events (handwashing with water) where soap was also used, across all timepoints; and the total number of observed HWWS events across all timepoints.

In addition, a series of indicators of intervention compliance were also assessed in the arm receiving the Surprise Soap intervention. These included the number of bars of Surprise Soap remaining at endline, whether a bar of Surprise Soap was wet on inspection at endline, the reported time in days required to reach the toy in the Surprise Soap, reported incidents of "toy cheats" (i.e. where Surprise Soap was broken to access the toy prematurely), and reported use of the Surprise Soap by other household members (children <5 years of age, and adults), and for other purposes than hand hygiene (bathing, laundry, washing dishes, or any other uses).

2.7. Data collection

All data collection activities were undertaken by a team of trained enumerators, recruited by Action Against Hunger, who had no role in the delivery of the intervention. The research team from the London School of Hygiene and Tropical Medicine (LSHTM) provided a three-day training to field supervisors, involving both classroom and practical sessions, and supervisors subsequently trained enumerators in the local

language. Basic background social and demographic data were collected at the time of recruitment using a verbally administered questionnaire. During the four weeks before intervention delivery, one enumerator returned to each enrolled household to conduct direct structured observations of child handwashing practices and to record data on household handwashing facilities using spot-check observations. Structured observations started at approximately 9:30AM and continued for 3 h – a period when most children would be home. Data were collected for all children aged 5–12 years present in the household during the observation period. Enumerators positioned themselves in an unobtrusive location in or near the household where they had the best view of the children and the handwashing facility (where available). Every instance of the five key handwashing occasions (as defined above) and the associated handwashing practice (hands not washed, washed with water only, washed with soap and water) was recorded. Any instances of HWWS that were not associated with these five key occasions was also recorded. To be recorded as 'washed with water only' or 'washed with soap and water' both hands had to be washed. If only one hand was washed this was recorded as 'hands not washed'. Structured observations were repeated 4 weeks, 12 weeks, and 16 weeks post intervention delivery. In intervention households only, directly after the 16-week structured observation, field workers also recorded information on intervention compliance. All data were collected using Open Data Kit (ODK) on android tablets and uploaded onto a dedicated encrypted server at the end of each data collection day for the research team at LSHTM to cross check the data daily.

2.8. Sample size and randomisation procedure

We calculated that a sample size of 200 households (i.e., clusters) was needed to detect an absolute difference in HWWS after key occasions of 10% between control and intervention groups (15% HWWS after key occasions in the control group, 25% in the intervention group), with 80% power ($\alpha = 0{\cdot}05$). We assumed an average of seven observed HWWS occasions (i.e., when hands could have been washed or not) per household per 3 hour observation period, a within-household intracluster correlation coefficient (ICC) of 0.21 (Biran et al., 2014), and a loss to follow-up (LTF) of 20%.

Each of the three IDP camps was considered as separate stratum and proportionate stratified random sampling was employed to select households across the three strata using complete lists of all households in the sites, randomised within Stata. If a household on the randomised list was non-eligible the next household on the list was approached, and so on until a total of 200 households were enrolled across each stratum. Within each stratum, households were randomly assigned to intervention or control group with a 1:1 ratio using a random number generator in Stata, Version 16.1 (StataCorp, 2019).

2.9. Blinding

The precise nature of the data being collected was not disclosed to participants, instead they were informed that the enumerators would be observing children's routines to build an understanding of how children's health and wellbeing can be improved in the area. Enumerators were informed that all participating households would receive a hand hygiene intervention, but they were not informed of the nature of the intervention received by intervention and control arms, and they had no role in the intervention delivery. Due to the nature of the intervention, no further blinding of study participants or enumerators was possible.

2.10. Statistical analysis

All statistical analyses were undertaken using Stata, Version 16.1 (StataCorp, 2019). We analysed the effect of the intervention on the proportion of key handwashing occasions accompanied by HWWS using a Poisson GEE model for rates, in which the number of handwashes with soap was offset by the total number of key occasions (possible handwashing occasions) per child. The proportion of all handwashes that used soap, was similarly assessed using a Poisson model for rates in which the total observed children's handwashes that used soap was offset by the number of observed handwashes per child. Finally, the total number of observed handwashes with soap was analysed using a Poisson GEE model for counts. In all models, clustering was accounted for at the highest level, the household (because children were nested within the household) and IDP camp was added as a fixed effect because randomisation was stratified across three IDP camps. To increase precision, adjusted rate (or count) ratios were found, adjusting for factors determined a priori to be associated with the outcome (age, sex, number of children aged 5–12 in the household, and number of people earning in the household.)

2.11. Ethics statement

The study was reviewed and approved by the London School of Hygiene and Tropical Medicine Ethics Review Committee (Ref: 22905) and the Research and Ethics Committee at the Ministry of Health, Somalia (Ref: MOH&HS/DGO/0014/2021). Written informed consent was sought from all participating households.

The trial is registered on the Open Science Framework (OSF), osf.io/va9yn.

3. Results

3.1. Participants and baseline data

200 households - 100 intervention and 100 control - were enrolled and completed the study between October 2021 to March 2022. No households were lost to follow up and complete data was obtained from all households (Fig. 2). Baseline prevalence of HWWS after key handwashing occasions was 7% in the intervention group and 5% in the control group. Child-level and household characteristics appeared well balanced between intervention and control group. Baseline characteristics are presented in Table 2.

5.2. Prevalence of handwashing with soap after intervention

The prevalence of HWWS after key occasions increased after baseline observations in both the intervention (+48 percentage points) and control group (+51 percentage points) and remained relatively stable throughout the 16-week follow up (Fig. 3).

3.3. Availability of a handwashing station and soap after intervention

The proportion of households with a handwashing station available increased in both groups, from 81% at baseline to 96% in the intervention group and 93% in the control group. Availability of soap at the handwashing station also increased in both groups. At endline, 99% of intervention households, and 98% of control households with a handwashing station were observed to have soap available at the station, up from baseline levels of 36% and 23%, respectively.

3.4. Primary outcome

There was no evidence of a difference in the proportion of key handwashing occasions that were accompanied by HWWS for children aged 5–12 between the intervention and control group at the 4-week follow-up, the 12-week follow-up, or the 16-week follow-up (16-week follow up: RR 1.0, 95% CI 0.9–1.2, p value = 0.6) (Table 3).

3.5. Secondary outcomes

There was no evidence of a difference in the proportion of all

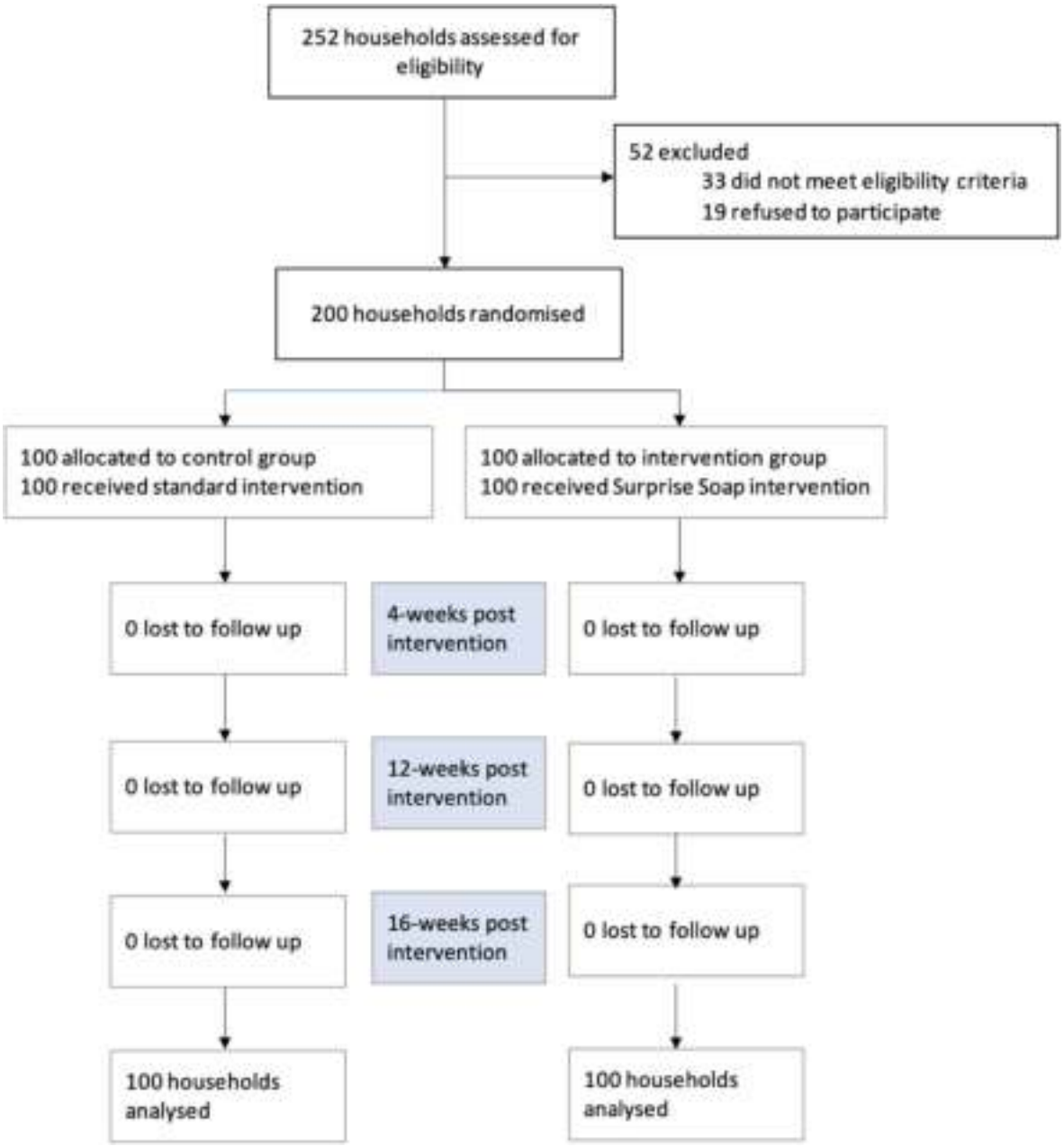

Fig. 2. Trial profile.

handwashes that used soap between the intervention and control group at the 4-week follow-up, the 12-week follow-up, or the 16-week follow-up (16-week follow-up: RR 1.1, 95% CI 1.0–1.3, p value = 0.2) (Table 4).

There was no evidence of a difference in the total number of handwashes with soap between the intervention and control group at the 4-week follow-up, the 12-week follow-up, or 16-week follow-up (CR: 1.1, 95% CI 0.9–1.4, p value = 0.2) (Table 5).

3.6. Surprise Soap compliance

At the 16-week follow up, all households reported that they had finished at least 1 bar of Surprise Soap, indicating they had all engaged with the intervention. 88% (n = 88) of households still had at least 1 bar of Surprise Soap remaining, with a mean of 2 Surprise Soaps remaining per household. Of these households, 91% (n = 80) had a bar of Surprise Soap that was wet on inspection indicating that most households were still engaging with the intervention 16-weeks later. Caregivers reported that it took approximately 5.5 days for children to reach the toy by washing their hands. 77% of households reported 'toy cheats', however, of the 17 Surprise Soaps received per household over the intervention period, only between 1 and 2 Surprise Soaps per household were reported to have been purposefully broken. 28% and 31% of households reported that children under the age of 5 and adults in the household also used the Surprise Soaps, respectively. Only 1 household reported that soap was used for purposes other than handwashing.

4. Discussion

In our trial, we found no evidence that the novel Surprise Soap intervention was more effective in increasing child HWWS than the standard approach of delivering health-based messages, information on how and when to wash hands, and providing bars of plain soap. These findings contrast with those of a previous proof-of-concept trial in an IDP camp in Iraq which found the Surprise Soap intervention to be

Table 2
Baseline characteristics.

Variable	Overall	Intervention	Control
Handwashing			
n (number of potential key handwashing occasions)	1052	517	535
HWWS accompanying key occasions (n, %)	66 (6.3%)	38 (7.4%)	28 (5.2%)
Child			
n (number of children observed)	571	284	287
Age, years (mean, sd)	8.2 ± 2.3	8.0 ± 2.3	8.3 ± 2.3
Sex, male (n, %)	268 (46.9%)	131 (46.1%)	137 (47.7%)
Household			
n (number of households)	200	100	100
Household head education score (mean, sd)	1.2 ± 0.4	1.2 ± 0.4	1.2 ± 0.4
Number earning income (mean, sd)	0.9 ± 0.7	0.8 ± 0.7	0.9 ± 0.7
Number household members (mean, sd)	7.4 ± 2.4	7.1 ± 2.4	7.6 ± 2.4
Number of children <5 (mean, sd)	1.5 ± 1.0	1.5 ± 1.0	1.4 ± 1.0
Number of children 5–12 (mean, sd)	2.6 ± 1.1	2.5 ± 1.2	2.8 ± 1.1
Length of time in residence, months (mean, sd)	30.9, 14.9	29.0, 13.5	32.8, 16.0
Handwashing station available (n, %)	162 (81.0%)	81 (81.0%)	81 (81.0%)
Soap available at station (n, %)	48 (29.6%)	29 (35.8%)	19 (23.5%)
Water available at station (n, %)	160 (98.7%)	81 (100%)	79 (97.5%)
Station reachable by children (n, %)	162 (100%)	81 (100%)	81 (100%)

approximately four times more effective than the standard intervention, which also comprised of household-level health-based messaging and provision of plain soap (Watson et al., 2021).

Although no significant difference in effectiveness was found between the two interventions in Somalia, both interventions – the experimental Surprise Soap intervention, and the standard handwashing intervention that served as an active control – were associated with a large increase in HWWS that was sustained over the 16-week follow-up. Our trial was not designed to assess the independent effects of these two interventions, only to assess whether the Surprise Soap intervention was more effective than the standard approach. However, the strong and sustained association observed suggests that both the standard and the Surprise Soap interventions were similarly effective in increasing and sustaining child HWWS.

Two key differences between the Somalia and Iraq contexts may explain the different results. The first is that children in Somalia had little past exposure to handwashing promotion (information shared by Action Against Hunger) of the type delivered in the active control arm, contrasting with high levels of exposure to such programmes in Iraq. As such, in the Somalia setting, both the Surprise Soap intervention and the standard handwashing intervention may have been similarly novel and therefore both were likely to have engaged children more compared to the populations in Iraq, where standard handwashing messaging was frequently encountered. Although it is often asserted that health messages do not strongly motivate behaviour change (Biran et al., 2009; White et al., 2020; Curtis et al., 2009), more recent studies have

Table 3
Effect of intervention on the proportion of key handwashing occasions accompanied by HWWS.

	Intervention	Control	Rate Ratio[a]	95% CI	P value
Baseline (n, %)	38 (7.4%)	28 (5.2%)	1.3	0.7–2.3	0.3
Week 4 (n, %)	280 (55.1%)	291 (56.4%)	1.0	0.9–1.1	0.6
Week 12 (n, %)	281 (55.0%)	251 (48.8%)	1.1	0.9–1.3	0.5
Week 16 (n, %)	306 (58.0%)	281 (55.3%)	1.0	0.9–1.2	0.6

Poisson for rates generalized estimating equations analyses accounting for clustering at the household level.

[a] Adjusted for age, sex, number of children aged 5–12 in the household, and number of household members earning an income.

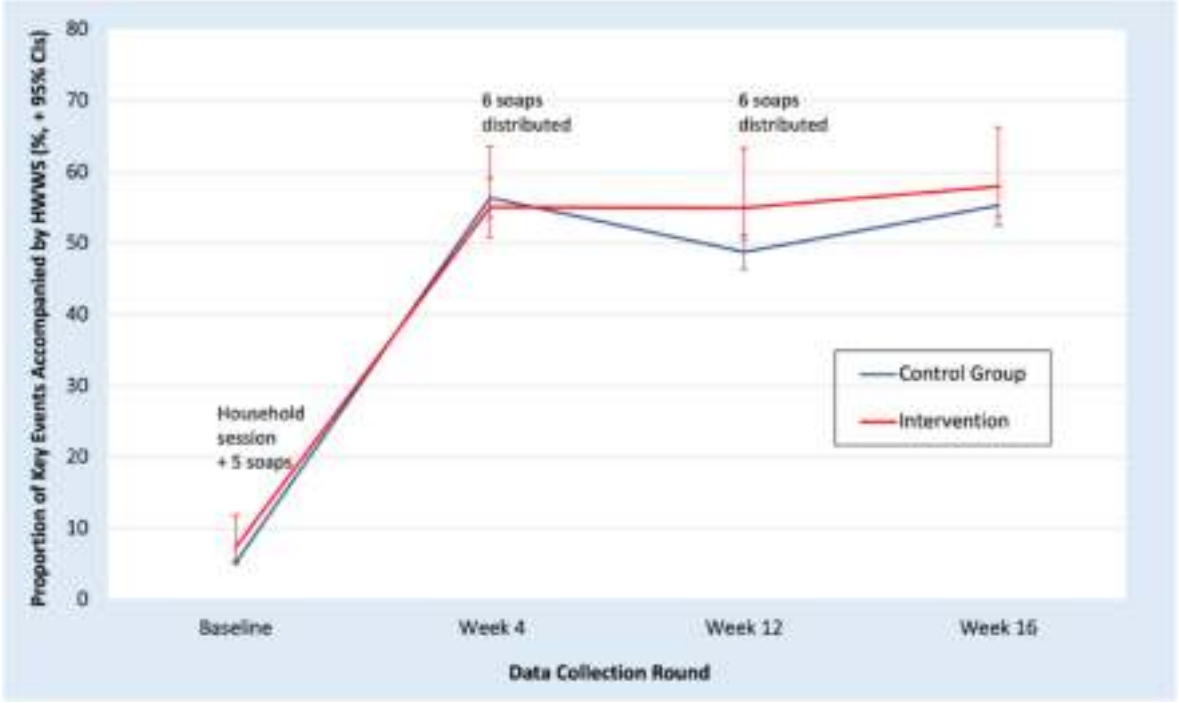

Fig. 3. Prevalence of handwashing with soap over the study period.

Table 4
Effect of intervention on the proportion of all handwashes that used soap.

	Intervention	Control	Risk Ratio[a]	95% CI	P value
Baseline (n, %)	53 (8.6%)	39 (6.3%)	1.3	0.8–2.1	0.3
Week 4 (n, %)	368 (58%)	362 (55.5%)	1.0	0.9–1.2	0.7
Week 12 (n, %)	342 (55.7%)	298 (48.3%)	1.1	0.9–1.3	0.3
Week 16 (n, %)	364 (57.2%)	321 (50.1%)	1.1	1.0–1.3	0.2

Poisson for rates generalized estimating equations analyses accounting for clustering at the household level.

[a] Adjusted for age, sex, number of children aged 5–12 in the household, and number of household members earning an income.

Table 5
Effect of intervention on the total number of handwashes with soap.

	Intervention	Control	Count Ratio[a]	95% CI	P value
Baseline (n)	53	39	1.3	0.8–2.1	0.3
Week 4 (n)	368	362	1.0	0.9–1.2	1.0
Week 12 (n)	342	298	1.1	0.9–1.4	0.3
Week 16 (n)	364	321	1.1	0.9–1.4	0.2

Poisson for counts generalized estimating equations analyses accounting for clustering at the household level.

[a] Adjusted for age, sex, number of children aged 5–12 in the household, and number of household members earning an income.

provided evidence that health messaging in handwashing interventions targeting older children can be effective (Watson et al., 2020, 2021; Okello et al., 2019; Khan et al., 2021). Our study indicates that health-messages can be effective in contexts where they provide new knowledge or present related information in a novel fashion. It also indicates that the effectiveness of the Surprise Soap intervention is not dependant on high levels of health-related handwashing knowledge – as was the case in Iraq – since the intervention appeared to be associated with increased HWWS in a population with low exposure to health-related handwashing promotion. The effectiveness of the Surprise Soap intervention however is likely to be a function of providing soap, information on key times to wash hands, and giving demonstrations of correct handwashing technique. These behaviour change techniques (BCTs) were employed in both the Surprise Soap and the standard intervention. All three BCTs have been found to contribute positively to intervention effectiveness and combining them in child targeted HWWS interventions has been recommended previously (Watson et al., 2021). Our results also indicate that future handwashing interventions should also incorporate these BCTs.

The second key difference between the two contexts is that soap availability in the household was much lower in Somalia than in Iraq. Providing any soap (plain or Surprise Soap) enables those, who are so inclined, to wash their hands with soap more often (Ashraf et al., 2017; Luby et al., 2009; Nizame et al., 2016). In Somalia, the baseline prevalence of HWWS was much lower than in Iraq (6.3% vs 28%), likely a function of the lack of access to soap and low exposure to handwashing promotion, so it is plausible that providing plain soap would be enough to motivate some children to practise more HWWS, whereas, in contexts where access to soap is already high, as in Iraq, providing further plain soap is unlikely to lead to an increase in HWWS (Phillips et al., 2015). Ensuring the physical environment enables HWWS should always be a key consideration when implementing any handwashing intervention.

It should be noted that the standard intervention used in this study was standard in terms of content, however, targeting children at the household level is not standard practice; children are typically targeted in schools within larger programmes (Watson et al., 2021). We chose to deliver the standard intervention at the household level to control for the effects of household delivery, hypothesising that targeting children in the household may give them more ownership of their handwashing practices and encourage caregivers to reinforce the messages. Though we cannot say for certain, the household-level delivery may be associated with the increase in HWWS observed in both groups. Additionally, like the Surprise Soap intervention, the standard intervention entailed a short, simple, one-off session. Both interventions were therefore relatively low-resource, quick to implement, and rapidly deployable, overcoming common challenges facing handwashing interventions (Saboori et al., 2011, Antwi-Agyei et al., 2017; Deroo et al., 2015; Alexander et al., 2013; Alexander et al., 2016) and making them feasible to deliver in emergency settings.

When considering whether to implement the Surprise Soap intervention, we urge practitioners to undertake formative work to understand the context – specifically the level of exposure to past handwashing promotion and availability of soap in the households. If they find that children have not had much exposure to handwashing promotion and soap availability is low, as in the IDP camps we worked in for this study, then it is probably more cost effective to implement a standard household-level intervention and distribute plain soap, perhaps switching to the Surprise Soap intervention when the standard intervention no longer increases or maintains higher rates of HWWS. However, if exposure and soap availability is high, the Surprise Soap intervention may be a more effective option, justifying the higher cost of bars of Surprise Soap (2 USD vs 1.5 USD for plain soap – costs in our study). Ultimately, what our study indicates is that, in emergencies, specifically targeting children with low-resource, rapidly deployable, handwashing promotion at the household level, creating a physically enabling environment (i.e., ensuring soap and water are available), and making sure they know when and how to practise HWWS is an important public health intervention.

This study has several limitations. Firstly, given the nature of the intervention, it was not possible to blind the enumerators to intervention status, which may have introduced observer bias. It was also not possible to blind the participants to intervention status. Due to time and budgetary restraints, we randomised at the household-level and not at camp-level, so it is likely that participant households were aware that some households received Surprise Soap and others plain soap, possibly leading to courtesy bias in the intervention group. Secondly, randomising at the household level presents the risk of contamination across arms which may have biased the estimate of intervention effect towards the null. However, it is unlikely that households in the intervention arm shared their bars of Surprise Soap with households in the control arm, so we perceive this risk to be low.

Thirdly, we used structured observations to measure handwashing. Although considered the 'gold standard' for measuring handwashing (Biran et al., 2008), this method is still at risk of social desirability bias (Ram et al., 2010), observer bias, and the 'Hawthorne Effect' or reactivity (McCambridge et al., 2014), where children modify their behaviour in response to their awareness of being observed (Grover et al., 2018; Ram et al., 2010). The large increases in HWWS prevalence in both study arms post intervention delivery may partly be attributed to this since, after receiving either of the interventions, households may have become aware that their handwashing practices were being observed. However, we used prolonged observation periods (3 h) and observation took place at multiple time points which likely reduces the risk of reactivity bias (Halder et al., 2013). During spot checks we also observed a large increase in the availability of soap at handwashing stations in both study groups. Given this is considered a proxy indicator of HWWS (Ram PK et al., 2014), it adds support to the hypothesis that the increased rates of HWWS observed in both groups were attributed to the intervention, as do the indicators of compliance we recorded for the intervention group which suggest most households were using Surprise Soap at endline.

Fourth, we employed an active control and saw no evidence of a difference in effectiveness between this and the intervention arm.

Although both interventions were associated with similarly large increases in HWWS, our trial design does not permit causal inference with regard to the independent effects; for this, a passive control group would be required. Finally, and this is not a limitation per se, but it should be noted that, because the plain soap, delivered as part of the standard intervention was identical to the Surprise Soap minus the toy, it was a different type of soap than the households would usually purchase (it was scented and colourful and, anecdotally, perceived to be of higher quality and more attractive). This may have provided an additional motivation for children to use it. As such, we cannot say for certain that distributing 'regular' plain soap within a standard household session would lead to the same results, especially considering that quality of handwashing materials has been reported as an important determinant of child handwashing in humanitarian settings (Watson et al., 2020).

5. Conclusions

Across selected IDP camps in the Kahda District of Somalia, where soap availability and exposure to hygiene promotion is low, it appears that well-designed, household-level handwashing interventions that directly target children and include the provision of soap, can increase children's HWWS and potentially reduce disease risk. In these camps, the Surprise Soap intervention offers no marginal benefit over a standard household-level handwashing intervention that would justify the additional costs, both were similarly effective. Knowledge of context, specifically related to hygiene promotion exposure and availability of handwashing hardware, should inform selection of future handwashing promotion approaches.

Declaration of competing interest

The authors declare no conflict of interests.

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References

Aiello, A.E., Coulborn, R.M., Perez, V., Larson, E.L., 2008. Effect of hand hygiene on infectious disease risk in the community setting: a meta-analysis. Am. J. Publ. Health 98, 1372–1381.

Alexander, K.T., Dreibelbis, R., Freeman, M.C., Ojeny, B., Rheingans, R., 2013. Improving service delivery of water, sanitation, and hygiene in primary schools: a cluster-randomized trial in western Kenya. J. Water Health 11, 507–519.

Alexander, K.T., Mwaki, A., Adhiambo, D., Cheney-Coker, M., Muga, R., Freeman, M.C., 2016. The life-cycle costs of school water, sanitation and hygiene access in Kenyan primary schools. Int. J. Environ. Res. Publ. Health 13.

Antwi-Agyei, P., Mwakitalima, A., Seleman, A., Tenu, F., Kuiwite, T., Kiberiti, S., Roma, E., 2017. Water, sanitation and hygiene (WASH) in schools: results from a process evaluation of the National Sanitation Campaign in Tanzania. J. Water, Sanit. Hyg. Dev. 7, 140–150.

Ashraf, S., Nizame, F.A., Islam, M., Dutta, N.C., Yeasmin, D., Akhter, S., Abedin, J., Winch, P.J., Ram, P.K., Unicomb, L., Leontsini, E., Luby, S.P., 2017. Nonrandomized trial of feasibility and acceptability of strategies for promotion of soapy water as a handwashing agent in rural Bangladesh. Am. J. Trop. Med. Hyg. 96, 421–429.

Azor-Martinez, E., Cobos-Carrascosa, E., Seijas-Vazquez, M.L., Fernández-Sánchez, C., Strizzi, J.M., Torres-Alegre, P., Santisteban-Martínez, J., Gimenez-Sanchez, F., 2016. Hand hygiene program decreases school absenteeism due to upper respiratory infections. J. Sch. Health 86, 873–881.

Biran, A., Rabie, T., Schmidt, W., Juvekar, S., Hirve, S., Curtis, V., 2008. Comparing the performance of indicators of hand-washing practices in rural Indian households. Trop. Med. Int. Health 13, 278–285.

Biran, A., Schmidt, W.P., Varadharajan, K.S., Rajaraman, D., Kumar, R., Greenland, K., Gopalan, B., Aunger, R., Curtis, V., 2014. Effect of a behaviour-change intervention on handwashing with soap in India (SuperAmma): a cluster-randomised trial. Lancet Global Health 2, 145–154.

Biran, A., Schmidt, W.P., Wright, R., Jones, T., Seshadri, M., Isaac, P., Nathan, N.A., Hall, P., Mckenna, J., Granger, S., Bidinger, P., Curtis, V., 2009. The effect of a soap promotion and hygiene education campaign on handwashing behaviour in rural India: a cluster randomised trial. Trop. Med. Int. Health 14, 1303–1314.

Blanton, E., Ombeki, S., Oluoch, G.O., Mwaki, A., Wannemuehler, K., Quick, R., 2010. Evaluation of the role of school children in the promotion of point-of-use water treatment and handwashing in schools and households–Nyanza Province, Western Kenya, 2007. Am. J. Trop. Med. Hyg. 82, 664–671.

Bresee, S., Caruso, B.A., Sales, J., Lupele, J., Freeman, M.C., 2016. 'A child is also a teacher': exploring the potential for children as change agents in the context of a school-based WASH intervention in rural Eastern Zambia. Health Educ. Res. 31, 521–534.

Cairncross, S., Hunt, C., Boisson, S., Bostoen, K., Curtis, V., Fung, I.C.H., Schmidt, W.P., 2010. Water, sanitation and hygiene for the prevention of diarrhoea. Int. J. Epidemiol. 39, 193–205.

Camp Coordination And Camp Management Cluster (Cccm), 2022. Detailed site assessment (DSA): Kahda district, Banadir region. Somalia (March 2022) [Online]. Available: https://reliefweb.int/report/somalia/detailed-site-assessment-dsa-kahda-district-banadir-region-somalia-march-2022. (Accessed 26 September 2022).

Connolly, M.A., Gayer, M., Ryan, M.J., Salama, P., Spiegel, P., Heymann, D.L., 2004. Communicable diseases in complex emergencies: impact and challenges. Lancet 364, 1974–1983.

Curtis, V., Cairncross, S., 2003. Effect of washing hands with soap on diarrhoea risk in the community: a systematic review. Lancet Infect. Dis. 3, 275–281.

Curtis, V.A., Danquah, L.O., Aunger, R.V., 2009. Planned, motivated and habitual hygiene behaviour: an eleven country review. Health Educ. Res. 24, 655–673.

Deroo, L., Walter, E., Graham, J., 2015. Monitoring and evaluation of WASH in schools programs: lessons from implementing organizations. J. Water, Sanit. Hyg. Dev. 5, 512–520.

Ejemot-Nwadiaro, R.I., Ehiri, J.E., Arikpo, D., Meremikwu, M.M., Critchley, J.A., 2015. Hand washing promotion for preventing diarrhoea. Cochrane Database Syst. Rev. 9. CDCD004265-CDCD004265.

Fewtrell, L., Kaufmann, R.B., Kay, D., Enanoria, W., Haller, L., Colford Jr., J.M., 2005. Water, sanitation, and hygiene interventions to reduce diarrhoea in less developed countries: a systematic review and meta-analysis. Lancet Infect. Dis. 5, 42–52.

Freeman, M.C., Stocks, M.E., Cumming, O., Jeandron, A., Higgins, J.P., Wolf, J., Pruss-Ustun, A., Bonjour, S., Hunter, P.R., Fewtrell, L., Curtis, V., 2014. Hygiene and health: systematic review of handwashing practices worldwide and update of health effects. Trop. Med. Int. Health 19, 906–916.

Gakidou, E., Cowling, K., Lozano, R., Murray, C., 2010. Increased educational attainment and its effect on child mortality in 175 countries between 1970 and 2009: a systematic analysis. Lancet 376, 959–974.

Grover, E., Hossain, M.K., Uddin, S., Venkatesh, M., Ram, P.K., Dreibelbis, R., 2018. Social Influence on Handwashing with Soap: Results from a Cluster Randomized Controlled Trial in Bangladesh. The American Journal of Tropical Medicine and Hygiene.

Halder, A.K., Molyneaux, J.W., Luby, S.P., Ram, P.K., 2013. Impact of duration of structured observations on measurement of handwashing behavior at critical times. BMC Publ. Health 13, 705.

Horton, S., Levin, C., 2016. Cost-effectiveness of interventions for reproductive, maternal, neonatal, and child health. In: Black, R.E., Laxminarayan, R., Temmerman, M., et al. (Eds.), Reproductive, Maternal, Newborn, and Child Health: Disease Control Priorities, third ed. The International Bank for Reconstruction and Development/The World Bank, Washington (DC). Volume 2.

Jamison, D., Bremen, J., Measham, A., Alleyne, G., Claeson, M., 2006. Disease Control Priorities in Developing Countries. World Bank, Washington DC.

Khan, S., Ashraf, H., Iftikhar, S., Baig-Ansari, N., 2021. Impact of hand hygiene intervention on hand washing ability of school-aged children. J. Fam. Med. Prim. Care 10, 642–647.

Kouadio, I.K., Aljunid, S., Kamigaki, T., Hammad, K., Oshitani, H., 2012. Infectious diseases following natural disasters: prevention and control measures. Expert Rev. Anti-infect. Ther. 10, 95–104.

Lamdin, D., 1996. Evidence of student attendance as an independent variable in education production functions. J. Educ. Res. 89, 155–162.

Luby, S.P., Halder, A.K., Tronchet, C., Akhter, S., Bhuiya, A., Johnston, R.B., 2009. Household characteristics associated with handwashing with soap in rural Bangladesh. Am. J. Trop. Med. Hyg. 81, 882–887.

Mccambridge, J., Witton, J., Elbourne, D.R., 2014. Systematic review of the Hawthorne effect: new concepts are needed to study research participation effects. J. Clin. Epidemiol. 67, 267–277.

Mohamed, N.A., Mohd Rani, M.D., Tengku Jamaluddin, T.Z.M., Ismail, Z., Ramli, S., Faroque, H., Abd Samad, F.N., Ariffien, A.R., Che Amir Farid, A.A.R., Isahak, I., 2020. Effect of hand hygiene intervention on the absenteeism of pre-school children in Klang Valley, Malaysia: a quasi-experimental study. World J. Pediat. 16, 416–421.

Morrissey, T., Hutchison, L., Winsler, A., 2014. Family income, school attendance, and academic achievement in elementary school. Dev. Psychol. 50, 741.

Nandrup-Bus, I., 2009. Mandatory handwashing in elementary schools reduces absenteeism due to infectious illness among pupils: a pilot intervention study. Am. J. Infect. Control 37, 820–826.

Nizame, F.A., Leontsini, E., Luby, S.P., Nuruzzaman, M., Parveen, S., Winch, P.J., Ram, P. K., Unicomb, L., 2016. Hygiene practices during food preparation in rural

Bangladesh: opportunities to improve the impact of handwashing interventions. Am. J. Trop. Med. Hyg. 95, 288–297.
Okello, E., Kapiga, S., Grosskurth, H., Makata, K., Mcharo, O., Kinungh, I.S., Dreibelbis, R., 2019. Factors perceived to facilitate or hinder handwashing among primary students: a qualitative assessment of the Mikono Safi intervention schools in NW Tanzania. BMJ Open 9, e030947.
Onyango-Ouma, W., Aagaard-Hansen, J., Jensen, B.B., 2005. The potential of schoolchildren as health change agents in rural western Kenya. Soc. Sci. Med. 61, 1711–1722.
Phillips, R.M., Vujcic, J., Boscoe, A., Handzel, T., Aninyasi, M., Cookson, S.T., Blanton, C., S Blum, L., Ram, P.K., 2015. Soap is not enough: handwashing practices and knowledge in refugee camps, Maban County, South Sudan. Conflict Health 9, 39.
Rabie, T., Curtis, V., 2006. Handwashing and risk of respiratory infections: a quantitative systematic review. Trop. Med. Int. Health 11, 258–267.
Ram, Pk, Sahli, M., Arnold, B., Colford Jm, J., Chase, C., Briceno, B., Orsola-Vidal, A., Pj, G., 2014. Validity of Rapid Measures of Handwashing Behavior; an Analysis of Data from Multiple Impact Evaluations in the Global Scaling up Handwashing Project. Water and Sanitation Program/The World Bank, Washington, DC.
Ram, P.K., Halder, A.K., Granger, S.P., Jones, T., Hall, P., Hitchcock, D., Wright, R., Nygren, B., Islam, M.S., Molyneaux, J.W., Luby, S.P., 2010. Is structured observation a valid technique to measure handwashing behavior? Use of acceleration sensors embedded in soap to assess reactivity to structured observation. Am. J. Trop. Med. Hyg. 83, 1070–1076.
Rheinländer, T., Samuelsen, H., Dalsgaard, A., Konradsen, F., 2015. Teaching minority children hygiene: investigating hygiene education in kindergartens and homes of ethnic minority children in northern Vietnam. Ethn. Health 20, 258–272.
Saboori, S., Mwaki, A., Porter, S., Okech, B., Freeman, M.C., Rheingans, R.D., 2011. Sustaining School Hand Washing and Water Treatment Programmes : Lessons Learned and to Be Learned. Waterlines, pp. 298–311.
Statacorp, 2019. Stata Statistical Software: Release, vol. 16. StataCorp LP, College Station, TX.
Stocks, M.E., Ogden, S., Haddad, D., Addiss, D.G., Mcguire, C., Freeman, M.C., 2014. Effect of water, sanitation, and hygiene on the prevention of trachoma: a systematic review and meta-analysis. PLoS Med. 11, e1001605.
Strunz, E.C., Addiss, D.G., Stocks, M.E., Ogden, S., Utzinger, J., Freeman, M.C., 2014. Water, sanitation, hygiene, and soil-transmitted helminth infection: a systematic review and meta-analysis. PLoS Med. 11, e1001620.
Talaat, M., Afifi, S., Dueger, E., El-Ashry, N., Marfin, A., Kandeel, A., Mohareb, E., El-Sayed, N., 2011. Effects of hand hygiene campaigns on incidence of laboratory-confirmed influenza and absenteeism in schoolchildren, Cairo, Egypt. Emerg. Infect. Dis. 17, 619–625.
Tidwell, J.B., Gopalakrishnan, A., Unni, A., Sheth, E., Daryanani, A., Singh, S., Sidibe, M., 2020. Impact of a teacher-led school handwashing program on children's handwashing with soap at school and home in Bihar, India. PLoS One 15, e0229655.
Toole, M.J., Waldman, R.J., 1997. The public health aspects of complex emergencies and refugee situations. Annu. Rev. Publ. Health 18, 283–312.
UNHCR, 2015. Global Trends: Forced Displacement in 2015 (Geneva).
Vos, T., Lim, S.S., Abbafati, C., Abbas, K.M., Abbasi, M., Abbasifard, M., Abbasi-Kangevari, M., Abbastabar, H., Abd-Allah, F., Abdelalim, A., Abdollahi, M., Abdollahpour, I., Abolhassani, H., Aboyans, V., Abrams, E.M., Abreu, L.G., Abrigo, M.R.M., Abu-Raddad, L.J., Abushouk, A.I., Acebedo, A., Ackerman, I.N., Adabi, M., Adamu, A.A., Adebayo, O.M., Adekanmbi, V., Adelson, J.D., Adetokunboh, O.O., Adham, D., Afshari, M., Afshin, A., Agardh, E.E., Agarwal, G., Agesa, K.M., Aghaali, M., Aghamir, S.M.K., Agrawal, A., Ahmad, T., Ahmadi, A., Ahmadi, M., Ahmadieh, H., Ahmadpour, E., Akalu, T.Y., Akinyemi, R.O., Akinyemiju, T., Akombi, B., Al-Aly, Z., Alam, K., Alam, N., Alam, S., Alam, T., Alanzi, T.M., Albertson, S.B., Alcalde-Rabanal, J.E., Alema, N.M., Ali, M., Ali, S., Alicandro, G., Alijanzadeh, M., Alinia, C., Alipour, V., Aljunid, S.M., Alla, F., Allebeck, P., Almasi-Hashiani, A., Alonso, J., Al-Raddadi, R.M., Altirkawi, K.A., Alvis-Guzman, N., Alvis-Zakzuk, N.J., Amini, S., Amini-Rarani, M., Aminorroaya, A., Amiri, F., Amit, A.M.L., Amugsi, D.A., Amul, G.G.H., Anderlini, D., Andrei, C.L., Andrei, T., Anjomshoa, M., Ansari, F., Ansari, I., Ansari-Moghaddam, A., Antonio, C. A.T., Antony, C.M., Antriyandarti, E., Anvari, D., Anwer, R., Arabloo, J., Arab-Zozani, M., Aravkin, A.Y., Ariani, F., Ärnlöv, J., Aryal, K.K., Arzani, A., Asadi-Aliabadi, M., Asadi-Pooya, A.A., Asghari, B., Ashbaugh, C., Atnafu, D.D., et al., 2020. Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet 396, 1204–1222.
Waddington, H., Snilstveit, B., 2009. Effectiveness and sustainability of water, sanitation, and hygiene interventions in combating diarrhoea. J. Dev. Effect. 1, 295–335.
Walker, D.G., Hutubessy, R., Beutels, P., 2010. WHO Guide for standardisation of economic evaluations of immunization programmes. Vaccine 28, 2356–2359.
Watson, J., Cumming, O., Aunger, R., Deola, C., Chase, R.P., Dreibelbis, R., 2020. Child handwashing in an internally displaced persons camp in Northern Iraq: a qualitative multi-method exploration of motivational drivers and other handwashing determinants. PLoS One 15, e0228482.
Watson, J., Cumming, O., Macdougall, A., Czerniewska, A., Dreibelbis, R., 2021. Effectiveness of behaviour change techniques used in hand hygiene interventions targeting older children - a systematic review. Soc. Sci. Med. 281, 114090.
Watson, J., Dreibelbis, R., Aunger, R., Deola, C., King, K., Long, S., Chase, R.P., Cumming, O., 2019. Child's play: harnessing play and curiosity motives to improve child handwashing in a humanitarian setting. Int. J. Hyg Environ. Health 222, 177–182.
Watson, J.A., Ensink, J.H.J., Ramos, M., Benelli, P., Holdsworth, E., Dreibelbis, R., Cumming, O., 2017. Does targeting children with hygiene promotion messages work? The effect of handwashing promotion targeted at children, on diarrhoea, soil-transmitted helminth infections and behaviour change, in low- and middle-income countries. Trop. Med. Int. Health 22, 526–538.
White, S., Thorseth, A.H., Dreibelbis, R., Curtis, V., 2020. The determinants of handwashing behaviour in domestic settings: an integrative systematic review. Int. J. Hyg Environ. Health 227, 113512.
Willmott, M., Nicholson, A., Busse, H., Macarthur, G.J., Brookes, S., Campbell, R., 2015. Effectiveness of hand hygiene interventions in reducing illness absence among children in educational settings: a systematic review and meta-analysis. Arch. Dis. Child.
Winter, J.C., Darmstadt, G.L., Lee, S.J., Davis, J., 2021. The potential of school-based WASH programming to support children as agents of change in rural Zambian households. BMC Publ. Health 21, 1812.
Wolf, J., Hunter, P.R., Freeman, M.C., Cumming, O., Clasen, T., Bartram, J., Higgins, J.P. T., Johnston, R., Medlicott, K., Boisson, S., Prüss-Ustün, A., 2018. Impact of drinking water, sanitation and handwashing with soap on childhood diarrhoeal disease: updated meta-analysis and meta-regression. Trop. Med. Int. Health 23, 508–525.

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Exposure to phthalates and DiNCH among preschool children in Sweden: Urinary metabolite concentrations and predictors of exposure

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ABSTRACT

Several plasticizing chemicals induce endocrine disrupting effects in humans, and the indoor environment is suggested to be a source of exposure. As children are particularly vulnerable to the effects from exposure to endocrine disrupting chemicals (EDCs), it is essential to monitor exposure to EDCs such as phthalates and non-phthalate plasticizers in indoor environments intended for use by children.

The aim of this study was to assess everyday plasticizer exposure among preschool-aged children in Sweden by measuring urinary plasticizer metabolite concentrations. In addition, it was investigated whether the concentrations would be altered as a result of the children spending part of the day at preschool, in comparison with weekend exposure, when they may spend more time in home environments or engage in various weekend and leisure activities. For this purpose, fourteen metabolites from eight phthalates (di-ethylhexyl phthalate, DEHP; di-n-butyl phthalate, DnBP; di-isobutyl phthalate, DiBP; butyl-benzyl phthalate, BBzP; di-iso-nonyl phthalate, DiNP; di-propylheptyl phthalate, DPHP; di-iso-decyl phthalate, DiDP; and di-ethyl phthalate, DEP) and one non-phthalate plasticizer (di-isononyl cyclohexane 1,2-dicarboxylate, DiNCH) were measured in 206 urine samples collected at four occasions, i.e. twice during the winter and twice during the spring from 54 children (mean 5.1 years, SD 0.94) enrolled at eight preschools in Sweden.

A detection frequency (DF) of 99.9% for the 14 metabolites indicates a widespread exposure to plasticizers among children in Sweden. Compared to previous Swedish and international studies performed during approximately the same time period, high urinary concentrations of monobenzyl phthalate (MBzP), a metabolite from the strictly regulated BBzP, were measured in this study (median 17 ng/mL). Overall, high urinary phthalate metabolite concentrations were observed in this study compared to the US CDC-NHANES from the same time period and similar age-group. Compared to European studies, however, similar concentrations were observed for most metabolites and the urinary concentrations from few participating children exceeded the human biomonitoring guidance values (HBM-GV) for children. After days with preschool attendance, lower urinary concentrations of metabolites originating from DEP and phthalates that are strictly regulated within the EU REACH legislation (DEHP, DnBP, and DiBP) and higher concentrations of metabolites originating from DiNP, DPHP, and DiDP, i.e. less or non-regulated phthalates were found compared the urinary concentrations of these metabolites in weekends. This may indicate that factors in the indoor environment itself are important for the extent of the plasticizer exposure. All the analyzed metabolites were measured in lower concentrations in urine collected from children attending preschools built or renovated after the year 2000, while no seasonal differences were observed in this study.

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Abbreviations

EDCs	endocrine disrupting chemicals
DEHP	di-ethylhexyl phthalate
DnBP	di-n-butyl phthalate
DiBP	di-isobutyl phthalate
BBzP	butyl-benzyl phthalate
DiNP	di-iso-nonyl phthalate
DPHP	di-propylheptyl phthalate
DiDP	di-iso-decyl phthalate
DEP	di-ethyl phthalate
DiNCH	di-isononyl cyclohexane 1,2-dicarboxylate
MEHP	mono-(2-ethylhexyl) phthalate
MEHHP	mono-(2-ethyl-5-hydroxylhexyl) phthalate
MEOHP	mono-(2-ethyl-5-oxohexyl) phthalate
MECPP	mono-(2-ethyl-5-carboxypentyl) phthalate
MCMHP	mono[2-(carboxymethyl)hexyl] phthalate
MBzP	monobenzyl phthalate
MBP	monobutyl phthalate
MnBP	mono-n-butyl phthalate
MiBP	mono-isobutyl phthalate
MHiNP	mono-(4-methyl-7-hydroxyloctyl) phthalate
MOiNP	mono-(4-methyl-7-oxo octyl) phthalate
MCiOP	mono-(4-methyl-7-carboxyheptyl) phthalate
MCiNP	monocarboxyisononyl phthalate
MPHHP	6-hydroxy monopropylheptyl phthalate
MEP	monoethyl phthalate
MOiNCH	2-(((4-methyl-7-oxyooctyl)oxy)carbonyl) cyclohexanecarboxylic acid
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
HBM-GV	human biomonitoring guidance values

1. Introduction

The many uses for phthalates and other plasticizing chemicals have led to their ubiquitous presence in various products in people's everyday lives. Because these chemicals are gradually released from materials and products, people are continuously exposed to them via, for example, food and beverages due to the transfer from food packaging materials, as well as indoor environments, including air and dust (Bi et al., 2018; Fromme et al. 2013, 2016; Larsson et al., 2017; Morgan et al., 2005; Wormuth et al., 2006; Zhu et al., 2019). The plasticizing chemicals can enter the human body by dermal contact, inhalation or ingestion (Giovanoulis et al. 2016, 2018; Gong et al., 2015).

Children may be at particular risk of exposure to various chemicals due to child-specific behaviors, including hand-to-mouth behaviors that cause direct abrasion of chemicals from biting and chewing on products as well as ingestion of chemicals present in dust (Hammel et al., 2019). Children also spend more time close to the floor, increasing their exposure to floor dust (Mercier et al., 2011). In addition, higher urinary concentrations have been observed for several phthalate metabolites in children who seldom wash their hands before eating, and if usually eating microwave-cooked meals, as well as if usually using plastic bottles and plastic food-contact materials (Huang and Wang 2017).

Exposure from different exposure routes can add up to potentially harmful levels in the human body of chemicals which may interfere with the endogenous endocrine system (Attina et al., 2016). This is a particular concern for children's health as children go through critical stages of development controlled by endocrine actions and therefore endocrine disrupting chemicals (EDCs) may be particularly harmful during early childhood. Health effects associated with EDC exposure have been reported not to follow a typical dose-response relationship (Vandenberg et al., 2012), to be sex-specific (Harley et al., 2019; Vafeiadi et al., 2018), and to include asthma and allergies, hormonal cancers, neurotoxic effects, reproductive disorders, thyroid malfunction, obesity, and diabetes type II (Bergman et al., 2013).

Due to endocrine disrupting effects, the phthalates diethyl hexyl phthalate (DEHP), butyl-benzyl phthalate (BBzP), di-n-butyl phthalate (DnBP), and di-isobutyl phthalate (DiBP) are strictly regulated in Sweden according to the current EU REACH legislation and usage within the EU is allowed only if an authorization had been granted before the sunset date February 21, 2015 (Echa, 2023). After the sampling in this study was completed, the European Commission has adopted the decision to amend the REACH Regulation and further restrict the usage of DEHP, BBzP, DnBP, and DiBP in consumer products on the EU market (European Commission, 2018). There have been several exceptions to the strict regulation to allow continued usage, for example in food packaging. Despite the exceptions, following the introduction of restrictions for certain phthalates in the EU legislation, associated metabolites have been reported to decrease in urine (Frederiksen et al., 2020; Larsson et al., 2017). Nevertheless, these restricted chemicals will remain present in indoor environments for a long time, as materials and products available on the market will not be replaced quickly and regulations differ around the globe. At the same time, monitoring studies of indoor environments report increased levels of substituting chemicals like the non-phthalate plasticizer di-isononyl cyclohexane 1,2-dicarboxylate (DiNCH) (Bui et al., 2016; Frederiksen et al., 2020; Schwedler et al., 2020a) and the substituting chemicals could possibly cause similar endocrine disrupting effects. As children spend the vast majority of their time indoors (Conrad et al., 2013; Gao et al., 2022), a better knowledge of plasticizer exposure associated with different indoor environments could be important for understanding how to limit children's total EDC exposure.

One proposed reason for differences in concentrations of plasticizers in indoor environments is the mass transfer balance. Indoor environment factors such as indoor temperature, ventilation, and airborne particle concentration can influence the mass transfer of phthalates from indoor sources (Wei et al., 2018). Differences in outdoor temperature may also lead to changes in behavior, such as spending more/less time outdoors as well as opening/closing windows and doors, which consequently also influences the indoor environment. This may be observed as seasonal differences of indoor phthalate concentrations in countries with significant differences in temperature etc.

In this study, we focus on children's exposure to widespread synthetic phthalate and non-phthalate plasticizers, which have short half-lives and are water-soluble or have water-soluble metabolites and therefore detectable in urine sampled close in time to exposure (Domínguez-Romero and Scheringer 2019). Hence, urinary metabolite concentrations are expected to vary in spot urine samples due to occasional activities prior to sampling, differences in food intake and personal care product usage, as well as characteristics of indoor environments where time is spent.

The aim of this study was to estimate the exposure to nine common plasticizers (DEHP, DnBP, DiBP, BBzP, DiNP, DPHP, DiDP, DEP and DiNCH) among preschool-aged children in Sweden by measuring 14 urinary metabolites and to explore whether preschool attendance, in relation to weekend exposure from home environments and leisure activities, alters the metabolite concentrations. A secondary aim was to assess whether attending a modern preschool, i.e. built or renovated after the year 2000, alters the urinary concentrations of plasticizer metabolites as building age and renovation has been associated with phthalates in the indoor environment (Den Hond et al., 2015). Furthermore, the participants were recruited from two different municipalities and repeated sampling was conducted to assess possible

geographical and seasonal differences, as previous studies have indicated some differences in phthalate exposure due to these factors (Arbuckle et al., 2014; Cutanda et al., 2015).

2. Method

2.1. Recruitment of study participants and categorization of the preschools

The study idea and set-up is presented in Fig. 1. Children were recruited from preschools in two municipalities in Sweden: *Eskilstuna*, a medium-sized Swedish city with about 70,000 inhabitants, and *Vingåker*, a place of residence for fewer than 10,000 people. Preschools in Sweden are non-compulsory and generally only available daytime on weekdays for enrolled children aged 1–6 years while the parents are at work. Municipal officials, in consultation with preschool managers, selected the preschools for recruitment of participating children from a practical point of view. A printed written invitation was distributed and in-person meetings were held at nine preschools for the recruitment of study participants and participants were recruited from eight of these preschools. Parents signed a consent form prior to sampling in which they agreed to participate in collecting urine samples in the home both midweek after preschool attendance and during the weekend. An ethical permit was granted by the regional ethical review board in Uppsala (Dnr, 2015/476). Participation in the study also included agreement in hand wipe sampling for other parts of the study and a questionnaire.

The preschool buildings were built between 1968 and 2008. Some reconstruction had been made at some of the preschools and information thereof was collected using a questionnaire handed out to the head of each preschool. Construction before or after the year 2000 has previously been reported to be significant in predicting the magnitude of the phthalate concentrations that would be found in the preschool indoor dust in Sweden (Larsson et al., 2017). The year 2000 has therefore been used as a cutoff in this study and the preschools have been categorized as *modern*, i.e. built or renovated after 2000, and *older*, i.e. built before 2000 and not renovated after 2000 (Fig. 1). The *preschool premises*-category was assessed as a possible predictor of exposure.

2.2. Urine sampling

Each participant was asked to collect four spot evening urine samples during two seasons; more specifically, two samples during a first sampling period (*winter*) and two samples during a second sampling period (*spring*). The samples were collected by parents at home in ethanol-rinsed polypropylene (PP) containers with high-density polyethylene (HD-PE) screw caps (Sarstedt), recommended by the contracted laboratory and provided by the study. According to instructions, in each sampling period one sample was collected in the evening after at least two consecutive days of no preschool attendance, preferably Sunday, and one sample was collected in the evening after at least one day of preschool attendance, preferably Wednesday. These samples, designated *weekend: home* and *midweek: preschool*, were collected with the aim of comparing plasticizer exposure associated with indoor environments encountered during leisure time versus plasticizer exposure during days of preschool attendance, as illustrated in Fig. 1. Urine samples from the first round of sampling were collected February 7–21, 2016 and February 28–March 21, 2016 in Vingåker and Eskilstuna, respectively. Urine samples from the second round of sampling were collected April 17–May 3, 2016 and May 8–22, 2016 in Vingåker and Eskilstuna, respectively. Each sample was kept cold in a refrigerator at home until the next morning when it was left at the preschools in AC-powered cooling boxes provided by the study. The samples were split later the same day and thereafter kept in sterile PP tubes (Sarstedt) at −20 °C prior to analysis.

First morning void is commonly collected in studies measuring urinary phthalate metabolites (Table S2). However, because one of the major objectives in this study was to differentiate the midweek plasticizer exposure from the weekend plasticizer exposure, i.e. the preschool and home indoor environments, respectively, and because several phthalate metabolites have relatively short half-lives (6–24 h), another approach was adopted. Because all children have an ongoing exposure from their home environment, we chose to collect the *midweek: preschool* urine samples in the evening approximately 3–6 h after exposure in the preschool environment. To standardize, also the *weekend: home* urine samples were collected in the evening.

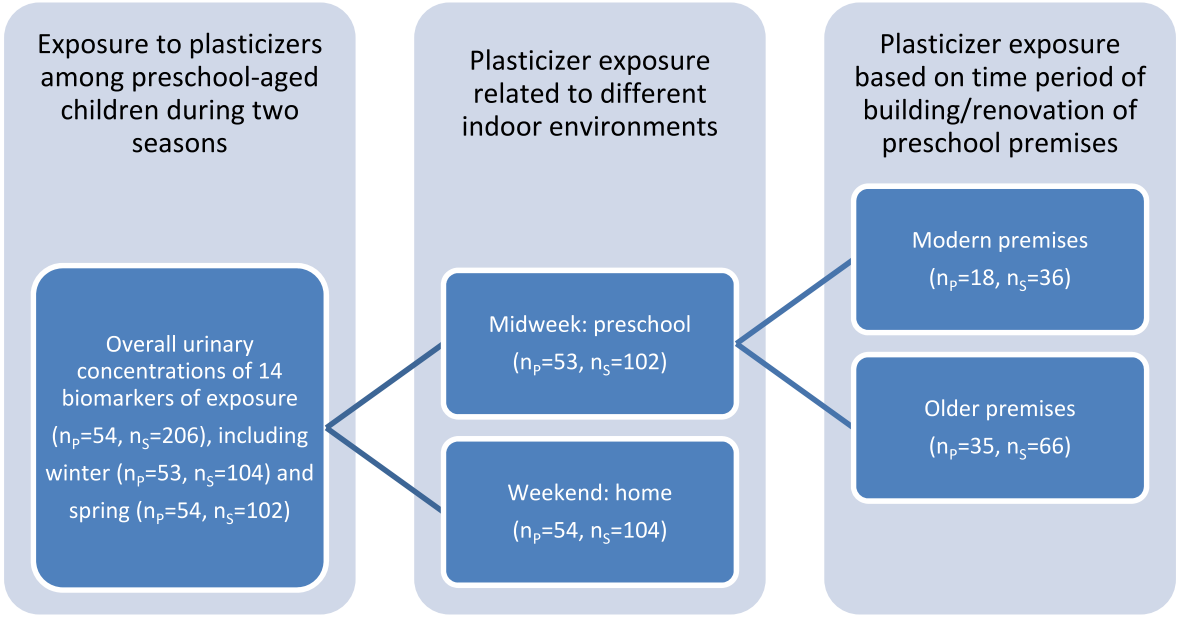

Fig. 1. Schematic overview of the study set-up. The number of participants (n_P) and the number of included samples (n_S) on which the analyzes are based are indicated in the figure.

Table 1
Chemical compounds analyzed in the present study.

Compound group	Parent compound		Measured metabolite	
Phthalates				
	DEHP	di-ethylhexyl phthalate	MEHP	mono-(2-ethylhexyl) phthalate
			MEHHP	mono-(2-ethyl-5-hydroxylhexyl) phthalate
			MEOHP	mono-(2-ethyl-5-oxohexyl) phthalate
			MECPP	mono-(2-ethyl-5-carboxypentyl) phthalate
			MCMHP	mono[2-(carboxymethyl) hexyl] phthalate
	BBzP	butyl-benzyl phthalate	MBzP	monobenzyl phthalate
	DnBP	di-n-butyl phthalate	MBP	monobutyl phthalate (sum of mono-n-butyl phthalate (MnBP) and mono-isobutyl phthalate (MiBP))
	DiBP	di-isobutyl phthalate		
	DiNP	di-iso-nonyl phthalate	MHiNP	mono-(4-methyl-7-hydroxyloctyl) phthalate
			MOiNP	mono-(4-methyl-7-oxo octyl) phthalate
			MCiOP	mono-(4-methyl-7-carboxyheptyl) phthalate
	DiDP[a]	di-iso-decyl phthalate	MCiNP	monocarboxyisononyl phthalate
			MPHHP	6-hydroxy monopropylheptyl phthalate
	DPHP[a]	di-propylheptyl phthalate	MCiNP	monocarboxyisononyl phthalate
			MPHHP	6-hydroxy monopropylheptyl phthalate
	DEP	di-ethyl phthalate	MEP	monoethyl phthalate
Non-phthalate plasticizer				
	DiNCH	di-isononyl cyclohexane 1,2-dicarboxylate	MOiNCH	2-(((4-methyl-7-oxyooctyl)oxy)carbonyl) cyclohexanecarboxylic acid

[a] The compounds DiDP and DPHP are commonly traded in mixtures and both are excreted as both MCiNP and MPHHP.

2.3. Chemical analysis

The analyzed urinary metabolites and their parent compounds are presented in Table 1. Samples were analyzed at the laboratory at the Division of Occupational and Environmental Medicine (OEM), Lund University, Sweden. Samples were analyzed according to the method presented by Gyllenhammar et al. (2017). Aliquots of 0.2 mL of urine were digested using β-glucoronidase (E-coli) and labeled internal standards (IS) of all analyzed compounds were added. The samples were analyzed using liquid chromatography connected to a hybrid triple quadrupole linear ion trap mass spectrometer (QTRAP 5500, AB SCIEX, Framingham, MA, USA; LC-MS/MS). The DnBP and DiBP metabolites mono-n-butyl phthalate (MnBP) and mono-isobutyl phthalate (MiBP) co-eluted and were quantified against a MnBP reference standard and IS. Accordingly, the DnBP and DiBP metabolite concentrations are reported as a sum of MnBP and MiBP, referred to as MBP.

The LOD was determined as the concentration corresponding to three times the standard deviation (SD) of the ratio of the peak at the same retention time as the analyzed compounds and the corresponding IS determined in the chemical blank samples. Each analytical batch was prepared in 96-well plates and includes standards, in-house prepared quality control (QC) samples and chemical blank samples. The relative standard deviation (RSD), i.e. coefficient of variation (CV), was between 2 and 12% for the analyzed metabolites (Table S1). The specific gravity (SG) of the urine samples was measured using a hand-held refractometer.

The laboratory in Lund participates in the HBM4EU QA/QC program and has qualified as a HBM4EU laboratory for the analysis of MEHP, MEHHP, MEOHP, MECPP, MBzP, MHiNP, MOiNP, MCiOP, (Figs. S1 and S2). The analysis of MEHP, MEHHP, MECPP, and MBzP is part of the German External QUality Assessment Scheme (G-EQUAS) from Erlangen Round Robin inter-laboratory control program.

2.4. Statistical analysis

Data were analyzed using IBM SPSS Statistics 25 (IBM Inc.). Urinary concentrations below the limit of detection (LOD) were replaced by LOD/$\sqrt{2}$. Descriptive statistics including mean, geometric mean (GM), median, and range were generated to evaluate children's exposure to plasticizers. To adjust for dilution, urinary metabolite concentrations were adjusted (Equation (1)) to the study mean specific gravity (SG) of 1.020, which was obtained as the grand mean, i.e. the average of the 54 participants' individual mean urine SG from their respective 2–4 urine samples (Singh et al., 2015). Due to skewness in the data, the data set was logarithmized prior to analysis. To allow for the repeated sampling design, linear mixed model analysis (LMM) with random intercept was used with restricted maximum likelihood (REML) estimation to explore if *midweek: preschool* is a predictor of exposure to any of the analyzed plasticizers, see Equations (2 and 3) for the level 1 (L1) and level 2 (L2) equations. This analysis was run with adjustment for the variables *season*, *sex*, and *municipality* and these variables were also tested separately with mixed model analysis as possible independent predictors of exposure. Furthermore, mixed model analysis was applied on the *midweek: preschool* urine data alone to evaluate possible statistical differences in urinary metabolite concentrations between samples collected from children attending *modern* and *older* preschools, respectively. No protocol was kept for activities outside the preschool nor for what indoor environments were visited prior to the *weekend: home* urine sampling and therefore the analysis of urine metabolites associated with building/renovation year includes solely *midweek: preschool* urine samples. The random factor was study participant in all analyses. The results from the LMM analysis are obtained as anti-logarithmic β-values and are expressed as exp(β) with 95% confidence intervals (CI). The level of statistical significance was set to 0.05 in all analyses. The LMM analysis adjusts for within- and between individual variability.

Within- and between-person variation in the dataset was assessed by using study participant as the only explanatory variable yielding within-person variation as the residuals in a variance analysis. The intraclass correlation coefficient (ICC) was calculated by dividing the between-person variation by the total variation (the sum of within- and between-person variation), yielding an ICC value within the range 0–1, where an ICC value >0.5 indicates that the between-person variation accounts for the majority of the exposure.

$$\text{Adjusted Conc}_{\text{Sample X}} = \text{Unadjusted Conc}_{\text{Sample X}}*(\text{SG}_{\text{Study grand mean}}-1)/(\text{SG}_{\text{Sample X}}-1) \quad (1)$$

$$\text{L1: } Y_{ij} = \ln([\text{Conc}]_{ij}) = \alpha_{0i}+\beta_1[\text{Indoor environment}]+\beta_2[\text{Sex}]+\beta_3[\text{Season}]+\beta_4[\text{Municipality}]+\varepsilon_{ij} \quad (2)$$

$$\text{L2: } \alpha_{0i} = \beta_0+\mu_i \quad (3)$$

where $\mu_i \sim N(0,\sigma_\mu^2)$, $\varepsilon_{ij} \sim N(0,\sigma_\varepsilon^2)$ and $Y_{ij} = \ln([\text{Concentration}]_{ij})$ is the logarithm of the measured concentration for the respective chemical in the j-th sample from the i-th participant; α_{0i} is the intercept for the i-th participant; $\beta_1, \ldots, \beta_n$ are the estimated fixed effects for the assessed variables; β_0 is the estimated overall intercept of the model; μ_i is a random factor specific to the i-th participant i.e. the deviation from β_0; and ε_{ij} is the residual in the j-th measurement from the i-th participant.

3. Results and discussion

A total of 206 urine samples were collected from 54 participating children (32 boys and 22 girls) with the median age of 5 years, according to the age reached during the year of sampling (mean 5.1 years, SD 0.94). Between one and 16 children participated from each of eight preschools. Up to four repeated measurements per child resulted from the applied study design (i.e. 2–4 samples per participant; $n_{2\ samples} = 2$, $n_{3\ samples} = 6$), and ICCs were calculated indicating poor to moderate ICCs (0.17–0.57). The poor ICCs were probably a result of the sampling strategy applied in this study with urine collection at weekend and midweek, exploring possible plasiticizer exposure differences due to preschool attendance versus leisure time. With one exception, urine samples were collected during a two-week period during each sampling period. The median number of days between a participant's sampling dates within the same sampling period was three (mean 4.5, SD 3.0). A few samples are missing (n = 10) because of illness during the sampling period or because of difficulty providing the urine on request.

3.1. Urinary concentrations of phthalate and DiNCH metabolites

The results from the measurements of urinary concentrations of phthalate metabolites indicate widespread exposure to a number of known or suspected EDCs among preschool-aged children, as all the analyzed chemicals were detected in urine samples from all participating children although not in all samples (Table 2). Only one metabolite (MCiNP) was not found in measurable concentrations in four individual samples, consequently the detection frequency (DF) was 99.9% for the 14 analyzed urinary metabolites in the 206 samples.

The highest unadjusted median urinary metabolite concentrations were measured for MBP (representing a sum of MnBP and MiBP; 51 ng/mL), MBzP (17 ng/mL), and MEP (16 ng/mL). Both MBP and MEP originate from phthalates with low molecular weight, i.e. DBP (sum of DiBP and DnBP) and DEP, respectively. Fragrances and personal care products might be a source for this phthalate exposure, as phthalates with low molecular weight are more volatile and often used in cosmetic and personal care products. Both MBP and MBzP originate from phthalates that are banned on the Swedish market according to the EU REACH legislation, i.e. DiBP, DnBP, and BBzP. Still, these metabolites were found in relatively high concentrations in this study compared to biomonitoring data from Germany, i.e. the fifth German Environmental Survey (GerEs V) (Schwedler et al., 2020b), and from the US, i.e. the National Health and Nutrition Examination Survey (CDC-NHANES) (CDC-NHANES 2016), and for MBzP also when compared with the European DEMOCOPHES study, i.e. DEMOnstration of a study to COordinate and Perform Human biomonitoring on a European Scale, (Den Hond et al., 2015) (Table S2), indicating noticeable exposure to the strictly regulated DnBP/DiBP and BBzP among children in Sweden. In the DEMOCOPHES study, an increased urinary MBzP concentration was found to be associated with polyvinyl chloride (PVC) floors and walls in the home, as well as high consumption of ice cream, and children in Sweden had on average three times the average European urinary MBzP concentration (Den Hond et al., 2015). The BBzP exposure among children in the present study also stands out in comparison to several other previous studies (Table S2). However, it is known that the sampling time point in relation to exposure (Aylward et al., 2014) may affect measurement results as short half-lives are recognized for phthalates, of which most are excreted within 24 h (Koch et al., 2017). At the same

Table 2
Descriptive statistics for specific gravity (SG) adjusted[a] and unadjusted (in italics) urinary metabolite concentrations (ng/mL) in 206 urine samples from 54 participants, whereof n(*weekend: home*) = 104 and n(*midweek: preschool*) = 102.

Parent and measured compound	Total (ng/mL)					*Weekend: home* (W) and *Midweek: preschool* (M) (ng/ml)				
	LOD (n > LOD)	Mean (SD)	GM (SD)	Median	Range		Mean (SD)	GM (SD)	Median	Range
DEHP										
MEHP		2.6 (2.2)	2.1 (2.0)	2.0	0.37–18	W	2.9 (2.6)	2.1 (2.1)	2.0	0.39–18
	0.09 (206)	*2.7 (2.8)*	*1.9 (2.4)*	*1.9*	*0.24–23*	M	2.4 (1.6)	2.0 (1.9)	1.9	0.37–8.6
MEHHP		21 (18)	16 (2.0)	15	2.9–160	W	24 (22)	18 (2.0)	16	3.8–160
	0.18 (206)	*21 (21)*	*15 (2.4)*	*15*	*1.4–200*	M	18 (13)	14 (2.0)	15	2.9–67
MEOHP		15 (13)	12 (2.0)	11	2.3–120	W	17 (16)	13 (2.1)	12	2.3–120
	0.25 (206)	*16 (15)*	*11 (2.4)*	*11*	*1.2–150*	M	13 (8.8)	11 (1.9)	10	2.7–42
MECPP		19 (13)	16 (1.9)	16	3.3–100	W	21 (15)	17 (1.9)	18	3.3–100
	0.21 (206)	*20 (17)*	*14 (2.3)*	*15*	*1.9–130*	M	18 (12)	15 (1.8)	14	4.1–56
MCMHP		6.2 (5.0)	5.0 (1.9)	4.7	0.94–39	W	6.9 (6.1)	5.4 (2.0)	4.7	1.3–39
	0.18 (206)	*6.3 (5.6)*	*4.6 (2.2)*	*4.6*	*0.78–43*	M	5.5 (3.6)	4.6 (1.8)	4.6	0.94–24
BBzP										
MBzP		31 (59)	19 (2.6)	17	0.83–770	W	36 (80)	19 (2.6)	16	1.6–770
	0.28 (206)	*31 (52)*	*17 (2.9)*	*17*	*0.54–540*	M	27 (24)	19 (2.6)	21	0.83–140
DnBP/DiBP										
MBP		69 (55)	56 (1.9)	55	11–510	W	84 (65)	68 (1.9)	69	20–510
	0.78 (206)	*69 (64)*	*51 (2.1)*	*51*	*6.7–610*	M	55 (36)	47 (1.8)	46	11–280
DiNP										
MHiNP		14 (15)	10 (2.2)	9.3	2.3–110	W	12 (13)	9.0 (2.0)	7.8	2.4–97
	0.15 (206)	*14 (17)*	*9.1 (2.6)*	*8.7*	*0.83–130*	M	16 (18)	11 (2.3)	10	2.3–110
MOiNP		6.7 (8.2)	4.5 (2.3)	4.0	0.90–77	W	5.3 (4.8)	4.0 (2.1)	3.7	0.90–32
	0.05 (206)	*6.7 (8.7)*	*4.1 (2.7)*	*3.8*	*0.25–89*	M	8.0 (10)	5.0 (2.5)	4.4	0.99–77
MCiOP		18 (20)	13 (2.1)	11	3.1–160	W	16 (17)	12 (2.0)	11	3.1–130
	0.15 (206)	*18 (22)*	*12 (2.5)*	*12*	*1.1–180*	M	19 (22)	13 (2.2)	13	3.3–160
DiDP/DPHP										
MCiNP		1.5 (1.7)	0.93 (2.6)	0.87	<LOD-12	W	1.2 (1.3)	0.80 (2.4)	0.77	<LOD-8.8
	0.07 (202)	*1.5 (2.1)*	*0.85 (2.8)*	*0.75*	*< LOD-17*	M	1.8 (2.0)	1.1 (2.7)	1.0	<LOD-12
MPHHP		4.9 (6.6)	3.0 (2.6)	2.7	0.49–49	W	3.8 (5.7)	2.4 (2.4)	2.2	0.50–49
	0.07 (206)	*5.1 (8.1)*	*2.7 (2.9)*	*2.4*	*0.13–58*	M	6.0 (7.2)	3.6 (2.6)	3.3	0.49–40
DEP										
MEP		47 (140)	20 (2.8)	16	3.3–1700	W	63 (180)	27 (2.8)	21	3.7–1700
	0.83 (206)	*42 (84)*	*18 (3.1)*	*16*	*2.1–590*	M	32 (68)	15 (2.6)	12	3.3–410
DiNCH										
MOiNCH		4.4 (11)	2.0 (2.9)	1.7	0.24–130	W	4.5 (13)	2.1 (2.7)	1.8	0.25–130
	0.03 (206)	*4.7 (14)*	*1.9 (3.3)*	*1.6*	*0.18–180*	M	4.3 (6.8)	2.0 (3.2)	1.6	0.24–37

[a] Adjusted to the study's SG grand mean.

time, the omnipresence of plasticizing chemicals suggests a continuous exposure to these compounds and they are therefore sometimes described as pseudo-persistent (Bui et al., 2016).

The results in this study also indicate widespread exposure to phthalates with high molecular weight (BBzP, DEHP, DiNP, DiDP, and DPHP), with only the DiDP/DPHP metabolite MCiNP measured below LOD in four individual samples. MCiNP was, as in previous studies (Table S2), the phthalate metabolite measured in the lowest median concentration (2.1 ng/mL), yet the contribution by its parent compounds to the total phthalate exposure should not be neglected.

As indoor dust may be a host matrix for emitted chemicals, settled dust was collected at the time of urine sampling at three of the preschools with participating children and has previously been analyzed for phthalates and non-phthalate plasticizers (Christia et al., 2019). In comparison with sampled indoor environments in various European countries, quite high levels were observed for several target chemicals, e.g. DEP, in indoor dust from these preschools. However, the highest indoor dust concentrations measured at the three Swedish preschools were from DiNP, DEHP, and DiDP. In that study, only dust sampled at Swedish offices had higher DiNP and DEHP mean concentrations (Christia et al., 2019). Those results may indicate that phthalates in dust can explain part of the exposure to several phthalates with high molecular weight as well as DEP observed in the present study.

3.1.1. Correlations

Correlation (Pearson) between measured urinary phthalate metabolites was assessed (Table S3). As expected, a high correlation was observed for DiDP and DPHP metabolites MCiNP and MPHHP as well as between metabolites originating from the same parent compound. More interestingly, the majority of the analyzed compounds were statistically significantly correlated with each other, even though with low correlation coefficients (0.15–0.36) (Table S3). This has been reported previously (Frederiksen et al., 2013; Langer et al., 2014) and may indicate a simultaneous exposure to most of the plasticizing chemicals. However, MEP was correlated to only one other metabolite and MOiNCH to none, whereas MBP was correlated only to metabolites also originating from strictly regulated phthalates.

3.2. Possible predictors of exposure to phthalates and DiNCH

3.2.1. The indoor environment

Urinary metabolites from all parent phthalates, except BBzP, were observed in statistically significant concentration differences between *weekend: home* and *midweek: preschool* (Table 3). Concentrations of the DEP metabolite MEP as well as the majority of the measured metabolites originating from strictly regulated phthalates were measured in statistically significantly higher urinary concentrations in the *weekend: home* samples. Conversely, the majority of urinary metabolites associated with the less or non-regulated phthalates were measured in statistically significantly higher concentrations in *midweek: preschool* samples. However, the results do not indicate a difference in exposure to BBzP nor DINCH between the assessed indoor environments (Table 3). The differences in urinary metabolite concentrations between the *midweek: preschool* and *weekend: home* samples were consistent irrespective of *season* in this study (data not shown).

Fromme et al. (2013) has also reported higher urinary metabolite concentrations for several regulated phthalates after the weekend than after preschool attendance among German children, but that study applied different sampling strategies. Still, this may indicate a higher exposure to strictly regulated phthalates associated with indoor environments such as homes rather than preschools. The results in the present study align with a previous Swedish study among preschool-aged children (Larsson et al., 2017), assuming that a Monday and Thursday morning urine sampling approach corresponds to the present study's sampling on Sunday and Wednesday evening.

In Sweden, public procurement is not optional but legally binding, and guidelines and officials' support in the enforcement of public procurement in line with present chemical regulation to prevent potentially hazardous chemicals from entering public premises might be reasons for the lower levels of urinary metabolites associated with strictly regulated compounds observed in *midweek: preschool* urine samples. Chemical regulation seems to affect exposure patterns, observed as time-trend decreases of regulated chemicals (Watkins et al., 2014). Further, distinct cleaning routines in public premises along with a coordinated and monitored selection of cleaning products as well as restrictions on perfumed products are likely to reduce exposure to recognized harmful chemicals. Other indoor environments, such as the homes, may include products and materials not subject to the same control as those in preschools, and this may have an impact on which emitted chemicals the residents are exposed to, e.g. from type of flooring or from building materials associated with both building age and renovation. In addition, home environments may contain privately imported goods from countries with weaker chemical legislation as well as a greater amount of electronic equipment, toys, personal care products such as cosmetic products, and fragranced products. DEP in particular is associated with cosmetic products and other personal care products and such products are probably mostly used during the morning and evening in the home environment. In this study, the largest difference in SG-adjusted urinary median metabolite concentrations between *midweek: preschool* and *weekend: home* samples, 12 ng/mL and 21 ng/mL, respectively, was

Table 3
Comparison of urinary metabolite concentrations in *weekend: home* urine samples (n = 104) and *midweek: preschool* urine samples (n = 102) using linear mixed model (LMM) analysis. *Weekend: home* exp(β) are presented in the table with 95% confidence intervals (CI). The metabolite concentrations presented are adjusted to the study's grand mean specific gravity (SG) and are based on a total of 206 urine samples collected from 54 participants. The regression model adjusts for *sex*, *season*, and *municipality*.

Compound group	Parent compound	Measured metabolite	*p*	exp(β)	95% CI	
Phthalates	DEHP	MEHP	0.40	1.07	0.92	1.24
		MEHHP	**0.010**	**1.23**	**1.05**	**1.44**
		MEOHP	**0.024**	**1.19**	**1.02**	**1.37**
		MECPP	0.12	1.11	0.97	1.28
		MCMHP	**0.044**	**1.14**	**1.00**	**1.29**
	BBzP	MBzP	0.97	1.00	0.84	1.19
	DnBP/DiBP	**MBP**	**<0.001**	**1.42**	**1.26**	**1.60**
	DiNP	**MHiNP**	**0.021**	**0.80**	**0.66**	**0.97**
		MOiNP	**0.026**	**0.79**	**0.64**	**0.97**
		MCiOP	0.13	0.87	0.73	1.04
	DiDP/DPHP	**MCiNP**	**0.001**	**0.74**	**0.62**	**0.89**
	DiDP/DPHP	**MPHHP**	**<0.001**	**0.66**	**0.54**	**0.81**
	DEP	**MEP**	**<0.001**	**1.70**	**1.40**	**2.07**
Non-phthalate plasticizer	DiNCH	MOiNCH	0.80	1.03	0.79	1.36

Bold values indicate statistically significant differences between the groups. *Midweek: preschool* serves as the reference category.

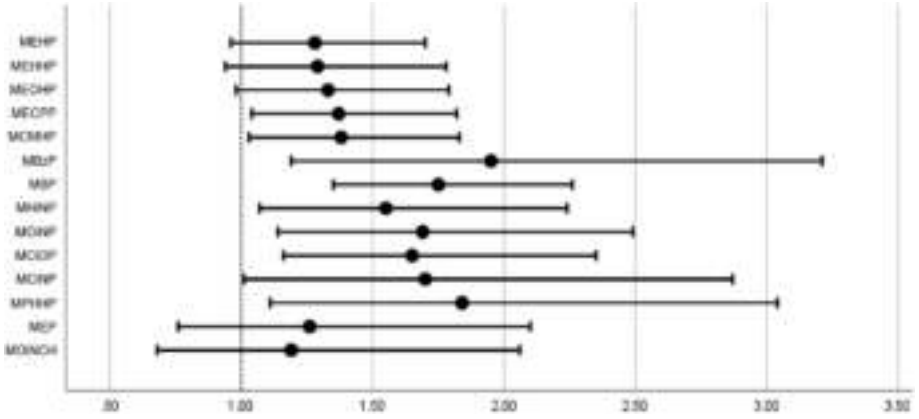

Fig. 2. The effects from attending *older* preschools on urinary metabolite concentrations with linear mixed model (LMM) analysis obtained antilogarithmic estimates (exp(β)) with 95% confidence intervals (CI) presented for urinary metabolite concentrations in study grand mean specific gravity (SG) adjusted *midweek: preschool* urine samples. The category including urine samples collected from children attending *modern* preschools serves as the reference category. A significant difference is identified for any metabolite with a 95% CI line which does not include the null effect exp(β)-value of 1.00.

observed for the DEP metabolite MEP (range 3.3–410 ng/mL and 3.7–1700 ng/mL, respectively). Although children may not be expected to be major consumers of cosmetic products, this study shows that there are sources of exposure to DEP in children's everyday life. This appears to be associated primarily with environments or activities outside the preschools. The results from the present study indicate a considerable difference in DEP exposure depending on the indoor environment itself or its associated activities, i.e. *midweek: preschool* and *weekend: home* in this study, seeing a 70% increase (i.e. exp(β) 1.70) in urinary MEP concentration in weekends (Table 3).

3.2.2. Modern vs older preschools

Except for DEP, all parent phthalate compounds were associated with statistically significantly higher urinary metabolite concentrations in samples collected from children attending *older* preschools, albeit three out of five DEHP metabolites were not statistically significantly higher as illustrated in Fig. 2 by the 95% CI including the value 1.00. Nearly doubled urinary metabolite concentrations were measured for some metabolites (exp(β) 1.95 and 1.83 for MBzP and MPHHP, respectively) in urine samples collected from children attending *older* preschools (Fig. 2).

As discussed previously, public procurement and chemical regulations will have an impact on what chemicals will be present in the indoor environment. It could therefore be expected that exposure to restricted chemicals would be lower in *modern* preschools, which for MnBP has been observed also by others (Ketema et al., 2021). As possible explanations for the observed pattern in Fig. 2, one could hypothesize that there has been a change in what chemicals are used in building materials and furniture or a change towards more easily cleaned premises as well as improved ventilation systems in the *modern* preschool buildings.

3.2.3. Municipality, sex, and season

The result from the present study suggests a higher exposure to DiDP, DPHP, and DiNCH among children in the municipality of *Eskilstuna*, as statistically significantly higher urinary metabolite concentrations were observed for MCiNP, MPHHP, and MOiNCH (Table 4). The plasticizers DiDP, DPHP, and DiNCH are all compounds often recognized as substituting plasticizers. The observed metabolite concentration differences might be a consequence of potential differences in procurement or in municipal cleaning routines. Taking on a rural-urban perspective for the municipal comparison, none of the statistically significant urinary metabolite concentration differences reported in various European studies within the DEMOCOPHES was observed in the present study (Cullen et al., 2017; Cutanda et al., 2015; Runkel et al., 2020). However, the difference in population density was smaller in the current study.

Furthermore, for the urinary metabolite concentrations of MCiNP and MPHHP the results from the mixed model analysis suggest sex differences as well, as shown by statistically significantly higher

Table 4
Sex, season, and municipality assessed as possible predictors of elevated metabolite concentrations in preschool children's urine samples. The antilogarithmic β-estimates (exp(β)) with 95% confidence intervals (CI) presented are assessed using linear mixed model (LMM) analysis based on 206 study grand mean specific gravity (SG) adjusted spot urine samples from 54 participants. exp(β) for *girls*, *winter*, and the municipality of *Vingåker* are presented.

		Sex[a]: girls				Season[b]: winter				Municipality[c]: Vingåker			
Parent compound	Metabolite	*p*	exp(β)[d]	95% CI		*p*	exp(β)[d]	95% CI		*p*	exp(β)[d]	95% CI	
DEHP	MEHP	0.71	1.05	0.80	1.39	0.21	0.91	0.78	1.057	0.27	1.16	0.88	1.53
	MEHHP	0.35	1.15	0.86	1.53	0.10	0.88	0.75	1.028	0.13	1.24	0.94	1.64
	MEOHP	0.73	1.05	0.78	1.41	0.074	0.87	0.75	1.014	0.19	1.21	0.91	1.61
	MECPP	0.57	1.078	0.83	1.40	0.14	0.90	0.78	1.033	0.44	1.11	0.85	1.43
	MCMHP	0.54	1.09	0.82	1.45	0.26	0.93	0.82	1.055	0.078	1.28	0.97	1.68
BBzP	MBzP	0.54	0.87	0.56	1.36	0.40	1.077	0.90	1.28	0.62	1.12	0.72	1.73
DBP	MBP	0.42	1.11	0.85	1.45	0.072	1.13	0.99	1.29	0.43	1.11	0.85	1.44
DiNP	MHiNP	0.70	1.06	0.79	1.41	0.76	1.031	0.85	1.25	0.51	1.097	0.83	1.45
	MOiNP	0.90	1.02	0.76	1.37	0.96	1.006	0.82	1.24	0.22	1.19	0.89	1.60
	MCiOP	0.58	1.082	0.82	1.44	0.80	1.024	0.85	1.23	0.44	1.11	0.84	1.47
DiDP/DPHP	MCiNP	**0.016**	**1.65**	**1.10**	**2.46**	0.92	0.99	0.82	1.19	**0.010**	**0.59**	**0.40**	**0.88**
	MPHHP	**0.022**	**1.55**	**1.07**	**2.24**	0.92	1.011	0.82	1.25	**0.005**	**0.60**	**0.42**	**0.85**
DEP	MEP	0.50	1.17	0.74	1.85	0.95	1.007	0.82	1.24	0.66	1.11	0.70	1.74
DiNCH	MOiNCH	0.67	1.08	0.75	1.57	0.26	1.17	0.89	1.53	< **0.001**	**0.53**	**0.39**	**0.73**

Bold values indicate statistically significant differences ($p < 0.05$) between the groups.
[a] *Boys* serves as the reference category.
[b] *Spring* serves as the reference category.
[c] *Eskilstuna* serves as the reference category.
[d] The results from the LMM analysis are obtained as anti-logarithmic β-values and are expressed as exp(β) with 95% CI.

concentrations in girls' urine samples in this study (Table 4). Whether this would be a consequence of sex differences in metabolization or in behavior or exposure is unclear. Although there were no more statistically significant sex differences observed, the majority of the analyzed metaboliteswere measured in higher urinary concentrations in girls. Higher phthalate metabolite concentrations have previously been observed in girls' urine samples in Nordic (Langer et al., 2014; Livsmedelsverket-Naturvårdsverket 2020) as well as in U.S. (NHANES) studies (Hatch et al., 2008; Hendryx and Luo 2018), although others report the opposite (Boas et al., 2010; Frederiksen et al., 2011) or no sex differences (Koch et al., 2011).

Unlike findings in other studies (Gao et al., 2017; Pilka et al., 2015), no significant seasonal urinary metabolite concentration differences were observed in the present study (Table 4). However, it is likely that this study did not capture large enough differences in indoor environment factors like temperature, ventilation, and airborne particle concentration to affect the mass transfer of phthalates and DiNCH from indoor sources, and thereby children's plasticizer exposure, when sampling during winter and spring. Sampling during the summer instead of the spring would have captured a larger difference in some of the factors controlling mass transfer of plasticizers, but because many children have long summer holidays in Sweden and because the oldest children leave for primary school during this period a different study design had to be chosen to facilitate the recruitment of study participants as well as the repeated measurements.

3.3. Health-based risk assessment

Human biomonitoring guidance values for the general population (HBM-GV_{GenPop}) as calculated by Lange et al. (2021) and relevant for this study are available for DEHP, BBzP, DnBP, DiBP, and DPHP (Table 5). These guidance values are to be used as an indicator of whether risk prevention efforts are needed by society at group level. The HBM-GV_{GenPop} for children 6–13 years of age in Table 5 are derived by mass balance equation calculations based on an established toxicity reference value (TRV) for each chemical. A corresponding HBM-GV_{GenPop} for the subgroup of children under 6 years of age has not been derived as it was considered not appropriate due to the lack of relevant toxicokinetic data (Lange et al., 2021). One should bear in mind that younger children may be more sensitive to exposure to chemicals. The evaluations of the concentrations in the present study in relation to the HBM-GV_{GenPop} for children indicate that some of the participating children are exposed to DBP well above the guidance values as MBP (MnBP + MiBP) was found in concentrations up to 0.61 mg/L, while the comparable HBM-GV_{GenPop} for children would be 0.28 mg/L for the sum of MnBP (0.12 mg/L) and MiBP (0.16 mg/L). Two participants in this study had urinary MBP concentrations above the HBM-GV_{GenPop} for children and one participant had a urinary concentration of MEHHP + MEOHP above the corresponding HBM-GV_{GenPop} for children (Table 5). The three samples exceeding the guidance values indicate that the strictly regulated phthalates DEHP, DnBP, and DiBP may still be relevant for children's accumulative exposure to EDCs. However, both the DBP and DEHP metabolite concentrations in this study (Table 2) were significantly lower in samples collected after a day with preschool attendance, likewise, the samples exceeding the guidance values were all *weekend: home* urine samples. As both DBP and DEHP are strictly regulated on the European market according to the EU REACH legislation the exposure is expected to decrease over time if future purchases are made from countries with legislation compatible with the EU chemical legislation, or at least from companies that do comply with it. To ensure this, public procurement can be a useful instrument. However, public procurement is not applicable for home environments, where children probably may spend most of their time. Therefore, in general, additional methods may be needed to reduce children's exposure to EDCs. Furthermore, it is important to ensure that substituting plasticizers will not cause adverse health effects similar to those associated with DBP and DEHP exposure.

Table 5
Health-based risk assessment of the biomarker concentrations using human biomonitoring guidance values for the general population (HBM-GV_{GenPop}) for children 6–13 years (mg/L) (Lange et al., 2021).

Parent compound	Metabolite (-s)	HBM-GV_{GenPop}	Present study		
			Range (Median)[a]	% > HBM-GV_{GenPop}	
				Total (n = 206)	*Weekend: home* (n = 104)
DEHP	MEHHP + MEOHP	0.34	0.0028–0.35 (0.025)	0.5	1
	MEHHP + MECPP	0.38	0.0038–0.33 (0.030)	–	–
BBzP	MBzP	2.0	0.00054–0.54 (0.017)	–	–
DnBP	MnBP	0.12	0.0067–0.61[b]	1	2
DiBP	MiBP	0.16	(0.051)		
DPHP	MPHHP	0.14	0.00013–0.058 (0.0024)	–	–

[a] Unadjusted.
[b] The sum of MnBP and MiBP.

4. Limitations

The present study has three main limitations. The first limitation is the rather small number of participants and the fact that the study participants were not evenly distributed between the preschools. This may have affected the generalizability of the results. Secondly, the sampling time point was standardized to serve the purpose of this study, i.e. participants collected weekend and midweek evening urine samples during winter and spring to assess exposure to plasticizers associated with home and preschool environments as well as seasonal differences in plasticizer exposure. Although the analyzed chemicals are sometimes referred to as pseudo-persistent, the sampling time point may be crucial for what metabolite concentrations will be found due to the short half-lives of phthalates and DiNCH. Among these chemicals, there is a range of half-lives and therefore the chosen sampling time point may not be optimal for all the metabolite measurements. In addition, the calculated poor to moderate ICCs indicate that single measurements may lead to misclassification, which in turn demonstrates that intraindividual differences could be a challenge when measuring urinary plasticizer metabolites. Finally, various lifestyle factors may have influenced the metabolite concentrations in the present study. Information about weekend activities was not collected, nor what indoor environments that had been visited during the weekend. Collection of such information along with more detailed information on housing conditions, for example type of flooring or renovation in the homes, could likely contribute to a better understanding of children's exposure to plasticizers. Furthermore, it would be valuable to assess additional indoor environments intended for children and to explore what characterizes indoor environments where the exposure to plasticizers is low.

5. Conclusions

The results from this study indicate that the exposure to plasticizing chemicals is ubiquitous among preschool-aged children in Sweden, even though several of the urinary metabolites analyzed in this study originate from chemicals recognized as EDCs and several are strictly regulated on the European market. In addition, the results suggest that there are significant differences in children's exposure to plasticizers associated with indoor environments, with lower exposure to strictly regulated phthalates as well as DEP, but higher exposure to less or non-regulated phthalates during a day with preschool attendance. Furthermore, in this study attending *older*, not recently renovated preschools

was associated with higher exposure to all the analyzed plasticizing chemicals. This may indicate that efficient chemical regulation and the mandatory public procurement in Sweden ensuring compliance with the current chemical legislation may contribute to reducing children's exposure to chemicals, i.e. the public indoor environments may benefit from being more controlled in the choice of building materials, furnishings, ventilation systems as well as cleaning routines and diets etc. There may also be less cosmetic products and perfumed items in the preschool indoor environment than elsewhere.

Few plasticizer metabolite concentrations exceeded the HBM-GV_{GenPop}, in the present study; however, the risk of mixture effects from various EDCs should not be neglected. The results indicate that risk assessments for chemicals need to be accelerated in order for harmful chemicals to be regulated as legal regulation appears to have noticeable effects on exposure. However, more efforts seem to be required to reduce children's exposure to strictly regulated phthalates outside of preschools.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114161.

References

Arbuckle, T.E., Davis, K., Marro, L., Fisher, M., Legrand, M., LeBlanc, A., et al., 2014. Phthalate and bisphenol a exposure among pregnant women in Canada–results from the mirec study. Environ. Int. 68, 55–65.

Attina, T.M., Hauser, R., Sathyanarayana, S., Hunt, P.A., Bourguignon, J.P., Myers, J.P., et al., 2016. Exposure to endocrine-disrupting chemicals in the USA: a population-based disease burden and cost analysis. Lancet Diabetes Endocrinol. 4, 996–1003.

Aylward, L.L., Hays, S.M., Smolders, R., Koch, H.M., Cocker, J., Jones, K., et al., 2014. Sources of variability in biomarker concentrations. J. Toxicol. Environ. Health B Crit. Rev. 17, 45–61.

Bergman, Å., Heindel, J.J., Jobling, S., Kidd, K.A., Zoeller, R.T., 2013. State of the Science of Endocrine Disrupting Chemicals 2012.

Bi, C., Maestre, J.P., Li, H., Zhang, G., Givehchi, R., Mahdavi, A., et al., 2018. Phthalates and organophosphates in settled dust and hvac filter dust of u.S. Low-income homes: association with season, building characteristics, and childhood asthma. Environ. Int. 121, 916–930.

Boas, M., Frederiksen, H., Feldt-Rasmussen, U., Skakkebæk, N.E., Hegedüs, L., Hilsted, L., et al., 2010. Childhood exposure to phthalates: associations with thyroid function, insulin-like growth factor i, and growth. Environ. Health Perspect. 118, 1458–1464.

Bui, T.T., Giovanoulis, G., Cousins, A.P., Magnér, J., Cousins, I.T., de Wit, C.A., 2016. Human exposure, hazard and risk of alternative plasticizers to phthalate esters. Sci. Total Environ. 541, 451–467.

CDC-NHANES, 2016. Biomonitoring Data Tables for Environmental Chemicals. Centers for Disease Control and Prevention.

Christia, C., Poma, G., Harrad, S., de Wit, C.A., Sjostrom, Y., Leonards, P., et al., 2019. Occurrence of legacy and alternative plasticizers in indoor dust from various eu countries and implications for human exposure via dust ingestion and dermal absorption. Environ. Res. 171, 204–212.

Conrad, A., Seiwert, M., Hünken, A., Quarcoo, D., Schlaud, M., Groneberg, D., 2013. The German environmental survey for children (geres iv): reference values and distributions for time-location patterns of German children. Int. J. Hyg Environ. Health 216, 25–34.

Cullen, E., Evans, D., Griffin, C., Burke, P., Mannion, R., Burns, D., et al., 2017. Urinary phthalate concentrations in mothers and their children in Ireland: results of the democophes human biomonitoring study. Int. J. Environ. Res. Publ. Health 14.

Cutanda, F., Koch, H.M., Esteban, M., Sánchez, J., Angerer, J., Castaño, A., 2015. Urinary levels of eight phthalate metabolites and bisphenol a in mother-child pairs from two Spanish locations. Int. J. Hyg Environ. Health 218, 47–57.

Den Hond, E., Govarts, E., Willems, H., Smolders, R., Casteleyn, L., Kolossa-Gehring, M., et al., 2015. First steps toward harmonized human biomonitoring in europe: demonstration project to perform human biomonitoring on a european scale. Environ. Health Perspect. 123, 255–263.

Domínguez-Romero, E., Scheringer, M., 2019. A review of phthalate pharmacokinetics in human and rat: what factors drive phthalate distribution and partitioning? Drug Metab. Rev. 51, 314–329.

Echa EU, 2023. Authorisation list. https://echa.Europa.Eu/sv/authorisation-list. (Accessed 6 March 2023).

European Commission, 2018. Commission Regulation (EU) 2018/2005.

Frederiksen, H., Aksglaede, L., Sorensen, K., Skakkebaek, N.E., Juul, A., Andersson, A.M., 2011. Urinary excretion of phthalate metabolites in 129 healthy Danish children and adolescents: estimation of daily phthalate intake. Environ. Res. 111, 656–663.

Frederiksen, H., Nielsen, J.K., Morck, T.A., Hansen, P.W., Jensen, J.F., Nielsen, O., et al., 2013. Urinary excretion of phthalate metabolites, phenols and parabens in rural and urban Danish mother-child pairs. Int. J. Hyg Environ. Health 216, 772–783.

Frederiksen, H., Nielsen, O., Koch, H.M., Skakkebaek, N.E., Juul, A., Jørgensen, N., et al., 2020. Changes in urinary excretion of phthalates, phthalate substitutes, bisphenols and other polychlorinated and phenolic substances in young Danish men; 2009-2017. Int. J. Hyg Environ. Health 223, 93–105.

Fromme, H., Lahrz, T., Kraft, M., Fembacher, L., Dietrich, S., Sievering, S., et al., 2013. Phthalates in German daycare centers: occurrence in air and dust and the excretion of their metabolites by children (lupe 3). Environ. Int. 61, 64–72.

Fromme, H., Schutze, A., Lahrz, T., Kraft, M., Fembacher, L., Siewering, S., et al., 2016. Non-phthalate plasticizers in German daycare centers and human biomonitoring of dinch metabolites in children attending the centers (lupe 3). Int. J. Hyg Environ. Health 219, 33–39.

Gao, F., Guo, Q., Wang, B., Cao, S., Qin, N., Zhao, L., et al., 2022. Distributions and determinants of time spent outdoors among school-age children in China. J. Expo. Sci. Environ. Epidemiol. 32, 223–231.

Gao, H., Zhu, Y.D., Xu, Y.Y., Zhang, Y.W., Yao, H.Y., Sheng, J., et al., 2017. Season-dependent concentrations of urinary phthalate metabolites among Chinese pregnant women: repeated measures analysis. Environ. Int. 104, 110–117.

Giovanoulis, G., Alves, A., Papadopoulou, E., Cousins, A.P., Schütze, A., Koch, H.M., et al., 2016. Evaluation of exposure to phthalate esters and dinch in urine and nails from a Norwegian study population. Environ. Res. 151, 80–90.

Giovanoulis, G., Bui, T., Xu, F., Papadopoulou, E., Padilla-Sanchez, J.A., Covaci, A., et al., 2018. Multi-pathway human exposure assessment of phthalate esters and dinch. Environ. Int. 112, 115–126.

Gong, M., Weschler, C.J., Liu, L., Shen, H., Huang, L., Sundell, J., et al., 2015. Phthalate metabolites in urine samples from beijing children and correlations with phthalate levels in their handwipes. Indoor Air 25, 572–581.

Gyllenhammar, I, Glynn, A, Jönsson, BA, Lindh, CH, Darnerud, PO, Svensson, K, et al., 2017. Diverging temporal trends of human exposure to bisphenols and plastizisers, such as phthalates, caused by substitution of legacy EDCs? Environ. Res. 153, 48–54.

Hammel, S.C., Levasseur, J.L., Hoffman, K., Phillips, A.L., Lorenzo, A.M., Calafat, A.M., et al., 2019. Children's exposure to phthalates and non-phthalate plasticizers in the home: the tesie study. Environ. Int. 132, 105061.

Harley, K.G., Berger, K.P., Kogut, K., Parra, K., Lustig, R.H., Greenspan, L.C., et al., 2019. Association of phthalates, parabens and phenols found in personal care products with pubertal timing in girls and boys. Hum. Reprod. 34, 109–117.

Hatch, E.E., Nelson, J.W., Qureshi, M.M., Weinberg, J., Moore, L.L., Singer, M., et al., 2008. Association of urinary phthalate metabolite concentrations with body mass index and waist circumference: a cross-sectional study of nhanes data, 1999-2002. Environ. Health 7, 27.

Hendryx, M., Luo, J., 2018. Children's environmental chemical exposures in the USA, nhanes 2003-2012. Environ. Sci. Pollut. Res. Int. 25, 5336–5343.

Huang, C.F., Wang, I.J., 2017. Changes in urinary phthalate metabolite levels before and after the phthalate contamination event and identification of exposure sources in a cohort of taiwanese children. Int. J. Environ. Res. Publ. Health 14.

Ketema, R.M., Ait Bamai, Y., Ikeda-Araki, A., Saito, T., Kishi, R., 2021. Secular trends of urinary phthalate metabolites in 7-year old children and association with building characteristics: hokkaido study on environment and children's health. Int. J. Hyg Environ. Health 234, 113724.

Koch, H.M., Wittassek, M., Brüning, T., Angerer, J., Heudorf, U., 2011. Exposure to phthalates in 5-6 years old primary school starters in Germany–a human biomonitoring study and a cumulative risk assessment. Int. J. Hyg Environ. Health 214, 188–195.

Koch, H.M., Rüther, M., Schütze, A., Conrad, A., Pälmke, C., Apel, P., et al., 2017. Phthalate metabolites in 24-h urine samples of the German environmental specimen bank (esb) from 1988 to 2015 and a comparison with us nhanes data from 1999 to 2012. Int. J. Hyg Environ. Health 220, 130–141.

Lange, R., Apel, P., Rousselle, C., Charles, S., Sissoko, F., Kolossa-Gehring, M., et al., 2021. The european human biomonitoring initiative (hbm4eu): human biomonitoring guidance values for selected phthalates and a substitute plasticizer. Int. J. Hyg Environ. Health 234, 113722.

Langer, S., Beko, G., Weschler, C.J., Brive, L.M., Toftum, J., Callesen, M., et al., 2014. Phthalate metabolites in urine samples from Danish children and correlations with phthalates in dust samples from their homes and daycare centers. Int. J. Hyg Environ. Health 217, 78–87.

Larsson, K., Lindh, C.H., Jonsson, B.A., Giovanoulis, G., Bibi, M., Bottai, M., et al., 2017. Phthalates, non-phthalate plasticizers and bisphenols in Swedish preschool dust in relation to children's exposure. Environ. Int. 102, 114–124.

Livsmedelsverket-Naturvårdsverket, 2020. Contaminants in Blood and Urine from Adolescents in sweden. Livsmedelsverkets samarbetsrapport), Uppsala.

Mercier, F., Glorennec, P., Thomas, O., Le Bot, B., 2011. Organic contamination of settled house dust, a review for exposure assessment purposes. Environ. Sci. Technol. 45, 6716–6727.

Morgan, M.K., Sheldon, L.S., Croghan, C.W., Jones, P.A., Robertson, G.L., Chuang, J.C., et al., 2005. Exposures of preschool children to chlorpyrifos and its degradation product 3,5,6-trichloro-2-pyridinol in their everyday environments. J. Expo. Anal. Environ. Epidemiol. 15, 297–309.

Pilka, T., Petrovicova, I., Kolena, B., Zatko, T., Trnovec, T., 2015. Relationship between variation of seasonal temperature and extent of occupational exposure to phthalates. Environ. Sci. Pollut. Res. Int. 22, 434–440.

Runkel, A.A., Snoj-Tratnik, J., Mazej, D., Horvat, M., 2020. Urinary phthalate concentrations in the slovenian population: an attempt to exposure assessment of family units. Environ. Res. 186, 109548.

Schwedler, G., Conrad, A., Rucic, E., Koch, H.M., Leng, G., Schulz, C., et al., 2020a. Hexamoll® dinch and dphp metabolites in urine of children and adolescents in Germany. Human biomonitoring results of the German environmental survey geres v, 2014-2017. Int. J. Hyg Environ. Health 229, 113397.

Schwedler, G., Rucic, E., Lange, R., Conrad, A., Koch, H.M., Pälmke, C., et al., 2020b. Phthalate metabolites in urine of children and adolescents in Germany. Human biomonitoring results of the German environmental survey geres v, 2014-2017. Int. J. Hyg Environ. Health 225, 113444.

Singh, G.K., Balzer, B.W., Desai, R., Jimenez, M., Steinbeck, K.S., Handelsman, D.J., 2015. Requirement for specific gravity and creatinine adjustments for urinary steroids and luteinizing hormone concentrations in adolescents. Ann. Clin. Biochem. 52, 665–671.

Vafeiadi, M., Myridakis, A., Roumeliotaki, T., Margetaki, K., Chalkiadaki, G., Dermitzaki, E., et al., 2018. Association of early life exposure to phthalates with obesity and cardiometabolic traits in childhood: sex specific associations. Front. Public Health 6, 327.

Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs Jr., D.R., Lee, D.H., et al., 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33, 378–455.

Watkins, D.J., Eliot, M., Sathyanarayana, S., Calafat, A.M., Yolton, K., Lanphear, B.P., et al., 2014. Variability and predictors of urinary concentrations of phthalate metabolites during early childhood. Environ. Sci. Technol. 48, 8881–8890.

Wei, W., Mandin, C., Ramalho, O., 2018. Influence of indoor environmental factors on mass transfer parameters and concentrations of semi-volatile organic compounds. Chemosphere 195, 223–235.

Wormuth, M., Scheringer, M., Vollenweider, M., Hungerbühler, K., 2006. What are the sources of exposure to eight frequently used phthalic acid esters in europeans? Risk Anal. 26, 803–824.

Zhu, Q., Jia, J., Zhang, K., Zhang, H., Liao, C., Jiang, G., 2019. Phthalate esters in indoor dust from several regions, China and their implications for human exposure. Sci. Total Environ. 652, 1187–1194.

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External Quality Assurance Schemes (EQUASs) and Inter-laboratory Comparison Investigations (ICIs) for human biomonitoring of polycyclic aromatic hydrocarbon (PAH) biomarkers in urine as part of the quality assurance programme under HBM4EU

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) were included as priority substances for human biomonitoring (HBM) in the European Human Biomonitoring Initiative (HBM4EU), which intended to harmonise and advance HBM across Europe. For this project, a specific Quality Assurance and Quality Control (QA/QC) programme applying Inter-laboratory Comparison Investigations (ICIs) and External Quality Assurance Schemes (EQUASs) was developed to ensure the comparability and accuracy of participating analytical laboratories. This paper presents the results of four ICI/EQUAS rounds for the determination of 13 PAH metabolites in urine, i.e. 1-naphthol, 2-naphthol, 1,2-dihydroxynaphthalene, 2-, 3- and 9-hydroxyfluorene, 1-, 2-, 3-, 4- and 9-hydroxyphenanthrene, 1-hydroxypyrene and 3-hydroxybenzo(a)pyrene. However, 4 PAH metabolites could not be evaluated as the analytical capacity of participating laboratories was too low. Across all rounds and biomarkers, 86% of the participants achieved satisfactory results, although low limits of quantification were required to quantify the urinary metabolites at exposure levels of the general population. Using high-performance liquid or gas chromatography coupled with mass spectrometry (HPLC-MS; GC-MS) and isotope dilution for calibration as well as performing an enzymatic deconjugation step proved to be favourable for the accurate determination of PAHs in urine. Finally, the HBM4EU QA/QC programme identified an international network of laboratories providing comparable results in the analysis of urinary PAH biomarkers, although covering all parameters initially selected was still too challenging.

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Abbreviations

PAHs	Polycyclic aromatic hydrocarbons
HBM	human biomonitoring
EQUAS	External Quality Assurance Scheme
ICI	Inter-laboratory Comparison Investigation
QA/QC	Quality Assurance and Quality Control
1-naphthol	1-hydroxynaphthalene
2-naphthol	2-hydroxynaphthalene
1,2-DHN	1,2-dihyroxynaphthalene
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
CM	control material
C	consensus value
A	assigned value
RSD	relative standard deviation
study RSDR	robust RSD for the CM of each round

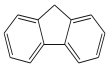
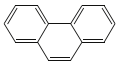
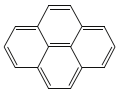
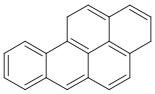
1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are emitted to the environment from pyrogenic and petrogenic sources and, to some extent, from biogenic sources (Wang et al., 2014). Pyrogenic PAHs are mainly generated during the incomplete combustion of organic materials, such as coal, oil, natural gas and wood (Kim et al., 2013). Due to the wide application of combustion processes for heating, transport and in many industrial processes, PAHs are ubiquitous in the environment (Ravindra et al., 2008), which implies an exposure of the general population to these substances by atmospheric pollution via inhalation or consumption of PAH-containing food (Waldman et al., 1991). Higher than average PAH exposure may result from smoking and consumption of grilled and smoked food (Li et al., 2012; St. Helen et al., 2012). Numerous PAHs have been identified as carcinogens or probable carcinogens (IARC, 2010) and several other adverse health outcomes have to be considered as a result of exposure to PAHs such as, for example, reproductive, developmental and immunologic toxicity of PAHs (Danish EPA, 2013; Kim et al., 2013; Vandráček and Machala, 2021). The severe genotoxic and non-genotoxic toxicity as well as the ubiquitous exposure of the population to PAHs has caused significant public health concern and initiated human biomonitoring (HBM) of the PAH exposure in the general population (Becker et al., 2003; Hudson-Hanley et al., 2021). As a first HBM parameter of exposure to PAHs, 1-hydroxypyrene (1-PYR) in urine was established in HBM studies and demonstrated its efficiency as a biomarker in both exposed workers and the general population (Göen et al., 1995; Hansen et al., 2008). Successively, additional HBM parameters have been included in studies of PAH exposure, primarily biomarkers of 2-, 3- and 4-rings PAHs and benzo[a]pyrene (Chetiyanukornkul et al., 2006; Li et al., 2008; Romanoff et al., 2006).

In the pan-European project HBM4EU (European Human Biomonitoring Initiative), several prominent PAH metabolites were considered as priority biomarkers for HBM studies (Louro et al., 2019; Vorkamp et al., 2021). HBM4EU was launched in 2016 as cooperation of 30 countries and European Commission authorities (https://www.hbm4eu.eu) in order to examine the exposure of EU citizens to a variety of chemicals (Vorkamp et al., 2021). The project's aim to harmonise and advance HBM across Europe for a better chemical risk management (Ganzleben et al., 2017) implied the establishment of a network of analytical laboratories which generated comparable data for the analysis of the prioritized human biomarkers in the HBM4EU Aligned Studies (Esteban López et al., 2021). For this purpose, a special Quality Assurance and Quality Control (QA/QC) scheme was designed and implemented in HBM4EU, consisting of several rounds of Inter-laboratory Comparison Investigations (ICIs) and External Quality Assurance Schemes (EQUASs). ICIs were used to compare the results of analytical laboratories applying for the analysis of HBM4EU samples (participating laboratories) among each other, while EQUASs compared the results of the participants to selected expert laboratories having documented experience with chemical analysis in previous HBM studies (Esteban López et al., 2021).

This paper presents the QA/QC programme developed in HBM4EU for thirteen PAH biomarkers in urine (Table 1) focussing on the results obtained, the methods applied and the limits of quantification (LOQs) reported by the participants and experts, but also points out the challenges in the analysis of some PAH biomarkers.

Table 1
PAH biomarkers in urine included in the QA/QC scheme in HBM4EU.

PAH parent compound	Number of fused rings	Metabolite	Abbreviation
Naphthalene		1-hydroxynaphthalene	1-naphthol
		2-hydroxynaphthalene	2-naphthol
		1,2-dihyroxynaphthalene	1,2-DHN
Fluorene		2-hydroxyfluorene	2-FLUO
		3-hydroxyfluorene	3-FLUO
		9-hydroxyfluorene	9-FLUO
Phenanthrene		1-hydroxyphenanthrene	1-PHEN
		2-hydroxyphenanthrene	2-PHEN
		3-hydroxyphenanthrene	3-PHEN
		4-hydroxyphenanthrene	4-PHEN
		9-hydroxyphenanthrene	9-PHEN
Pyrene		1-hydroxypyrene	1-PYR
Benzo [a] pyrene		3-hydroxybenzo[a]pyrene	3-BaP

2. Materials and methods

2.1. Design of the HBM4EU ICI/EQUAS programme for PAH biomarkers in urine

From March 2018 to November 2019, four rounds of tailor-made ICI and EQUAS exercises were organised in the frame of the HBM4EU QA/QC programme to assess the proficiency of laboratories for the analysis of urinary PAH biomarkers. The overall organisation and the specific conditions of the ICI/EQUAS exercises for all substance groups in the HBM4EU project is described in Esteban López et al. (2021). To ensure high quality of the analysis in the HBM4EU Aligned Studies (Gilles et al., 2021), laboratories had to participate successfully in at least two ICIs/EQUASs for PAH biomarkers. In each round, two control materials (CMs) were provided, spiked with all PAH biomarkers at two concentrations, i. e. CM_{low} and CM_{high}. While the 1st round was organised as ICI, the following three rounds were conducted as EQUASs. The implementation of EQUASs increased international comparability by involving experienced European and international laboratories as experts. In addition, the participants' performance could be assessed even if an evaluation as ICI was impossible due to a too small number of participants (<7) or too heterogeneous results. For the four biomarkers 1,2-DHN, 9-FLUO, 9-PHEN and 3-BaP, an evaluation was still not possible (Suppl. Table 1), because the number of quantitative results submitted by expert laboratories (EQUAS) was too low (<3) and/or their results were too inhomogeneous.

2.2. Recruiting of participants

Details of the recruiting of laboratories that participated in the HBM4EU QA/QC programme are specified in Esteban López et al. (2021). The first call to identify participants resulted in a list of 29 laboratories interested in conducting analyses of PAH biomarkers in HBM4EU. After the second call, this list increased to 42 laboratories, of which 29 laboratories from 14 countries finally participated in the ICIs/EQUASs (Suppl. Table 2).

In a letter of invitation, the participants were asked to report their LOQs which should be low enough to enable the detection of PAH biomarkers in samples of the general population. Laboratories could participate in the ICIs/EQUASs for all offered PAH biomarkers (n = 13) or for less.

2.3. Expert laboratories

The selection of the six expert laboratories was conducted by the HBM4EU Quality Assurance Unit (QAU) according to specific selection criteria (see Nübler et al., 2021, 2022a,b). Two international expert laboratories were selected, as well as four European experts, three of which were also considered as participating laboratories.

2.4. Control materials (CMs)

Preparation: Native (non-spiked) CM (CM_{native}) consisted of filtrated human urine (from smokers) with addition of 1 g sodium azide (Merck, Darmstadt, Germany) per liter urine. Spiking of the CMs was conducted using native compounds for most parameters, while the degradation-sensitive PAH metabolites 1-naphthol, 1,2-DHN and 3-BaP were spiked as glucuronide conjugate, as recommended by Zobel et al. (2017). The respective stock solutions (Suppl. Table 3) in acetonitrile (Merck, Darmstadt, Germany) were diluted accordingly and added to CM_{native} to achieve the final concentrations for CM_{low} and CM_{high} (Suppl. Table 4). A volume of 5 mL of each CM was filled in vials with caps (82 × 13 mm, polypropylene, Sarstedt, Nümbrecht, Germany) and stored at ≤ −18 °C until shipment. Homogeneity and stability of all CMs were monitored by analysing randomly selected samples under the conditions specified in the Supplementary Material (Suppl. Tables 5-7).

Homogeneity testing: Ten randomly selected vials of CMs of each concentration were taken from the storage shortly after preparation. After thawing and re-homogenising, CMs were analysed in duplicate under the conditions given in Suppl. Table 5. The analysis of all samples for one round was conducted as one batch and the results were evaluated according to ISO 13528:2015 (Fearn and Thompson, 2001; Thompson, 2000).

Stability testing: Stability testing of the CMs followed ISO 13528 (Statistical methods for use in proficiency testing by inter-laboratory comparison, 2015) and the International Harmonised Protocol for the Proficiency Testing of Analytical Laboratories (Thompson et al., 2006). Samples were stored at −18 °C (representative for storage at the participants' laboratories) and at −80 °C (considering maximum stability). Samples were thawed and treated like the samples shipped to the participating laboratories. Stability was determined by parallel analysis of six randomly selected samples of each concentration after storage at either −18 °C or −80 °C for the time interval between shipment and deadline for result submission (on average 4–5 weeks per round). To assess the stability, the means of the six samples at −18 °C and −80 °C were compared using the t-Test. In the 1st round, stability assessment was slightly different, because ten samples were analysed on the day of sample preparation and compared to the analysis of ten samples stored at - 18 °C for a time interval of 25 days.

Distribution: In the 1st and 2nd round, CM_{low} and CM_{high} were sent under ambient conditions and protected from light and included six vials of 5 mL urine each. From the 3rd round on, only one vial of each CM (5 mL) was dispatched to the participants on dry ice. The switch to dry ice was made at the request of some participants whose CMs were traveling longer due to shipping delays, although no effect of the transport conditions on the integrity of the biomarkers was found in the first two rounds. At the time of shipment, participants received a letter with instructions on how to handle the samples, including storing the samples under frozen (−18 °C) conditions until analysis. Furthermore, participants were asked to conduct single analysis of each sample and to use the same method as they were going to use for the analysis of the samples in HBM4EU Aligned Studies. In the 2nd round, three samples of CM_{low} and three samples of CM_{high} were sent to selected expert laboratories, which were requested to conduct duplicate analysis. From the 3rd round on, the experts received six samples of each CM and could perform single analysis.

2.5. Performance assessment

The implementation and evaluation of the ICIs/EQUASs for PAH biomarkers followed the requirements defined in the standard operating procedures available in the online library of the HBM4EU website (https://www.hbm4eu.eu/online-library/). Procedures for the assessment of laboratory performance in ICIs/EQUASs were the same for different biomarker groups in the HBM4EU project and have been described previously (Esteban López et al., 2021; Nübler et al., 2021, 2022a,b).

In an ICI, at least seven quantitative results from participants were required to calculate the robust mean of the participants' results as consensus value (C). In this case, robust statistics were used to reduce the influence of outliers on C. The evaluation of EQUAS results was based on quantitative results reported by a minimum of three designated expert laboratories. The mean-of-means of the experts' results was used as assigned value (A), when the uncertainty of A was not higher than 17.5% (for details see Esteban López et al., 2021 and Nübler et al., 2021). For the determination of A, classical statistics were applied due to the low number of experts.

To assess the proficiency of the participants' results (x), Z-scores were calculated using the target relative standard deviation (σ_T) of 25% and C (ICI) or A (EQUAS) according to the equation:

$$Z = \frac{x - C \text{ or } A}{\sigma_T * C \text{ or } A} \quad (1)$$

Table 2
Detailed ICI/EQUAS results.

PAHs	Round	CM	Consensus/Assigned value (ng/mL)	RSDexperts (%)	Study RSDR (%)	Labs reporting results	Satis (%)	Quest (%)	Unsat (%)
1-naphthol	**1**	**low**	4.16	ne	22	12	100	0	0
		high	8.71	ne	14	12	100	0	0
	2	**low**	2.01	32	44	17	76	6	18
		high	3.09	24	32	17	82	6	12
	3	**low**	3.04	14	30	16	69	12	19
		high	6.76	11	35	16	88	6	6
	4	**low**	2.77	34	24	15	80	13	7
		high	10.6	29	25	15	93	7	0
2-naphthol	**1**	**low**	6.50	ne	17	13	100	0	0
		high	13.9	ne	12	13	100	0	0
	2	**low**	9.35	17	22	20	85	10	5
		high	14.3	16	22	20	85	10	5
	3	**low**	1.28	10	34	16	75	0	25
		high	4.02	10	25	16	75	12.5	12.5
	4	**low**	5.34	18	14	14	86	7	7
		high	9.90	21	16	14	93	7	0
1,2-DHN	**1**	**low**	ne	ne	ne	0	ne	ne	ne
		high	ne	ne	ne	0	ne	ne	ne
	2	**low**	ne	ne	ne	0	ne	ne	ne
		high	ne	ne	ne	0	ne	ne	ne
	3	**low**	–	–	–	–	–	–	–
		high	–	–	–	–	–	–	–
	4	**low**	–	–	–	–	–	–	–
		high	–	–	–	–	–	–	–
2-FLUO	**1**	**low**	0.780	ne	25	7	86	0	14
		high	1.59	ne	23	7	100	0	0
	2	**low**	0.436	29	28	10	100	0	0
		high	0.827	29	23	10	90	10	0
	3	**low**	0.134	19	15	8	62.5	25	12.5
		high	0.299	14	14	8	87.5	12.5	0
	4	**low**	0.503	10	41	8	50	25	25
		high	0.995	9	44	8	50	25	25
3-FLUO	**1**	**low**	ne	ne	ne	3	ne	ne	ne
		high	ne	ne	ne	3	ne	ne	ne
	2	**low**	0.214	29	33	6	83	0	17
		high	0.389	ne	20	6	100	0	0
	3	**low**	0.073	8	26	5	60	20	20
		high	0.194	4	33	6	66	17	17
	4	**low**	0.170	17	75	3	67	0	33
		high	0.401	11	42	4	75	0	25
9-FLUO	**1**	**low**	ne	ne	ne	2	ne	ne	ne
		high	ne	ne	ne	2	ne	ne	ne
	2	**low**	ne	ne	ne	4	ne	ne	ne
		high	ne	ne	ne	4	ne	ne	ne
	3	**low**	ne	ne	ne	5	ne	ne	ne
		high	ne	ne	ne	5	ne	ne	ne
	4	**low**	ne	ne	ne	2	ne	ne	ne
		high	ne	ne	ne	2	ne	ne	ne
1-PHEN	**1**	**low**	0.325	ne	25	7	86	14	0
		high	0.495	ne	31	7	86	14	0
	2	**low**	0.476	20	23	11	82	18	0
		high	0.788	20	23	11	100	0	0
	3	**low**	0.232	19	44	11	73	18	9
		high	0.303	20	26	11	82	9	9
	4	**low**	0.230	25	24	8	75	25	0
		high	0.414	25	17	8	87.5	12.5	0
2-PHEN	**1**	**low**	0.218	ne	18	7	100	0	0
		high	0.349	ne	10	7	100	0	0
	2	**low**	0.308	ne	43	10	100	0	0
		high	0.732	18	39	11	75	25	0
	3	**low**	0.146	10	13	11	82	9	9
		high	0.196	12	25	11	82	9	9
	4	**low**	0.125	ne	17	9	100	0	0
		high	0.274	ne	15	9	89	0	11
3-PHEN	**1**	**low**	0.282	ne	7	7	100	0	0
		high	0.438	ne	12	7	100	0	0
	2	**low**	0.271	34	37	11	82	9	9
		high	0.482	33	35	11	82	9	9
	3	**low**	0.160	23	30	11	82	9	9
		high	0.227	18	38	11	91	0	9

(*continued on next page*)

Table 2 (*continued*)

PAHs	Round	CM	Consensus/Assigned value (ng/mL)	RSDexperts (%)	Study RSDR (%)	Labs reporting results	Satis (%)	Quest (%)	Unsat (%)
	4	**low**	0.188	9	12	8	100	0	0
		high	0.353	8	13	8	100	0	0
4-PHEN	**1**	**low**	0.104	ne	24	7	86	0	14
		high	0.140	ne	27	7	86	0	14
	2	**low**	0.129	ne	21	12	92	8	0
		high	0.246	ne	21	12	92	8	0
	3	**low**	0.074	ne	43	11	91	0	9
		high	0.133	ne	27	11	100	0	0
	4	**low**	0.091	ne	35	7	100	0	0
		high	0.193	ne	21	8	100	0	0
9-PHEN	**1**	**low**	ne	ne	ne	5	ne	ne	ne
		high	ne	ne	ne	5	ne	ne	ne
	2	**low**	ne	ne	ne	7	ne	ne	ne
		high	ne	ne	ne	7	ne	ne	ne
	3	**low**	ne	ne	ne	4	ne	ne	ne
		high	ne	ne	ne	5	ne	ne	ne
	4	**low**	ne	ne	ne	4	ne	ne	ne
		high	ne	ne	ne	4	ne	ne	ne
1-PYR	**1**	**low**	0.121	ne	27	13	100	0	0
		high	0.212	ne	25	13	100	0	0
	2	**low**	0.113	36	30	25	62	15	23
		high	0.258	34	31	26	73	19	8
	3	**low**	0.038	ne	64	18	66	17	17
		high	0.041	ne	39	18	83	0	17
	4	**low**	0.113	8	21	19	68	21	11
		high	0.253	25	19	19	90	5	5
3-BaP	**1**	**low**	ne	ne	ne	1	ne	ne	ne
		high	ne	ne	ne	1	ne	ne	ne
	2	**low**	ne	ne	ne	3	ne	ne	ne
		high	ne	ne	ne	3	ne	ne	ne
	3	**low**	–	–	–	–	–	–	–
		high	–	–	–	–	–	–	–
	4	**low**	–	–	–	–	–	–	–
		high	–	–	–	–	–	–	–

Satis: satisfactory results; quest: questionable results; unsat: unsatisfactory results; ne = no evaluation as ICI or/and EQUAS was possible due to the lack of participants/experts or too high inhomogeneity of the results of the participants/experts.

In ICIs/EQUASs, the absolute values of Z-scores were interpreted as follows:

$|Z| \leq 2 \Rightarrow$satisfactory

$2 < |Z| < 3 \Rightarrow$questionable

$|Z| \geq 3 \Rightarrow$unsatisfactory

A laboratory had to achieve satisfactory Z-scores in both CMs to pass an ICI/EQUAS for the respective biomarkers. After successful participation in at least two ICIs/EQUASs, a laboratory was qualified for the analysis of these biomarkers in HBM4EU Aligned Studies.

If in an EQUAS round, the calculation of A was not possible, because less than three expert results were reported or the expert results were not in sufficient agreement (uncertainty of A >17.5%), the results were evaluated as ICI, using C for the calculation of Z-scores, when the criteria for an ICI evaluation were fulfilled. If the requirements for an EQUAS and an ICI evaluation were not met, no evaluation was conducted for the respective CM level.

2.6. Statistical evaluation

For statistical analyses, IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY, IBM Corp. released 2019, was used. Paired samples were analysed with the Wilcoxon test, while the Mann-Whitney-U-Test was applied for independent groups. The significance level was set at 0.05. Statistical analyses of Z-scores were conducted using the absolute values as they were also applied for passing an ICI/EQUAS round. Boxplots ranged from the 25th to the 75th percentile with the median as horizontal line, the arithmetic as square, the 5th–95th percentiles as whiskers and the minimum and maximum values as crosses.

3. Results and discussion

3.1. Selection of spiked concentrations and testing of homogeneity and stability

The selection of the range of concentrations of the CMs was crucial to assess the proficiency of the participants. They should be in line with the expected exposure levels in the general population which was the target population in HBM4EU Aligned Studies. To best reflect the levels of the respective PAH biomarkers in the general population, the urine was spiked in a range between 0.003 and 9 ng/mL for CM_{low} and between 0.006 and 30 ng/mL for CM_{high} (Suppl. Table 4). Together with the content of CM_{native} (urine of smokers), the resulting levels in the prepared CMs detected in the homogeneity analyses (Suppl. Table 8) were lowest for 3-BaP, followed by 1-PYR, 3-FLUO, all PHENS, 2- and 9-FLUO and highest for the naphthols and 1,2-DHN. The spike levels were chosen from urinary levels of PAH metabolites published for European populations (Bartolomé et al., 2015; Nilsson et al., 2013; Urbanocova et al., 2020). HBM studies on the PAH exposure in the general populations of Spain, Poland, Serbia and Italy showed similar levels (Bartolomé et al., 2015; Nilsson et al., 2013).

Homogeneity analyses (see Section 2.4) detected no major outliers (three minor outliers marked in Suppl. Table 8) and CMs were considered sufficiently homogeneous. The results of the stability testing (see Section 2.4) are shown in Suppl. Table 9. The data did not indicate any degradation during storage and transport. Nevertheless, the means from the day of sample preparation and the means after being stored for 25 days at −18 °C showed some significant differences in the 1st round. However, the differences showed no preferred direction and were still

within the range of the day-to-day precision so that the respective biomarkers in the CMs were considered sufficiently stable. Once samples for stability testing were all measured on the same day, for two different temperatures (from the 2nd round on), no more deviation was detected and thus constant concentrations of the PAH biomarkers between preparation and analysis could be ensured over all rounds.

3.2. Participation rates

In the 1st exercise for PAH biomarkers, 19 out of 29 (66%) invited laboratories participated (Suppl. Table 10). To the 2nd round, 42 laboratories were invited and 27 laboratories participated, leading to a participation rate of 64%. It subsequently decreased to 59% and 50% in the 3rd and the 4th round, respectively. Seven laboratories participated in all four rounds, and eight laboratories participated in three ICIs/EQUASs. Over all rounds, 35 individual laboratories participated and 30 of them reported quantitative results for at least one PAH biomarker (Suppl. Fig. 1). Eleven participants submitted quantitative results for at least seven biomarkers, while the maximum of reported biomarkers (all except for 1,2-DHN) was only reached by two participants (Suppl. Fig. 1; Suppl. Fig. 2). 1-PYR, 1- and 2-naphthol were quantified by most participants, while the fewest participants submitted quantitative results for the FLUO biomarkers, 3-BaP and 1,2-DHN (Fig. 1). This distribution pattern may be based on the history of chemical analysis of specific PAH metabolites in HBM, as 1-PYR and the naphthols were the first biomarkers, followed by the assessment of OH-PHEN isomers and the successively increased application of a broader biomarker spectrum in the last two decades (Hansen et al., 2008; Klotz et al., 2018; Grimmer et al., 1993; Li et al., 2008). In the 2nd and 3rd round, all six experts participated, while in the 4th round one expert laboratory was missing.

3.3. Applied methods by participants and experts

An overview of the methods for the analysis of PAH biomarkers used by participants and experts is shown in Suppl. Fig. 3 and 4. The laboratories of three of the six experts and of around one third of the participants were ISO17025 accredited. Almost all participants and experts used enzymatic deconjugation with β-glucuronidase or β-glucuronidase/(aryl)sulfatase (with only one exception using steam distillation with sulfonic acid). In cases in which an extraction was performed, solid-phase extraction (SPE) was preferred over liquid-liquid extraction (LLE). Roughly one third of participants and experts applied SPE clean-up and/or derivatisation. For detection, most of the participants and experts used HPLC (Suppl. Fig 4 A) coupled to tandem mass spectrometry (Suppl. Fig. 4 B) and isotope-labelled internal standards which were mainly added to the sample before deconjugation. Around one third of the participants and experts either prepared a matrix-matched calibration curve or used solvent standards. Predominantly, multi-level calibration was applied and a correction of the recovery rate was largely dispensed with.

3.4. Laboratory performance results

The outcome of the four ICI/EQUAS rounds for the PAH biomarkers is shown in Table 2. Individual Z-scores of the participants who submitted quantitative results for CM_{low} and CM_{high} in the respective rounds are shown in Suppl. Fig. 5-13. In all rounds and for most biomarkers, the majority of participants obtained results that could be classified as "satisfactory". In the 1st round, conducted as ICI, no evaluation was possible for 3-FLUO, 1,2-DHN, 3-BaP, 9-PHEN and 9-FLUO, because less than the required seven laboratories participated for these biomarkers. The inclusion of experts' results from the 2nd round on enabled the assessment of 3-FLUO (either as EQUAS or as ICI with included experts' results) in all subsequent rounds (2nd-4th round). In contrast, participation for 1,2-DHN was also very low in the 2nd round (only one laboratory), so it was decided to exclude this biomarker from subsequent ICIs/EQUASs. The number of participants for 3-BaP in the 2nd round would have been sufficient for an evaluation as ICI, but too few laboratories had been able to quantitatively detect the biomarkers in the exposure range of the general population provided in the CMs. Additionally, only one expert was available for the analysis of 3–BaP so that this biomarker was also no longer offered in the 3rd and the 4th round. For 9-PHEN and 9-FLUO, the number of quantitative results from participants and/or from experts was too low to enable an evaluation from the 2nd to the 4th round.

For the other biomarkers, satisfactory Z-scores ranged from 50% (in both CMs of 2-FLUO in the 4th round) to 100% (in 21 CMs belonging to 9 biomarkers, mainly in the 1st round) and were on average obtained by 86% of the participants. The two highest concentrations of the CMs were applied for most of the continuously evaluable biomarkers (1- and 2-naphthol, 2-FLUO, 1- to 4-PHEN and 1-PYR) in the 1st round, the lowest in the 3rd round. Accordingly, we found that the average performance of the participants and the inter-laboratory comparability was best in the 1st round and lowest in the 3rd round (Supp. Fig. 14, 15), reflecting some difficulties in the detection of lower concentrations. In addition, 63% of participants who could separate and quantify the hydroxyphenanthrene isomers 1-, 2-, 3- and 4-PHEN used GC instead of HPLC (38%), whereas overall only 28% of all participants used GC. Successful separation and quantification of the hydroyfluorene isomers 2-, 3- and 9-FLUO was achieved by four particpants, all using GC (three with triple quadrupole and one with single quadrupole detection). The five participants who measured only two FLUO isomers (2- and 3-FLUO)

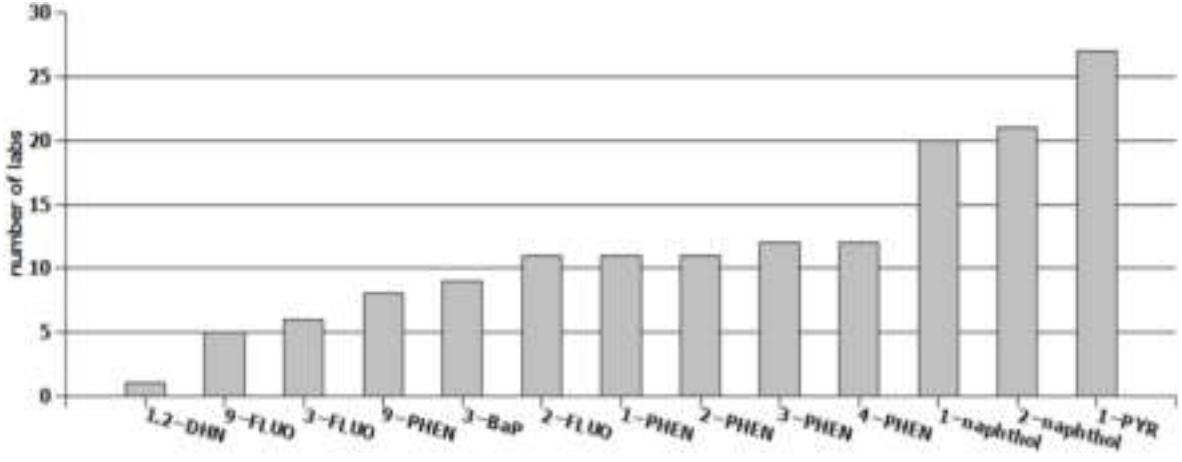


Fig. 1. Overview of the number of labs reporting quantitative results for each of the 13 PAH biomarkers.

and/or sums of isomers (mainly sum of 2- and 3-FLUO) applied HPLC or UPLC with triple quadrupole detection.

Statistical analyses showed that for most biomarkers, the differences in the Z-scores observed between CM_{low} and CM_{high} were not significant (except for 1-PYR). This might be a concentration effect since over all rounds, CMs and biomarker groups, the lowest levels of A and C were obtained for 1-PYR (Fig. 2A), which is in accordance with its low exposure level in the general population (Bartolomé et al., 2015). Several significant differences were found in the obtained Z-scores between the biomarkers over all CMs and rounds, which may be related to the differences in the values of A and C for all evaluable biomarkers. The median Z-scores of both naphthols and of the evaluable PHEN biomarkers (1- to 4-PHEN) were significantly lower compared to the median Z-scores of 1-PYR and the group of 2- and 3-FLUO (Fig. 2B). The inter-laboratory reproducibility over all rounds – represented by the lower mean study RSD_R – was also higher for the naphthols and the PHENs than for the FLUOs and 1-PYR (Fig. 2C). For 1- to 4-PHEN, these results were unexpected because the analysis of these biomarkers includes the challenge of chromatographic separation of isomers, potentially leading to higher uncertainty.

3.5. Comparison of the different assessment schemes

In the 1st round, the performance of the participants was exclusively evaluated based on the mean results of at least seven participants (ICI). As a consequence, 10 of the 26 CMs in this round could not be evaluated, because the required number of participants was not reached (see section 3.4). The introduction of the assessment as EQUAS from the 2nd round enabled an evaluation of 77% of the 70 CMs in total, 78% of which were evaluated by using the mean of the experts' results (EQUAS) and 22% were still evaluated based on the participants' results (ICI). In the 42 CMs evaluated as EQUAS, the results of the experts were clearly more homogeneous than the results of the participants. While the mean study RSD_R of the participants ranged from 7 to 75% and was on average 29%, the relative standard deviation of the expert values ranged between 4% and 36% and was on average 19%. The mean inter-laboratory variability in the present ICI/EQUAS programme for 1-PYR (32%) was similar to the data presented in Göen et al. (2012), where the deviation from the target value in the range of the reference value accepted in G-EQUAS (German-EQUAS) was 38%.

To compare the different test designs (ICI and EQUAS), the corresponding Cs of all CMs that had been evaluated as EQUAS were calculated from the participants' results (Suppl. Table 11). The difference between A of the experts and C of the participants was mainly within the range of the standard deviation (SD) of A or C (with two exceptions, marked). The difference between A and C was 13% on average and distinctly higher in the low concentration ranges of A (Suppl. Fig. 16). This demonstrates that the different evaluation schemes resulted in comparable Z-score results and thus enabled an equivalent evaluation of the performance.

3.6. Reported LOQs and corresponding laboratory performance

The reported LOQs of the participants mainly ranged from 0.001 to 2 μg/L (Suppl. Table 12) and the average LOQs over all rounds were lowest for 2-PHEN (0.024 μg/L) and highest for 1-naphthol (0.512 μg/L). The LOQ range of the expert laboratories (0.002–0.78 μg/L) was distinctly smaller than that of the participating laboratories. For both naphthols, for the three FLUO biomarkers and for 3-BaP, a lower mean LOQ over all rounds was reported by the experts (3 rounds) compared to the participants (4 rounds). The average LOQ for 1-PYR was nearly the same for expert laboratories and participants, while the participants reported lower LOQs for the five PHEN biomarkers and 1,2-DHN than the experts over all rounds. During their participation, most participants reported constant LOQs for most or all biomarkers. Twelve laboratories could improve their LOQs for some biomarkers and five participants reported higher LOQs in successive rounds. A comparison of the participants' performance according to their reported LOQs revealed significantly lower Z-scores in the groups with lower LOQs (Suppl. Fig. 17) for the FLUO and the PHEN biomarkers as well as for 1-PYR. For the naphthols, the same tendency was visible but not significant, which might be attributed to the higher spiked concentrations for these biomarkers. Overall, this suggests that a high sensitivity of the PAH biomarker analysis method was particularly beneficial for the detection of FLUOs, PHENs and 1-PYR in urine, which are present in the general population at low concentration ranges.

A total of 14 participants (out of 30 participants with quantitative results in total) were unable to detect one or several biomarkers in the ICIs/EQUASs. Interestingly, the 35 reported LOQ values for the non-quantifiable biomarkers ('<LOQ' results) were significantly lower than the LOQs of participants that were able to report results for all biomarkers for which they participated (Suppl. Fig. 18). This might in part be explained by the fact that many of the '<LOQ' results for the FLUO and PHEN biomarkers were due to the inability of the laboratories to separate the isomers so that only sums of isomers had been reported but could not be evaluated (last column in Suppl. Table 13). 2- and 3-FLUO, 3- and 9-FLUO, 1- and 9-PHEN, 2- and 3-PHEN and 4- and 9-PHEN could not be separated by some of the participants. The highest number of '<LOQ' results was reported for 3-BaP, 9-PHEN and 1-PYR. While 1-PYR, as the first established PAH parameter, was analysed by most (15–26) participants in each round, the number of participants for 3-Ba-P (5–8) and 9-PHEN (4–10) was quite low (see also Suppl. Table 10). Furthermore, 3-BaP and 9-PHEN are among the less stable PAH biomarkers and are likely to be more difficult to calibrate (Gaudreau et al., 2016), which could have contributed to their poorer detection rate. For 1-PYR, participants applying an internal standard in general (Suppl. Fig. 19 D) and in particular an isotope-labelled internal standard (Suppl. Fig. 20 D) reported significantly lower LOQs than participants without this quantification method. Z-scores were significantly lower when an internal standard was used for the detection of both naphthols and of the group 1- to 4-PHEN (Suppl. Fig. 21 A, C). The same tendencies were shown for 2- and 3-FLUO and 1-PYR (Suppl. Fig. 21 B, D). The application of an isotope-labelled internal standard also resulted in significantly lower Z-scores for the naphthols (Suppl. Fig. 22 A) and for 3-PHEN (Suppl. Fig. 22 B). However, an internal standard with isotopic label was also used by most participants who had been unable to detect one or several biomarkers in the CMs. Most participants with undetected biomarkers were not able to improve their performance in follow-up rounds. Only one participant who firstly reported only the sum of 2- and 3-PHEN could manage the separation of both isomers in the following ICIs/EQUASs, but without indicating specific changes in the applied method. This is in accordance with the observed overall good performance of the participants for the group of 1- to 4-PHEN.

4. Conclusions

The HBM4EU QA/QC programme has given an overview of the European inter-laboratory comparability for the analysis of urinary PAH biomarkers at concentration levels relevant for general populations. Over four rounds, two different proficiency test schemes, ICI and EQUAS, were applied and resulted in a comprehensive evaluation of the participants' performance. Initially, 13 PAH metabolites in urine were offered for analysis. However, 1,2-DHN, 3-BaP, 9-PHEN and 9-FLUO could finally not be evaluated as the analytical capacity of participating laboratories was too low. For the nine evaluable biomarkers (1-naphthol, 2-naphthol, 2-FLUO, 3-FLUO, 1- to 4-PHEN and 1-PYR), the overall analytical quality was very high as 86% of participants achieved satisfactory results over all rounds. As expected, rounds with higher spiked concentrations resulted in a better overall performance, although the results for the low concentration range were also mainly satisfactory (mean of 83% over all rounds). Moreover, the study revealed significantly lower absolute Z-scores and a better inter-laboratory reproducibility for the naphthols and the PHEN biomarkers compared to the FLUO biomarkers and 1-PYR. Twenty-three participating laboratories

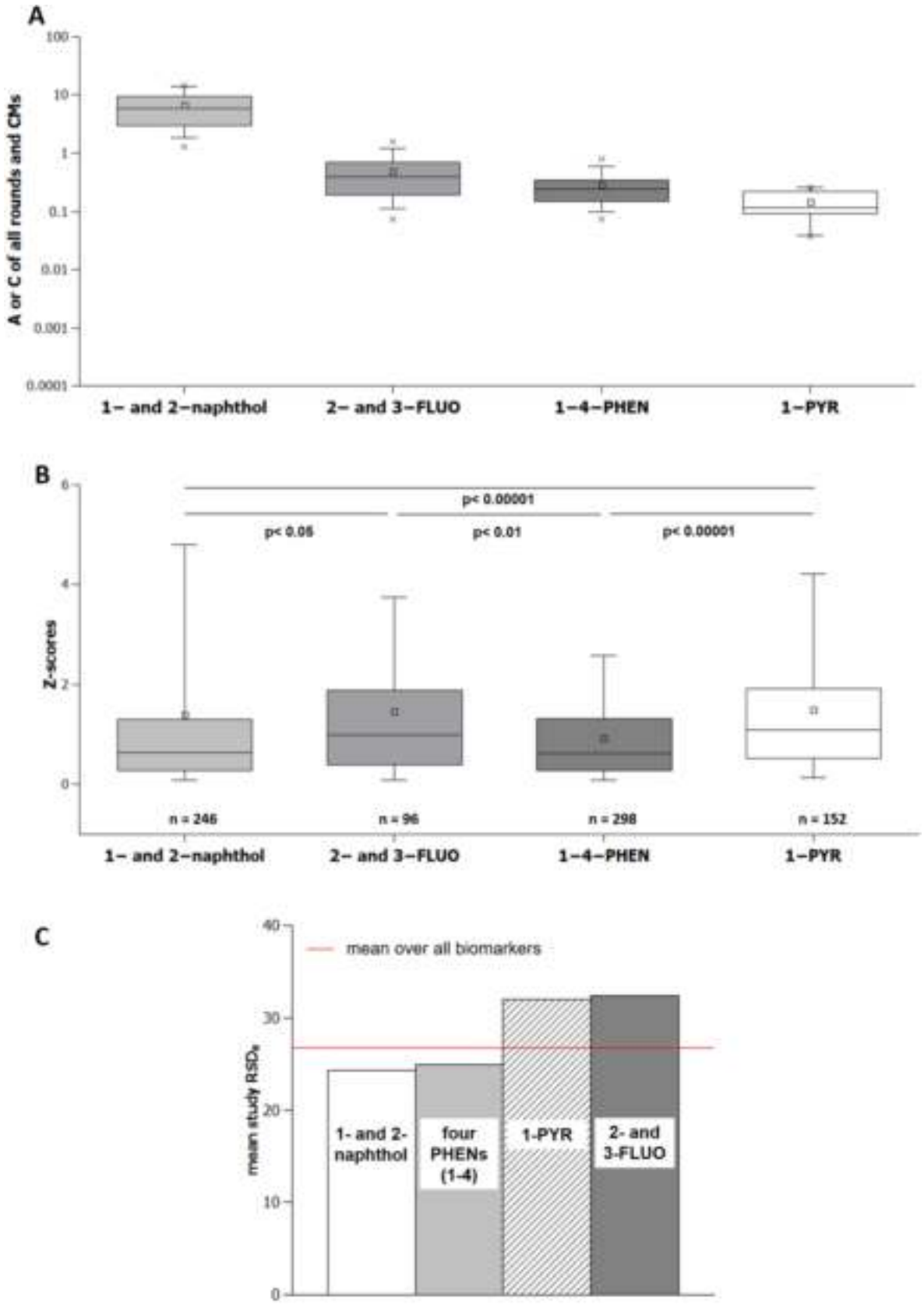


Fig. 2. Comparison of the outcome over all ICI/EQUAS rounds for 4 different PAH biomarker groups: range of C and A in logarithmic scale (A), Z-scores compared with Mann-Whitney *U* test (B) and mean study RSDR values (C).

from 12 European countries obtained successful results for at least one PAH biomarker, 12 of which were successful for at least three biomarkers, mainly for 1-, 2-naphthol and 1-PYR. These biomarkers are best established in practice in contrast to many other PAH biomarkers which still seem to represent a major challenge for some laboratories. Particular difficulties were found, among others, in the separation of the isomers of FLUOs and PHENs, for which the use of GC seemed clearly advantageous. The results of the proficiency tests call for an improvement in analytical performance in some participating laboratories. Nevertheless, this study succeeded in identifying a European network of highly qualified analytical laboratories as basis for joint population studies on urinary PAH biomarkers within the HBM4EU project. To extend the number of analysing laboratories as well as their capacities to analyse more PAH biomarkers with high sensitivity and performance may require a specific training programme.

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Availability of data and materials

All data generated or analysed during this study are included in this article and its supplement.

Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114169.

References

Becker, K., Schulz, C., Kaus, S., Seiwert, M., Seifert, B., 2003. German Environmental Survey 1998 (GerES III): environmental pollutants in the urine of German population. Int. J. Hyg Environ. Health 206, 15–24. https://doi.org/10.1078/1438-4639-00188.

Bartolomé, M., Ramos, J.J., Cutanda, F., Huetos, O., Esteban, M., Ruiz-Moraga, M., Calvo, E., Pérez-Gómez, B., González, O., Bioambient, E.S., Castaño, A., 2015. Urinary polycyclic aromatic hydrocarbon metabolites levels: the BIOAMBIENT.ES project. Chemophere 135, 436–446. https://doi.org/10.1016/j.chemosphere.2014.12.008.

Chetiyanukornkul, T., Toriba, A., Kameda, T., Tang, N., Hayakawa, K., 2006. Simultaneous determination of urinary hydroxylated metabolites of naphthalene, fluorene, phenanthrene, fluoranthene and pyrene as multiple biomarkers of exposure to polycyclic aromatic hydrocarbons. Anal. Bioanal. Chem. 386, 712–718. https://doi.org/10.1007/s00216-006-0628-6.

Danish EPA, 2013. Polyaromatic hydrocarbons (PAH). Evaluation of health hazards and estimation of a quality criterion in soil. Danish Ministry of the Environment. Environmental Project 1523 (2013).

Esteban López, M., Göen, T., Mol, H., Nübler, S., Zarrabi, K., Koch, H.M., Dvorakova, D., Hajslova, J., Antignac, J.P., Vaccher, V., Elbers, I., Thomsen, C., Vorkamp, K., Pedraza-Díaz, S., Kolossa-Gehring, M., Castaño, A., 2021. The European Human Biomonitoring platform - design and implementation of a QA/QC programme for selected priority chemicals. Int. J. Hyg Environ. Health 234, 113740. https://doi.org/10.1016/j.ijheh.2021.113740.

Fearn, T., Thompson, M., 2001. A new test for 'sufficient homogeneity'. Analyst 126, 1414–1417.

Ganzleben, C., Antignac, J.P., Barouki, R., Castaño, A., Fiddicke, U., Klánová, J., Lebret, E., Olea, N., Sarigiannis, D., Schoeters, G.R., Sepai, O., Tolonen, H., Kolossa-Gehring, M., 2017. Human biomonitoring as a tool to support chemicals regulation in the European Union. Int. J. Hyg Environ. Health 220, 94–97. https://doi.org/10.1016/j.ijheh.2017.01.007.

Gaudreau, É., Bérubé, R., Bienvenu, J.F., Fleury, N., 2016. Stability issues in the determination of 19 urinary (free and conjugated) monohydroxy polycyclic aromatic hydrocarbons. Anal. Bioanal. Chem. 408, 4021–4033. https://doi.org/10.1007/s00216-016-9491-2.

Gilles, L., Govarts, E., Rambaud, L., Vogel, N., Castaño, A., Esteban López, M., Rodriguez Martin, L., Koppen, G., Remy, S., Vrijheid, M., Montazeri, P., Birks, L., Sepai, O., Stewart, L., Fiddicke, U., Loots, I., Knudsen, L.E., Kolossa-Gehring, M., Schoeters, G., 2021. HBM4EU combines and harmonises human biomonitoring data across the EU, building on existing capacity - the HBM4EU survey. Int. J. Hyg Environ. Health 237, 113809. https://doi.org/10.1016/j.ijheh.2021.113809.

Göen, T., Gündel, J., Schaller, K.H., Angerer, J., 1995. The elimination of 1-hydroxypyrene in the urine of the general population and workers with different occupational exposure to PAH. Sci. Total Environ. 163, 195–201. https://doi.org/10.1016/0048-9697(95)04484-I.

Göen, T., Schaller, K.H., Drexler, H., 2012. External quality assessment of human biomonitoring in the range of environmental exposure levels. Int. J. Hyg Environ. Health 215 (2), 229–232. https://doi.org/10.1016/j.ijheh.2011.08.012.

Grimmer, G., Dettbarn, G., Jacob, J., 1993. Biomonitoring of polycyclic aromatic hydrocarbons in highly exposed coke plant workers by measurement of urinary phenanthrene and pyrene metabolites (phenols and dihydroydiols). Int. Arch. Occup. Environ. Health 65, 189–199.

Hansen, A.M., Mathiesen, L., Pedersen, M., Knudsen, L.E., 2008. Urinary 1-hydroxypyrene (1-HP) in environmental and occupational studies - a review. Int. J. Hyg Environ. Health 211, 471–503. https://doi.org/10.1016/j.ijheh.2007.09.012.

Hudson-Hanley, B., Smit, E., Branscum, A., Hystad, P., Kile, M.L., 2021. Trends in urinary metabolites of polycyclic aromatic hydrocarbons (PAHs) in the non-smoking U.S. population, NHANES 2001-2014. Chemosphere 276, 130211. https://doi.org/10.1016/j.chemosphere.2021.130211.

IARC, 2010. Some Non-heterocyclic polycyclic aromatic hydrocarbons and some related exposures. In: International Agency for Research on Cancer. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, 92. World Health Organization, Geneva, p. 2010.

Kim, K.H., Jahan, S.A., Kabir, E., Brown, R.J.C., 2013. A review of airborne polycyclic aromatic hydrocarbons (PAHs) and their human health effects. Environ. Int. 60, 71–80. https://doi.org/10.1016/j.envint.2013.07.019.

Klotz, K., Drexler, H., Hartwig, A., MAK Commission, 2018. Naphthalene – BAT value documentation. The MAK Collection for Occupational Health and Safety 3, 860–877. https://doi.org/10.1002/3527600418.bb9120e2218.

Li, Z., Sandau, D.D., Romanoff, L.C., Caudill, S.P., Sjödin, A., Needham, L.L., Patterson Jr., D.G., 2008. Concentration and profile of 22 urinary polycyclic aromatic hydrocarbon metabolites in the US population. Environ. Res. 107, 320–331.

Li, Z., Romanoff, L., Bartell, S., Pittman, E.N., Trinidad, D.A., McClean, M., Webster, T.F., Sjödin, A., 2012. Excretion profiles and half-lives of ten urinary polycyclic aromatic

hydrocarbon metabolites after dietary exposure. Chem. Res. Toxicol. 25, 1452–1461. https://doi.org/10.1021/tx300108e.

Louro, H., Heinälä, M., Bessems, J., Buekers, J., Vermeire, T., Woutersen, M., van Engelen, J., Borges, T., Rousselle, C., Ougier, E., Alvito, P., Martins, C., Assunção, R., Silva, M.J., Pronk, A., Schaddelee-Scholten, B., Del Carmen Gonzalez, M., de Alba, M., Castaño, A., Viegas, S., Humar-Juric, T., Kononenko, L., Lampen, A., Vinggaard, A.M., Schoeters, G., Kolossa-Gehring, M., Santonen, T., 2019. Human biomonitoring in health risk assessment in Europe: current practices and recommendations for the future. Int. J. Hyg Environ. Health 222, 727–737. https://doi.org/10.1016/j.ijheh.2019.05.009.

Nilsson, R., Antic, R., Berni, A., Dallner, G., Dettbarn, G., Gromadzinska, J., Joksic, G., Lundin, C., Palitti, F., Prochazka, G., Rydzynski, K., Segerbäck, D., Soucek, P., Tekle, M., Seidel, A., 2013. Exposure to polycyclic aromatic hydrocarbons in women from Poland, Serbia and Italy – relation between PAH metabolite excretion, DNA damage, diet and genotype (the EU DIEPHY project). Biomarkers 18 (2), 165–173. https://doi.org/10.3109/1354750X.2012.762807.

Nübler, S., Esteban Lopez, M., Mol, H., Schäfer, M., Zarrabi, K., Antignac, J.P., Hajslova, J., Koch, H.M., Thomsen, C., Vorkamp, K., Castaño, A., Göen, T., 2021. Interlaboratory comparison Investigations (ICI) and external quality assurance schemes (EQUAS) for cadmium in urine and blood:results from the HBM4EU project. Int. J. Hyg Environ. Health 234, 113711. https://doi.org/10.1016/j.ijheh.2021.113711.

Nübler, S., Schäfer, M., Haji-Abbas-Zarrabi, K., Marković, S., Marković, K., Esteban López, M., Castaño, A., Mol, H., Koch, H.M., Antignac, J.P., Hajslova, J., Thomsen, C., Vorkamp, K., Göen, T., 2022a. Interlaboratory Comparison Investigations (ICIs) for human biomonitoring of chromium as part of the quality assurance programme under HBM4EU. J. Trace Elem. Med. Biol. 70, 126912 https://doi.org/10.1016/j.jtemb.2021.126912.

Nübler, S., Esteban López, M., Castaño, A., Mol, H., Haji-Abbas-Zarrabi, K., Schäfer, M., Müller, J., Hajslova, J., Dvorakova, D., Antignac, J.P., Koch, H.M., Haug, L.S., Vorkamp, K., Göen, T., 2022b. Interlaboratory Comparison Investigations (ICIs) and External Quality Assurance Schemes (EQUASs) for human biomonitoring of perfluoroalkyl substances (PFASs) in serum as part of the quality assurance programme under HBM4EU. Sci. Total Environ. 847, 157481 https://doi.org/10.1016/j.scitotenv.2022.157481.

Ravindra, K., Sokhi, R., Van Grieken, R., 2008. Atmospheric polycyclic aromatic hydrocarbons: source attribution, emission factors and regulation. Atmos. Environ. 42 (2008), 2895–2921. https://doi.org/10.1016/j.atmosenv.2007.12.010.

Romanoff, L.C., Li, Z., Young, K.J., Blakely III, N.C., Patterson Jr., D.G., Sandau, C.D., 2006. Automated solid-phase extraction method for measuring urinary polycyclic aromatic hydrocarbon metabolites in human biomonitoring using isotope-dilution gas chromatography high-resolution mass spectrometry. J. Chromatogr. B 835, 47–54. https://doi.org/10.1016/j.jchromb.2006.03.004.

StHelen, G., Goniewicz, M.L., Dempsey, D., Wilson, M., Jacob, P., Benowitz, N.L., 2012. Exposure and kinetics of polycyclic aromatic hydrocarbons (PAHs) in cigarette smokers. Chem. Res. Toxicol. 25, 952–964. https://doi.org/10.1021/tx300043k.

Thompson, M., 2000. Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing. Analyst 125, 385–386.

Thompson, M., Ellison, R., Wood, R., 2006. The international harmonized protocol for the proficiency testing of analytical chemistry laboratories. Pure Appl. Chem. 78 (1), 145–196.

Urbanocova, K., Dvorakova, D., Gramblicka, T., Sram, R.J., Hajslova, J., Pulkrabova, J., 2020. Comparison of polycyclic aromatic hydrocarbon metabolite concentrations in urine of mothers and newborns. Sci. Total Environ. 723, 138116 https://doi.org/10.1016/j.scitotenv.2020.138116.

Vandráček, J., Machala, M., 2021. The role of metabolism in toxicity of polycyclic aromatic hydrocarbons and their non-genotoxic modus of action. Curr. Drug Metabol. 22, 584–595. https://doi.org/10.2174/1389200221999201125205725.

Vorkamp, K., Castaño, A., Antignac, J.P., Boada, L.D., Cequier, E., Covaci, A., Esteban López, M., Haug, L.S., Kasper-Sonnenberg, M., Koch, H.M., Pérez Luzardo, O., Osīte, A., Rambaud, L., Pinorini, M.T., Sabbioni, G., Thomsen, C., 2021. Biomarkers, matrices and analytical methods targeting human exposure to chemicals selected for a European human biomonitoring initiative. Environ. Int. 146, 106082 https://doi.org/10.1016/j.envint.2020.106082.

Waldman, J.M., Lioy, P.J., Greenberg, A., Butler, J.P., 1991. Analysis of Human Exposure to Benzo[a]pyrene via Inhalation and Food Ingestion in the Total Human Environmental Exposure Study (THEES), 1. J Expo Anal Environ Epidemiol, pp. 193–225.

Wang, Z., Yang, C., Parrott, J.L., Frank, R.A., Yang, Z., Brown, C.E., Hollebone, B.P., Landriault, M., Fieldhouse, B., Liu, Y., Zhang, G., Hewitt, L.M., 2014. Forensic source differentiation of petrogenic, pyrogenic, and biogenic hydrocarbons in Canadian oil sands environmental samples. J. Hazard Mater. 271, 166–177. https://doi.org/10.1016/j.jhazmat.2014.02.021.

Zobel, M., Göen, T., Belov, V., Klotz, K., 2017. Reliable quantification of 1,2-dihydroxynaphthalene in urine using a conjugated reference compound for calibration. Anal. Bioanal. Chem. 409, 6861–6872. https://doi.org/10.1007/s00216-017-0651-9.

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High prevalence of *Escherichia coli* co-harboring conjugative plasmids with colistin- and carbapenem resistance genes in a wastewater treatment plant in China

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ABSTRACT

Emergence and dissemination of resistance to last-resort antibiotics such as carbapenem and colistin is a growing, global health concern. Wastewater treatment plants (WWTPs) link human activities and the environment, can act as reservoirs and sources for emerging antibiotic resistance, and likely play a large role in antibiotic resistance transmission. The aim of this study was to investigate occurrence and characteristics of colistin- and carbapenem-resistant *Escherichia coli* (CCREC) in wastewater and sludge samples collected over a one-year period from different functional areas of an urban WWTP in Jinan city, Shandong, China. A total of 8 CCREC were isolated from 168 samples with selective agar and PCR, corresponding to high prevalence of 4.8%, co-harboring carbapenem resistance genes (bla_{NDM}) and colistin resistance gene (*mcr-1*) and subsequently whole-genome sequenced. Additionally, all isolates were multidrug-resistant by antimicrobial susceptibility testing and carried a variety of antibiotic resistance genes. Two isolates carrying virulence genes associated with avian pathogenic *E. coli* were identified, one belonging to the high-risk clone O101:H9-ST167. Southern blotting was used to characterize CCREC isolates and plasmids carrying bla_{NDM}-genes or *mcr-1* could be transferred to a recipient strain *E. coli* J53 by *in vitro* conjugation assays. Resistance to other antibiotic classes were sporadically co-transferred to the transconjugant. Transposition of and *mcr-1*-carrying element from a transferable IncHI2-plasmid was observed among two CCREC clones isolated within 4 days of each other. The occurrence of multidrug-resistant CCREC capable of transferring their antibiotic resistance genotypes via conjugative plasmids is alarming. WWTPs bring bacteria from different sources together, providing opportunities for horizontal exchange of DNA among compatible hosts. Further dissemination of the colistin-, carbapenem-, or both colistin- and carbapenem resistant *E. coli* could lead to a serious threat to public health.

1. Introduction

During the last two decades, the number of difficult-to-treat infections on account of multidrug-resistant and extended spectrum β-lactamase-(ESBL)-producing Enterobacterales has rised to a remarkable degree, which has resulted in an increased usage of the antibiotics carbapenems and colistin (Peyclit et al., 2019). As last-resort antibiotic therapies, carbapenems play a crucial part in the treatment of these infections whereas for carbapenem-resistant Enterobacterales (CRE) infections, colistin is the last-resort antibiotic (Oliveira et al., 2020). Carbapenems and colistin are categorized as critically important drugs and so the emergence of resistance to these antibiotics is a serious public health concern (WHO, 2019). In 2015, in China, a plasmid-mediated colistin resistance gene, *mcr-1*, was first identified in *E. coli* isolated from a pig (Liu et al., 2016). Shortly after, the gene was reported among numerous bacterial species in different ecological niches in more than 70 countries on 5 continents (Nang et al., 2019). In China, the widespread dissemination of *mcr*-1 has been attributed to selective pressure generated by colistin used in animal husbandry, which promotes the spread of *mcr-1*-carrying plasmids (Shen et al., 2016). Therefore, the use of colistin as a feed additive was banned from May 1, 2017, in China, reducing its annual use by more than 8000 tons (Walsh and Wu, 2016). Nevertheless, misuse and overuse of colistin still occurs.

Colistin- and carbapenem-resistant *Escherichia coli* (CCREC) co-

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producing *mcr-1* and carbapenem resistance genes (such as bla_{NDM}-genes) have been increasingly reported among isolates from animals, humans, and the environment (Bilal et al., 2021; Feng et al., 2021; Nukui et al., 2019; Wang et al., 2021). Infections caused by such strains are also increasing (Bao et al., 2022; Bilal et al., 2021; Hameed et al., 2021; Lin et al., 2020; Yan et al., 2021). These infections constitute a major therapeutic challenge. As CCREC are often multidrug-resistant besides, no standardized treatment strategies are available.

Wastewater treatment plants (WWTPs) link human activities and the environment and can act as reservoirs and sources from which antibiotic resistance emergence (Guo et al., 2017; Wright, 2019). As WWTPs collect and treat municipal, domestic, hospital, and slaughterhouse wastewater, concentrated populations of microbes from diverse sources coupled with diverse selection pressures exerted from compounds in waste from different origins (e.g., antibiotic pollution from hospital waste) create favorable conditions for the transfer of antibiotic resistance genes (ARGs) and proliferation of antibiotic-resistant bacteria (ARB) (Magiorakos et al., 2012). WWTPs reduce the number of bacteria, but do not eliminate them entirely, so ARGs, ARB, and potential pathogens may disseminate into the environment via the outlet water or sludge, and subsequently contact with bacteria in the environment, poultry and humans (Beattie et al., 2020; Nguyen et al., 2021).

Therefore, it is crucial to monitor WWTPs for the detection of colistin- and carbapenem-resistant *Escherichia coli*. The objective of this study was to explore the occurrence of CCREC in sewage and sludge samples from seven functional units of a large WWTP in Eastern China, during the different seasons of 2018, and characterize isolates of CCREC in terms of antimicrobial susceptibility, ARGs, virulence genes and sequence type. Additionally, the mobile genetic elements on which the CCREC carry their colistin- and carbapenem resistance genes were also the focus of our study.

2. Materials and methods

2.1. Sample collection

This sampling was carried out from February to November in 2018 in a WWTP in Eastern China. The WWTP is located in the north of Jinan city, Shandong province, and mainly receives municipal wastewater, domestic sewage, live poultry market sewage and hospital wastewater. It has a maximum treatment capacity of 3×10^5 m^3 per day and treats wastewater through an anaerobic/anoxic/oxic (A/A/O) process. Our seasonal sampling of this large WWTP was conducted during the winter (February 1, February 5), spring (May 24, May 28), summer (August 16, August 21), and autumn (November 22, November 26) of 2018. The sampling sites consisted of seven different functional areas: the water inlet, the anaerobic tank, the aerobic tank, the activated sludge, the sludge thickener, the water outlet and the mud cake storage area (Fig. 1), and the samples were taken at 8 a.m., 2 p.m., and 6 p.m. throughout one day. A total volume of 1 L of mixed sewage and sludge samples were collected in each functional unit. No sludge sample was collected in the outlet, and no sewage sample was collected in the mud cake storage area. Totally, 168 samples were collected in this study.

2.2. Screening for colistin- and carbapenem-resistant E. coli

The sample processing method is described in a previous study (Zou et al., 2022). To isolate putative CCREC, 100 μL of the culture solutions were spread on MacConkey agar containing 2 mg/L meropenem and 2 mg/L colistin. Isolates identified as *E. coli* by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker, Bremen, Germany) were selected for further analysis and subsequently screened for carriage of carbapenem resistance genes (bla_{NDM}, bla_{KPC}, bla_{IMP}, bla_{OXA} and bla_{VIM}-genes) by using PCR followed by DNA sequencing (Biosune, Shanghai, China) (Poirel et al., 2011). The isolates were also screened by multiplex PCR, as previously described (Rebelo et al., 2018), to detect the colistin resistance genes *mcr-1, mcr-2, mcr-3, mcr-4*, and *mcr-5*.

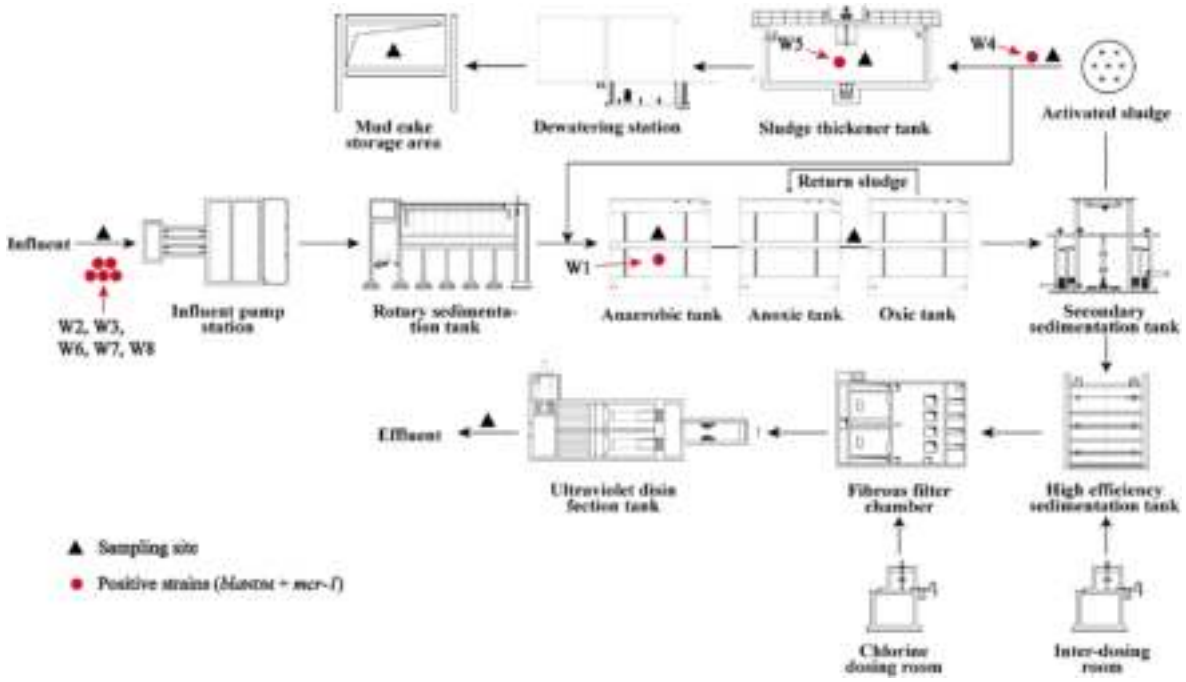

Fig. 1. Treatment process flow chart of the study wastewater treatment plant and sampling sites (black triangles). Sampling sites positive for carbapenem- and colistin-resistant *E. coli* (CCREC) are denoted by red circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.3. Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MIC) of putative CCREC were determined by using agar dilution according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (https://clsi.org) for imipenem (IPM), meropenem (MEM), piperacillin/tazobactam (PTZ), ampicillin (AMP), cefotaxime (CTX), cefoxitin (FOX), trimethoprim/sulfamethoxazole (SXT), fosfomycin (FOS), gentamicin (GEN), amikacin (AMK), tetracycline (TET), and ciprofloxacin (CIP), and interpreted by using the CLSI clinical breakpoints (CLSI 2022), whereas MIC determinations for tigecycline (TGC) and colistin (CL) were used broth microdilution, and interpreted by using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (EUCAST, 2022) (available at http://www.uscast.org/clinical_breakpoints/). In the antimicrobial susceptibility testing, *E. coli* ATCC 25922 was used as a quality control strain.

2.4. Plasmid characterization and conjugation assays

The sizes of plasmid carrying carbapenem and colistin resistance genes were determined by using S1-nuclease pulsed-field gel electrophoresis (PFGE) and Southern blot method (Ji et al., 2019; Zhao et al., 2021). The transferability of plasmid with colistin and carbapenem resistance genes was performed by using standard conjugation (Ngbede et al., 2021). The recipient strain was *E. coli* J53 (sodium azide-resistant). Transconjugants were cultivated on LB agar containing with meropenem (2 mg/L) and sodium azide (150 mg/L), colistin (2 mg/L) and sodium azide (150 mg/L), or colistin (2 mg/L), meropenem (2 mg/L) and sodium azide (150 mg/L). Transconjugants were verified by using PCR and MICs were determined as described above.

2.5. Whole-genome sequencing and bioinformatic analysis

Genomic DNA was extracted and purified by using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany), and then sequenced by using the Illumina NovaSeq 6000 System (Illumina, San Diego, USA), assembled by using SPAdes 3.11 (http://cab.spbu.ru/software/spades/) and annotated via PATRIC (https://www.patricbrc.org/). Multilocus sequence typing (MLST), *E. coli* serotype, antibiotic resistance genes, virulence genes, and plasmid incompatibility groups were analyzed by using online tools available at the Center for Genomic Epidemiology (www.genomicepidemiology.org). Single nucleotide polymorphism (SNP) analysis was based on CSIPhylogeny (https://cge.food.dtu.dk/services/CSIPhylogeny/) and clonal relationship was defined as isolates differing by $\leq$ 10 SNPs (Schurch et al., 2018). Genome data was uploaded to the BacWGSTdb webserver (http://bacdb.cn/BacWGSTdb/analysis_single.php), and 9 strains differences from strains in this study in less than 500 SNPs were screened out. The genetic environments of bla_{NDM}-genes and *mcr-1* were annotated by using Easyfig 2.2.3. In the BioProject database, accession: PRJNA834566 (W1-W8 respectively correspond to Library ID W2-W9 in the database), the datasets generated were deposited.

3. Results

3.1. Detection of colistin- and carbapenem-resistant E. coli

A total of 168 samples were collected from the study WWTP and screened for CCREC. Eight isolates of CCREC (W1-W8) were verified to be colistin-and carbapenem-resistant by MIC determination and to carry colistin- and carbapenem resistance genes. Among the tested functional units in the WWTP, the largest number of CCREC isolates were detected in the water inlet (n = 5). One isolate each was detected in the anaerobic tank, sludge thickening tank, and activated sludge. No isolate was detected from the aerobic tank, water outlet or the mud cake storage area. There was no observable variation in the detection rate of CCREC during winter (n = 2), spring (n = 2), summer (n = 2) and autumn (n = 2).

3.2. Antimicrobial susceptibility testing

All isolated CCREC were multidrug-resistant (Magiorakos et al., 2012) and resistant to colistin, meropenem, imipenem, piperacillin-tazobactam, ampicillin, cefotaxime, cefoxitin, trimethoprim-sulfamethoxazole, fosfomycin, gentamicin, and tetracycline (Table 1). Four and seven isolates were resistant to amikacin and ciprofloxacin, respectively. All were susceptible to tigecycline. The MICs of colistin were 4 mg/L for most isolates (n = 7), however, isolate W4 exhibited an MIC of 32 mg/L.

3.3. Whole-genome sequencing and ARGs

The ARGs of the CCREC isolates are presented in Fig. 2. The sequenced isolates harbored a plethora of ARGs encoding resistance to drugs of various antibiotic classes. All isolates carried carbapenem resistance genes and *mcr-1* (as per the inclusion criteria). The most common carbapenemase resistance gene was bla_{NDM-5}, which was carried by eight isolates. One isolate (W7) carried bla_{NDM-4}. In addition, bla_{CTX-M} genes were very commonly carried by isolates (5/8), including $bla_{CTX-M-14}$ (W5, W7, W8), $bla_{CTX-M-15}$ (W8), and $bla_{CTX-M-65}$ (W3, W4). Genes encoding high-level aminoglycosides resistance (*armA* and *rmtB*) were carried by W3, W4 and W6.

Genetic environment analysis revealed that the bla_{NDM-5}-genes among W2, W3, W4 and W5 were associated with IS*5* and the bleomycin resistance gene *ble*MBL, directly located at the downstream of bla_{NDM-5} (IS*5*-bla_{NDM-5}-*ble*MBL). The bla_{NDM-5}-genes of other three isolates and bla_{NDM-4} of W7 were only adjacent to *ble*MBL (Fig. 3a). Fig. 3b shows the genetic context of *mcr-1* among the CCREC isolates. A genetic motif consisting of *mcr-1* adjacent to *pap2* was observed among all isolates. For isolate W1 and W2, *mcr-1* were flanked upstream or downstream by IS*Apl1*. Notably, in isolates W3 and W4, *mcr-1* was located on identical (100% shared nucleotide identity) 141,135 bp long contigs.

3.4. Strain typing and genetic diversity

In silico serotyping of the CCREC isolates revealed 4 serotypes among the isolates; O51:H10 (W2), O101:H21 (W3, W4), O101:H9 (W6, W8), and O9:H4 (W5). Isolate W1 and W7 were typed as H25 and H10, respectively, with the O-serotype getting no hit in the typing scheme (Table 2). MLST analysis showed that the isolates belonged to six different sequence types (STs) (Table 2): ST156 (W1), ST181 (W2), ST1284 (W3, W4), ST46 (W5), ST167 (W6), and ST617 (W7, W8).

According to the SNP analysis, isolates W3 and W4 (ST1284) differed by 6 SNPs (Fig. 4). W3 was isolated from the inlet sample collected at 2:00 p.m. on May 24, 2018, and W4 was isolated from the activated sludge tank sample at 8:00 a.m. on May 28, 2018. Metadata of the 9 genomes from BacWGSTdb associated with the genomes of CCREC in our study are shown in Table S2. The closest related strain to W7 (differing by 83 SNPs) was YMC_2017_02_MS631 (GenBank: SBHK01), which was isolated in 2017 from stool of a patient in Seoul, South Korea (Fig. 4). W6 was closely related to three isolates from patients (differing by 73–90 SNPs) in Sichuan Province in China, four isolates from patients (differing by 65–66 SNPs) in Zhejiang Province in China, and one isolate from chicken in Shandong Province in China (differing by 66 SNPs). W6 was homologous (differing by 90 SNPs) to SCNJ06, which was found in sputum from a cancer patient in Sichuan in October 2018. Unlike the above-mentioned strains, SCNJ06 collected after the isolation of W6.

3.5. Plasmid diversity and transferability

S1-PFGE and Southern hybridization revealed that bla_{NDM-5} was located on IncX3 plasmids (~46 kb) except W6, W7 and W8 (Fig. S1)

Table 1
Antimicrobial susceptibility profiles of carbapenem- and colistin-resistant *E. coli* isolated from a large WWTP in Shandong Province in 2018.

Isolate	Source	Time	Minimal inhibitory concentration (MIC mg/L)													
			PTZ	AMP	CTX	FOX	IMP	MEM	SXT	FOS	GEN	AMK	TGC	TET	CIP	CL
W1	Anaerobic tank	Feb. 1st- 6 p.m.	**>128/4**	**>256**	**>128**	**>512**	**>32**	**>32**	**8/152**	**>512**	**>128**	4	0.25	**32**	**8**	**4**
W2	Inlet	Feb. 5th- 8 a.m.	**>128/4**	**>256**	**>128**	**>512**	**>32**	**>32**	**8/152**	**512**	**64**	2	0.125	**16**	**>32**	**4**
W3	Inlet	May 24th- 2 p.m.	**>128/4**	**>256**	**>128**	**>512**	**>32**	**>32**	**8/152**	**512**	**>128**	**>256**	0.25	**32**	**>32**	**4**
W4	Activated sludge	May 28th- 8 a.m.	**>128/4**	**>256**	**>128**	**>512**	**>32**	**>32**	**8/152**	**>512**	**>128**	**>256**	0.25	**32**	**>32**	**32**
W5	Sludge thickening tank	Aug. 21st- 6 p.m.	**>128/4**	**>256**	**128**	**512**	**16**	**16**	**8/152**	**>512**	**32**	8	0.5	**32**	0.5	**4**
W6	Inlet	Aug. 21st- 2 p.m.	**>128/4**	**>256**	**>128**	**>512**	**32**	**32**	**8/152**	**>512**	**>128**	**>256**	1	**32**	**>32**	**4**
W7	Inlet	Nov. 22nd- 6 p.m.	**>128/4**	**>256**	**>128**	**>512**	**>32**	**>32**	**8/152**	**>512**	**>128**	**>256**	1	**32**	**>32**	**4**
W8	Inlet	Nov. 26th- 2 p.m.	**>128/4**	**>256**	**>128**	**>512**	**>32**	**32**	**8/152**	**>512**	**>128**	8	0.25	**16**	**>32**	**4**
ATCC 25922			1/4	4	0.06	4	0.06	0.03	0.25/4.75	2	0.5	2	0.125	2	0.01	1

MICs in bold indicated resistant isolates.
PTZ, piperacillin/tazobactam; AMP, ampicillin; CTX, cefotaxime; FOX, cefoxitin; IMP, imipenem; MEM, meropenem; SXT, trimethoprim/sulfamethoxazole; FOS, fosfomycin; GEN, gentamicin; AMK, amikacin; TGC, tigecycline; TET, tetracycline; CIP, ciprofloxacin; CL, colistin.

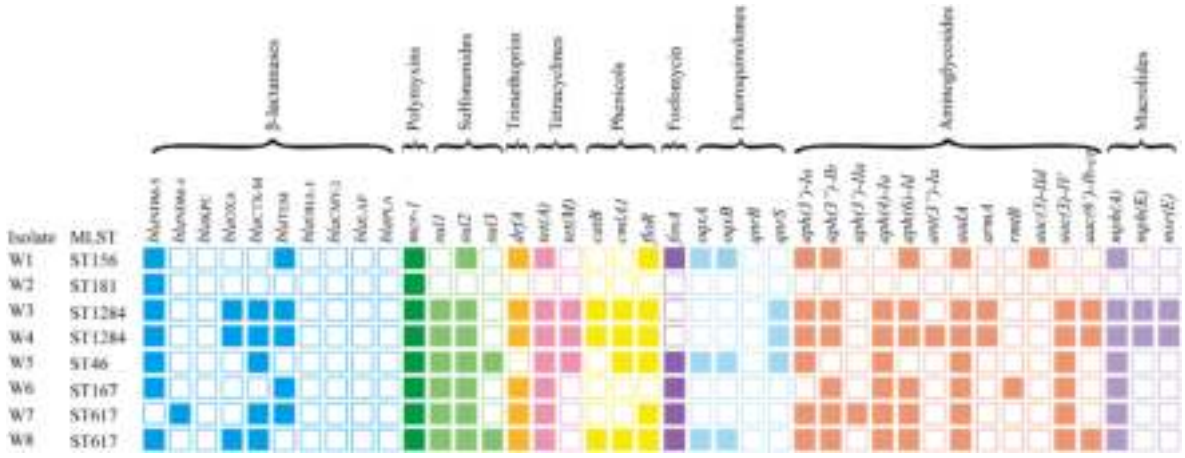

Fig. 2. Antibiotic resistance genes and multilocus sequence typing (MLST) of carbapenem- and colistin-resistant *E. coli* isolated from a large WWTP in Shandong Province in 2018. Filled squares indicate carriage of genes.

(Table 2). bla_{NDM-5} in W8 was determined to be located on an IncHI2 plasmid (~274 kb) whereas for W6, the bla_{NDM-5} was located on a plasmid (~150 kb), of which the replicon type could not be determined. bla_{NDM-4} in W7 could not be located to any plasmid by S1-PFGE, Southern hybridization or sequence analysis (Fig. S3). S1-PFGE and Southern hybridization revealed that *mcr-1* was located on plasmids in six isolates. The gene was carried on p0111-type plasmids in W2, on IncHI2 plasmids in W3 and W5, and on approximately 100 kb or 120 kb large plasmids, which could not be typed, in W1 or W6 and W8 (Fig. S2).

The conjugation experiments demonstrated that all identified bla_{NDM-5}-carrying plasmids and all identified *mcr-1*-carrying plasmids all could be successfully transferred to the recipient *E. coli* J53 according to plasmid conjugation experiments (Table 3). bla_{NDM-4} of W7, and *mcr-1* of W4, and W7 could not be transferred to *E. coli* J53. When using agar plates selective for both carbapenem and colistin resistance to isolates transconjugants, all isolates capable of transferring the *mcr-1*-carrying plasmid also transferred the carbapenemase gene-carrying plasmid, conferring both the colistin and carbapenem resistance phenotype to the transconjugants. Furthermore, resistance to all other antibiotics for which the donor strains were resistant to, were sporadically transferred along with the colistin and carbapenem resistance genes to the transconjugants in the conjugation experiments.

3.6. Virulence genes

Virulence genes detected among the CCREC isolates are presented in Table 2. Notably, one isolate (W1) carried the gene encoding, *astA*, which encodes the heat-stable enterotoxin 1 and is associated with enteroaggregative *E. coli*. Two isolates (W1 and W6) carried the genes *hlyF, iroN, iss, ompT* and *iutA*, which are associated with avian pathogenic *E. coli* (APEC).

4. Discussion

Carbapenems are last-resort antibiotics used to treat infections caused by multidrug-resistant and ESBL-producing Enterobacterales whereas colistin is a last-resort antibiotic used against carbapenem- and multidrug-resistant Enterobacterales. Infections caused by such strains are often very difficult to treat due to their antibiotic resistance profiles. Their increasing prevalence globally, due to the dissemination of carbapenem and colistin resistance genes, accordingly represents a serious public health concern (Serwecińska, 2020). CCREC carrying

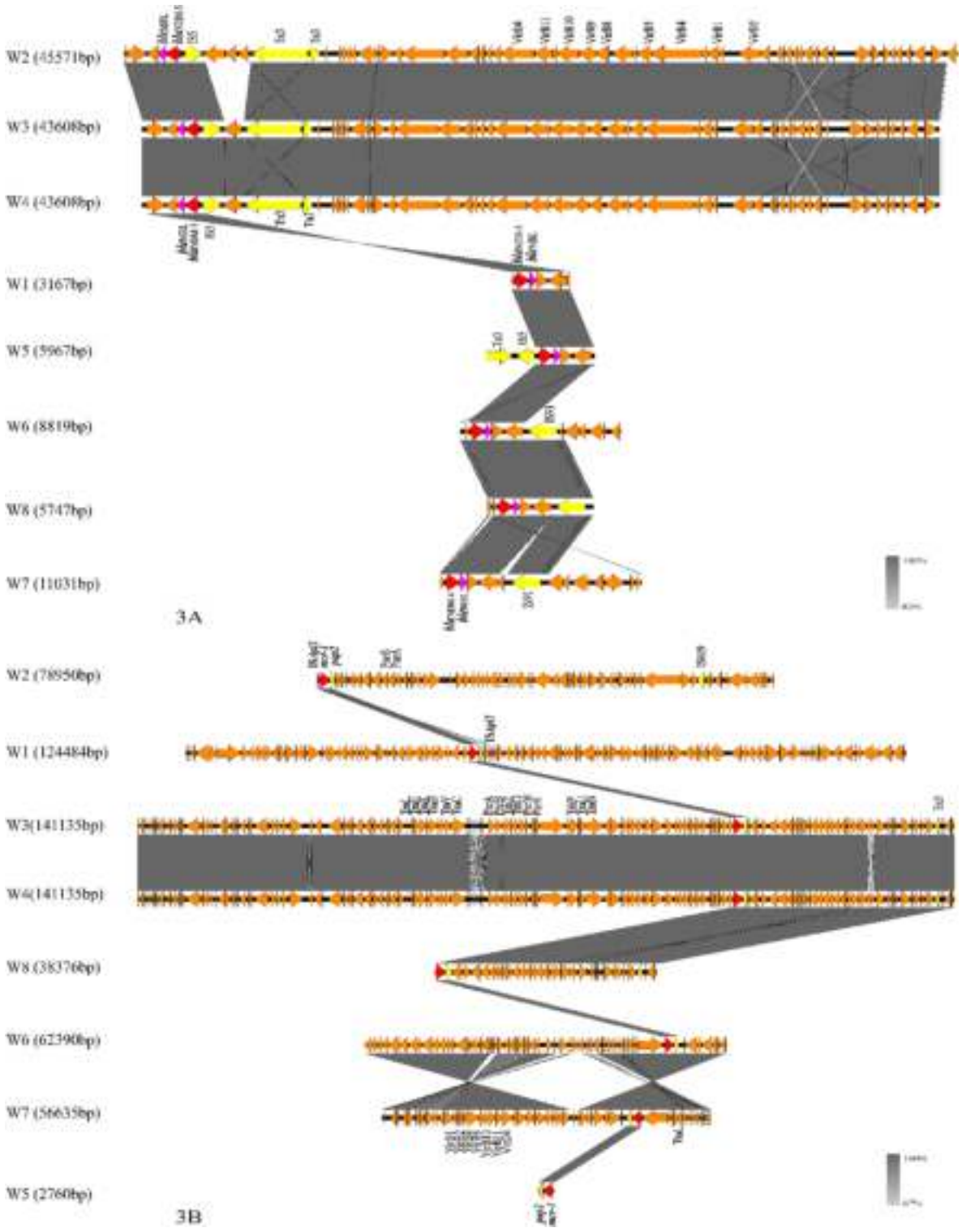

Fig. 3. Genetic environment of bla_{NDM} (3 A) and *mcr-1* (3 B) carried by carbapenem- and colistin-resistant *E. coli* isolated from a large WWTP in Eastern China in 2018. The arrow direction indicates the direction of transcription. Shared regions with high sequence similarity are shown in gray. Red arrows indicate antibiotic resistance genes; Yellow arrows indicate mobile genetic factors and orange arrows indicate other genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

carbapenem resistance genes and *mcr-1* are still fairly rare in clinical contexts but are sporadically found in Southeast Asian countries such as China (Zheng et al., 2016) and Vietnam (Berglund et al., 2017). The prevalence of CCREC from non-clinical sources has also been reported as correspondingly low. Shen et al. (2018) screened 3859 stool specimens collected from healthy volunteers from 19 provinces in China and could isolate CCREC carrying bla_{NDM}-genes and *mcr-1* from 0.36% (n = 14) of the samples whereas Liu et al. (2019) found a similar prevalence of 0.23% (n = 5) among 2147 food samples collected from Shenzhen, China. Sporadic findings of CCREC carrying carbapenemase genes have also been reported in low numbers from other sources such as animal feces from a large animal breeding area in northern China (Gu et al.,

Table 2
Sequence and serotypes of carbapenem- and colistin-resistant *E. coli* isolated in 2018 form sewage or sludge from a wastewater treatment plant in Eastern China, and profiles of plasmids containing carbapenem resistance genes and *mcr-1* genes, carried by the isolates.

Isolate	MLST	Serotype	Replicon type of bla_{NDM}-carrying plasmid	Replicon type of *mcr-1*-carrying plasmid	Carbapenem resistance genes	Plasmid profile	Virulence genes
W1	ST156	H25[b]	IncX3[a] (~46 kb)	N.D.[a] (~100 kb)	bla_{NDM-5}	IncFIB, IncFIC(FII), IncX3, IncY	*astA, cma, cvaC, gad, hlyF, iroN, iss, iucC, iutA, lpfA, ompT, papC, sitA, terC, traT*
W2	ST181	O51:H10	IncX3[a] (~46 kb)	p0111[a] (~98 kb)	bla_{NDM-5}	IncI1-I(α), IncX3, Col440I, p0111	*cia, fyuA, gad, irp2, terC*
W3	ST1284	O101:H21	IncX3[a] (~46 kb)	IncHI2[a] (~274 kb)	bla_{NDM-5}	IncFIB, IncHI2, IncHI2A, IncN, IncX3, IncY	*capU, fyuA, gad, irp2, iss, terC, traT*
W4	ST1284	O101:H21	IncX3[a] (~46 kb)	–	bla_{NDM-5}	IncFIB, IncHI2, IncHI2A, IncN, IncX3, IncY	*capU, fyuA, gad, irp2, iss, terC, traT*
W5	ST46	O9:H4	IncX3[a] (~46 kb)	IncHI2[a] (~274 kb)	bla_{NDM-5}	IncFIB(K), IncFII, IncHI2, IncHI2A, IncN, IncX3	*cma, gad, ompT, sitA, terC*
W6	ST167	O101:H9	N.D.[a] (~150 kb)	N.D.[a] (~120 kb)	bla_{NDM-5}	IncFIB, IncFIC(FII), IncI2(Δ)	*capU, cma, fyuA, gad, hlyF, hra, iroN, irp2, iss, iucC, iutA, ompT, sitA, terC, traT*
W7	ST617	H10[b]	–	–	bla_{NDM-4}	IncFIB, IncFIC(FII), IncN, IncI1-I(α), IncI2(Δ), IncX1, IncX4, Col156	*cma, cvaC, gad, hra, iss, iucC, iutA, papA_F19, sitA, terC, traT*
W8	ST617	O101:H9	IncHI2[a] (~274 kb)	N.D.[a] (~120 kb)	bla_{NDM-5}	IncFIA, IncFIB, IncFII, IncHI2, IncHI2A, IncN, Col440I	*fyuA, gad, hra, irp2, iss, iucC, iutA, sitA, terC, traT*

-: bla_{NDM} and *mcr-1* could not be located to plasmids.
N.D.: non-determinable; the size of the bla_{NDM}-carrying or *mcr-1*-carrying plasmid could be determined by Southern hybridization, but the corresponding plasmid replicon type could not be determined.
[a] These plasmids were able to transfer to the recipient strain in the conjugation experiments.
[b] The O-serotype of this isolate could not be determined.

2022), and from fresh leaf rape and spinach in China (Liu and Song, 2019). However, occurrence of CCREC in wastewater have rarely been reported; Han et al. (2020) isolated an *E. coli* strain carrying both *mcr-1* and bla_{NDM-5} in 2017 from wastewater from a WWTP treating waste from pharmaceutical factory in Zhejiang Province, China. In our study, CCREC were screened for and isolated from samples collected from a WWTP in the north of Jinan city, Shandong province, Eastern China in 2018. A considerably large portion of the samples, 8 out of 168 samples (4.8%) were positive for CCREC carrying *mcr-1* and could indicate an increasing occurrence of these strains.

WWTPs are often considered to be potential reservoirs of ARB and ARGs, while also providing the ideal opportunity for ARB and wild type strains from different sources to exchange genetic material (Guo et al., 2017; Wright, 2019). Aside from antibiotics, as stressors in the WWTP environment, environmental contaminants including metals and biocides can exert selective pressure contributing to persistence and enrichment of ARGs, as genes encoding resistance to these compounds may be co-located on plasmids carrying ARGs (Li et al., 2017; Pal et al., 2015; Ju et al., 2019). The effluent of WWTPs can thus act as an important source of antibiotics, ARB, and ARG contamination to the wider environment, as effluent can be discharged into surface water or recycled, either of which could lead to exposure via different routes (e.g., ground water). Aside from the influent water, CCREC were also isolated from samples collected from the anaerobic tank, sludge thickening tank, and activated sludge. Sludge is a potential source from which ARGs can disseminate, as excess sludge is often used as fertilizer, thus providing bacteria captured in the sludge matrix an opportunity to transmit into the surrounding environment (Sabri et al., 2020). Crops cultivated from sludge can also be contaminated by ARB and provide a dissemination route to humans directly through consumption or indirectly by transmission via domesticated animals feeding on the produce.

CCREC isolated in the WWTP in the current study were related to strains previously isolated from clinical and animal sources, from Zhejiang, Sichuan, and Shandong provinces in China, and South Korea, clearly illustrating the wide dissemination of these strains. These isolates were carbapenem-resistant, and two of them (YMC_2017_02_MS631 and L743) also carried the *mcr-1* gene. Interestingly, isolates W3 (from inlet) and W4 (from activated sludge) were collected within 4 days of each other and were found to be clonally related with only 6 SNPs difference between each other. Both isolates shared an identical contig on which *mcr-1* was located. Nonetheless, whereas W3 was shown to carry *mcr-1* on a transferable IncHI2-plasmid, *mcr-1* in W4 could not be located to a plasmid and could not transferred to the recipient strain. W4 was isolated 4 days after W3, suggesting that the *mcr-1*-carrying contig in W3 may have undergone a transposition event and migrated to another, non-transferable plasmid, or even to the chromosome, while in the WWTP. This scenario illustrates that the WWTP can serve as a hub in which mobile genetic elements can undergo rearrangements and transpositions, potentially resulting in improved transferability to a diverse array of plasmids and bacteria to transfer to.

All isolates were resistant to the tested carbapenems imipenem and meropenem, colistin, trimethoprim-sulfamethoxazole, fosfomycin, gentamicin and tetracycline. The resistance prevalence was also high for ciprofloxacin (87.5%) whereas all isolates were susceptible to tigecycline. Notably, four isolates were highly resistant to amikacin (MICs >256 mg/L), which for isolates W3, W4 and W6 could be attributed to carriage of the 16 S rRNA methylase genes *armA* and *rmtB*. Interestingly, these three isolates managed to confer amikacin resistance to the transconjugants in the conjugation experiments carried out for the carbapenem resistance genes and *mcr-1*, indicating that these genes are also carried on highly transmissible plasmids which can be co-transferred with the plasmids encoding carbapenem and colistin resistance. Overall, the antibiotic resistance profiles of the CCREC analyzed in this study are worrisome and indicate that very few therapeutic options exist for treating infections caused by these strains (Xie et al., 2022; Zhou et al., 2019). Although all isolates were susceptible to tigecycline, this drug is generally not considered to be useful for urinary tract infections and is restricted for use in young children (European Medicines Agency, 2021).

In this study, bla_{NDM-5} was the most prevalent carbapenem resistance gene among CCREC. It was carried by seven out of eight isolates, and among five of these, the gene was carried on IncX3 plasmids. Different genera of Enterobacteriaceae carrying IncX3 plasmids harboring bla_{NDM-5} have been reported from clinical and environmental samples in different geographical regions of China, as well as (Liu et al., 2019). For example, in Zhejiang Province, bla_{NDM-5} has been found to be transferred between *E. coli* and *Citrobacter freundii* strains by the same IncX3 plasmid

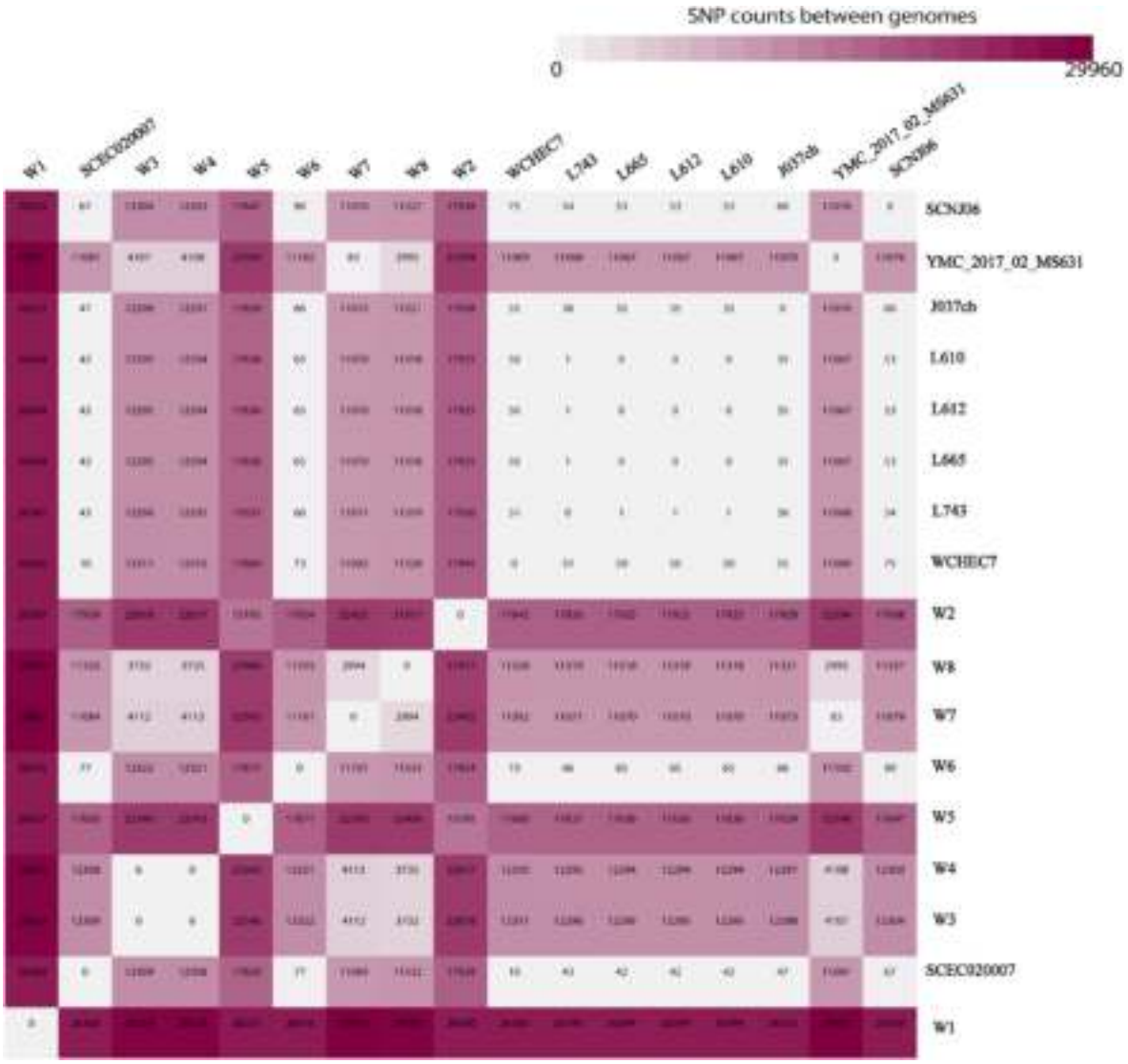

Fig. 4. The distance matrix of phylogenetic analysis of 8 strains in this study compared with closely related strains based on single nucleotide polymorphisms (SNPs). The darker the box, the larger the SNPs that differ between the two genomes. SNP values less than 10 indicate that the two strains are clones. The genome sequence of *E. coli* K-12 MG1655 (NC_000913.3) was used as a reference.

in several hospitals (Li et al., 2018). In this study, one CCREC isolate was found to carry $bla_{\text{NDM-5}}$ on an IncHI2 plasmid. IncHI2 plasmids carrying $bla_{\text{NDM-5}}$ have also been reported to be widely spread. For example, isolates of *E. coli* carrying $bla_{\text{NDM-5}}$ on these types of plasmids were recovered from pigs in Guangdong, China (Ma et al., 2021). *E. coli* harboring IncHI2 plasmids carrying $bla_{\text{NDM-5}}$ have also been isolated from the influent of a WWTP in Seoul, South Korea (Shin et al., 2021). In this study, genetic environment analysis showed that most isolates contained a conserved motif around $bla_{\text{NDM-5}}$, consisting of IS*5*-$bla_{\text{NDM-5}}$-*ble*MBL (Fig. 3a). This motif has also been reported from other sources, including among $bla_{\text{NDM-5}}$-positive *E. coli* isolated from a hospital in Eastern China (Sun et al., 2019), among *Morganella morganii* isolated from patients with liver cancer in Zhejiang Province (Guo et al., 2019), and *Klebsiella pneumoniae* and *E. coli* isolated from a teaching hospital in Shanghai (Zhang et al., 2016). The common features of the plasmids carrying $bla_{\text{NDM-5}}$ in this study with plasmids found to have disseminated the gene across different regions in China speak to their high transmissibility. This was further verified by the conjugation experiments which showed that all CCREC isolates could transfer their $bla_{\text{NDM-5}}$-carrying plasmid to the recipient strain, yielding the carbapenem resistance phenotype in the process (Table 3).

E. coli carrying *mcr-1* on IncHI2 plasmids have been isolated from various animal and clinical sources as well as from retail ready-to-eat food (Zhang et al., 2021), and in swine in Northeastern China (Cheng et al., 2021). Accordingly, these plasmids are likely to have the potential to spread via *E. coli* transmitted to humans through the food chain or further disseminate to other bacteria through horizontal transmission. *mcr-1* of isolate W2 was carried on p0111-type plasmids. p0111-plasmids carrying *mcr-1* have also been detected among *Escherichia* spp. isolated from chicken in Guangdong Province (Li et al., 2020), in *E. coli* originating from aquaculture products imported from Vietnam to the Czech Republic (Kalova et al., 2021) and Enterobacterales from patients with intra-abdominal infections in Zhejiang Province (Jiang et al., 2020).

An open reading frame including the motif *mcr-1-pap2* was present among all isolates in this study (Fig. 3b). The presence of these genetic characteristics in plasmid- or chromosomally mediated *mcr-1* in *E. coli* is common and have been frequently reported from various environmental sources. For example, chromosomally mediated *mcr-1* in *E. coli* carried *mcr-1-pap2* had high prevalence in healthy residents in Vietnam (Yamaguchi et al., 2020). Such *mcr-1-pap2* structures have also been found among *Salmonella* spp. and *E. coli* from retail food products, and

Table 3
Antimicrobial susceptibility profiles of transconjugants of recipient strain *E. coli* J53 with donors W1-W8. W1-W8 were selected on medium containing sodium azide with either meropenem (M1-M8), colistin (C1-C8) or both meropenem and colistin (M1+C1-.

Isolate	Minimal inhibitory concentration (mg/L)													
	PTZ	AMP	CTX	FOX	IMP	MEM	SXT	FOS	GEN	AMK	TGC	TET	CIP	CL
M1	**>128/4**	**256**	**>128**	**>512**	**>32**	**32**	**8/152**	**512**	**32**	4	0.25	**32**	2	**4**
M2	**>128/4**	**256**	**>128**	**>512**	**32**	**32**	**>8/152**	64	8	1	0.125	2	0.125	0.5
M3	**>128/4**	**256**	**>128**	**512**	**>32**	**16**	**8/152**	**512**	**>128**	**64**	0.25	**32**	**16**	2
M4	**>128/4**	8	**>128**	**>512**	**>32**	**>32**	0.5/9.5	64	8	**64**	0.25	0.06	0.125	2
M5	**128/4**	**256**	**128**	**512**	**16**	**16**	**8/152**	**>512**	**128**	8	0.5	**32**	0.125	0.5
M6	**128/4**	**256**	**>128**	**512**	**32**	**32**	**8/152**	**256**	**128**	**64**	0.5	**32**	0.5	**4**
M8	**>128/4**	**256**	**64**	**>512**	**>32**	**32**	**8/152**	64	8	2	0.25	**32**	2	0.5
C1	**>128/4**	**256**	**>128**	**512**	2	2	**8/152**	**256**	**128**	4	0.5	**32**	**16**	**4**
C2	**>128/4**	**256**	**128**	**512**	**4**	**4**	**8/152**	64	8	2	0.5	2	0.125	**4**
C3	**>128/4**	**256**	**>128**	**512**	**4**	**8**	**8/152**	**256**	**128**	**64**	0.5	**32**	**16**	**4**
C5	64/4	**256**	**128**	**512**	2	2	**8/152**	128	**128**	16	0.5	**16**	0.125	**4**
C6	**>128/4**	**256**	**128**	**>512**	**8**	**16**	**8/152**	**>512**	**128**	**64**	0.5	**32**	**16**	**8**
C8	**>128/4**	**256**	**>128**	**>512**	2	1	**8/152**	64	**32**	16	0.25	**32**	**16**	**4**
M + C1	**128/4**	**256**	**>128**	**512**	**8**	**32**	**8/152**	**256**	**32**	4	0.5	**32**	**4**	**8**
M + C2	**128/4**	**256**	**>128**	**512**	**8**	**16**	2/38	64	8	2	0.5	2	0.125	**4**
M + C3	**128/4**	**256**	**>128**	**512**	**4**	**16**	**8/152**	**256**	**128**	**64**	0.5	**32**	**16**	**4**
M + C5	**>128/4**	**256**	**128**	**>512**	**16**	**>32**	0.5/9.5	**256**	**>128**	8	0.5	**32**	0.25	**4**
M + C6	**128/4**	**256**	**>128**	**512**	**16**	**16**	**8/152**	**256**	**128**	**64**	0.25	**16**	**16**	**4**
M + C8	**128/4**	**256**	**>128**	**512**	**4**	**8**	**8/152**	64	8	4	0.5	**16**	**16**	**4**
J53	2/4	2	0.06	0.06	0.06	0.03	0.016	1	0.5	1	0.25	0.5	0.008	0.5
ATCC 25922	1/4	4	0.06	4	0.06	0.03	0.25/4.75	2	0.5	2	0.125	2	0.01	1

M8+C8).
MICs in bold indicated resistant isolates.

samples from hospital patients across China (Kawahara et al., 2021; F. Li et al., 2021). The insertion sequence IS*Apl1* is often observed in the vicinity of *mcr-1*, either as a remnant of, or forming a complete composite transposon with another, flanking IS*Apl1* element (Snesrud et al., 2016). However, IS*Apl1* was not observed around *mcr-1* among the isolates in this study except for directly upstream or downstream of *mcr-1* in isolate W1 and W2. The presence of IS*Apl1* in W5 could not be analyzed due to the short length of *mcr-1*-containing contigs (Fig. 3). There is insertion sequence IS*Apl1* upstream and downstream of *mcr-1* called intact structures, and it has previously been reported that the lack of intact complex transposon structures in some *mcr-1* bearing isolates may help stabilize genes on plasmids and thus facilitate their spread. (Snesrud et al., 2016). For the CCREC isolates in which *mcr-1* could be localized to a plasmid, conjugation experiments were successfully carried out, and the plasmids could be transferred to the recipient strain. The resulting MICs of colistin for the transconjugants were 4–8 mg/L, demonstrating the capacity of these plasmids to disseminate the colistin resistance phenotype (Table 3).

Virulence genes and serotypes of the CCREC isolated in this study are presented in Table 2. Notably, isolates W1 and W6 carried *hlyF, iroN, iss, ompT* and *iutA*, five genes which are linked to, and considered predictors of highly virulent APEC (Azam et al., 2020). W1 additionally carried the EAST-1 heat-stable toxin gene *astA*, which is typically associated with EAEC, an *E. coli* pathotype which can cause pediatric diarrhea. Isolate W6 was determined to belong to the high-risk serotype O101:H9, and ST167, an ST which has disseminated globally and is frequently associated with human infection and NDM-type carbapenemases (Garcia--Fernandez et al., 2020; Johnson et al., 2008). Outbreaks caused by high-risk clones of ST167 CCREC have occurred in several different regions in China (W. Li et al., 2021; Huang et al., 2022). It is very seriously disturbing that these virulence factors are harbored by bacteria with such extensive antibiotic resistance profiles, as any infections caused by these are likely to be very difficult to treat.

5. Conclusion

In this study, *E. coli* carrying colistin- and carbapenem resistance genes were isolated from wastewater and sludge samples collected from a WWTP in Eastern China, and subsequently characterized with antimicrobial susceptibility testing and whole-genome sequencing. The isolates were resistant to a large number of antibiotics, severely limiting treatment options for infections caused by these bacteria. Most plasmids carrying genes conferring resistance to colistin and carbapenems belonged to widely spread families of plasmids which could be successfully transferred to a recipient strain via conjugation, demonstrating their potential to further facilitate the dissemination of this resistance phenotype. Additionally, resistance to several other clinically important antibiotics could be co-transferred in the conjugation experiments. Aside from resistance profiles, a few of the CCREC isolates were also carrying virulence genes associated with APEC and belonged to serotypes and STs considered to constitute high-risk clones.

WWTPs provide an ideal environment for ARB to co-mingle with clinical and environmental bacteria, exchange genetic material, and disseminate to other environments. The high prevalence in the study WWTP of the CCREC strains described in this study suggest an urgent risk of further dissemination of the colistin-, carbapenem-, and multidrug resistance genotypes, which could lead to a serious threat to public health. Thus urgent, preventative actions are called for. To enable the design of efficient interventions to this end, improved monitoring of the epidemiologic situation and investigation of the mechanisms of environmental transmission of antibiotic resistance are imperative.

CRediT authorship contribution statement

Di Wang: performed the experiments, Formal analysis, Writing – original draft. **Huiyun Zou:** contributed to the sampling; All authors reviewed and revised the manuscript, Investigation, Methodology. **Ling Zhao:** contributed to the sampling, All authors reviewed and revised the manuscript, Investigation, Methodology. **Qi Li:** performed the experiments. **Min Meng:** contributed to the sampling, All authors reviewed and revised the manuscript, Investigation, Methodology. **Xuewen Li:** designed the study, Funding acquisition, Investigation, Methodology. **Björn Berglund:** Formal analysis, Writing – original draft.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a

potential conflict of interest.

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Appendix A. Supplementary data

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References

Azam, M., Mohsin, M., Johnson, T.J., Smith, E.A., Johnson, A., Umair, M., Sajjad Ur, R., 2020. Genomic landscape of multi-drug resistant avian pathogenic Escherichia coli recovered from broilers. Vet. Microbiol. 247, 108766 https://doi.org/10.1016/j.vetmic.2020.108766.

Bao, D., Xu, X., Wang, Y., Zhu, F., 2022. Emergence of a multidrug-resistant Escherichia coli Co-carrying a new mcr-1.33 variant and bla NDM-5 genes recovered from a urinary tract infection. Infect. Drug Resist. 15, 1499–1503. https://doi.org/10.2147/IDR.S358566.

Beattie, R.E., Skwor, T., Hristova, K.R., 2020. Survivor microbial populations in post-chlorinated wastewater are strongly associated with untreated hospital sewage and include ceftazidime and meropenem resistant populations. Sci. Total Environ. 740 https://doi.org/10.1016/j.scitotenv.2020.140186.

Berglund, B., Hoang, N.T.B., Tärnberg, M., Le, N.K., Welander, J., Nilsson, M., Khu, D.T. K., Nilsson, L.E., Olson, L., Le, H.T., Larsson, M., Hanberger, H., 2017. Colistin- and carbapenem-resistant Klebsiella pneumoniae carrying mcr-1 and blaOXA-48 isolated at a paediatric hospital in Vietnam. J. Antimicrob. Chemother. 73 (4), 1100–1102. https://doi.org/10.1093/jac/dkx491.

Bilal, H., Rehman, T.U., Khan, M.A., Hameed, F., Jian, Z.G., Han, J., Yang, X., 2021. Molecular Epidemiology of mcr-1, bla KPC-2, and bla NDM-1 harboring clinically isolated Escherichia coli from Pakistan. Infect. Drug Resist. 14, 1467–1479. https://doi.org/10.2147/IDR.S302687.

Cheng, P., Yang, Y.Q., Cao, S., Liu, H.B., Li, X.T., Sun, J.C., Zhang, X.Y., 2021. Prevalence and characteristic of swine-origin mcr-1-positive Escherichia coli in northeastern China. Front. Microbiol. 12 https://doi.org/10.3389/fmicb.2021.712707.

CLSI, 2022. CLSI M100-Ed32:2022 Performance Standards for Antimicrobial Susceptibility Testing, 32th Edition. 2022. http://em100.edaptivedocs.net/GetDoc.aspx?doc=CLSI%20M100%20ED32.

Eucast, 2022. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint Tables for Interpretation of MICs and Zone Diameters. Version 12.0, 2022. http://www.eucast.org.

European Medicines Agency, 2021. Tygacil : EPAR Product Information. https://www.ema.europa.eu/en/medicines/human/EPAR/tigecycline-accord.

Feng, J., Xiang, Q., Ma, J., Zhang, P., Li, K., Wu, K., Yang, Z., 2021. Characterization of carbapenem-resistant Enterobacteriaceae cultured from retail meat products, patients, and porcine excrement in China. Front. Microbiol. 12, 743468 https://doi.org/10.3389/fmicb.2021.743468.

Gu, C., Li, X., Zou, H., Zhao, L., Meng, C., Yang, C., Berglund, B., 2022. Clonal and plasmid-mediated dissemination of environmental carbapenem-resistant Enterobacteriaceae in large animal breeding areas in northern China. Environ. Pollut. 297, 118800 https://doi.org/10.1016/j.envpol.2022.118800.

Guo, J., Li, J., Chen, H., Bond, P.L., Yuan, Z., 2017. Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic resistance genes and mobile genetic elements. Water Res. 123, 468–478. https://doi.org/10.1016/j.watres.2017.07.002.

Guo, X.B., Rao, Y.T., Guo, L.H., Xu, H., Lv, T., Yu, X., Zheng, B.W., 2019. Detection and genomic characterization of a Morganella morganii isolate from China that produces NDM-5. Front. Microbiol. 10 https://doi.org/10.3389/fmicb.2019.01156.

Hameed, M.F., Chen, Y., Wang, Y., Shafiq, M., Bilal, H., Liu, L., Ge, H., 2021. Epidemiological characterization of colistin and carbapenem resistant Enterobacteriaceae in a tertiary: a hospital from anhui province. Infect. Drug Resist. 14, 1325–1333. https://doi.org/10.2147/IDR.S303739.

Han, H., Liu, W., Cui, X., Cheng, X., Jiang, X., 2020. Co-existence of mcr-1 and bla NDM-5 in an Escherichia coli strain isolated from the pharmaceutical industry, WWTP. Infect. Drug Resist. 13, 851–854. https://doi.org/10.2147/IDR.S245047.

Huang, J., Zhu, J., Gong, D., Wu, L., Zhu, Y., Hu, L., 2022. Whole genome sequence of EC16, a blaNDM-5-, blaCTX-M-55-, and fosA3-coproducing Escherichia coli ST167 clinical isolate from China. J Glob Antimicrob Resist 29, 296–298. https://doi.org/10.1016/j.jgar.2022.04.001.

Ji, X., Zheng, B., Berglund, B., Zou, H., Sun, Q., Chi, X., Nilsson, L.E., 2019. Dissemination of extended-spectrum beta-lactamase-producing Escherichia coli carrying mcr-1 among multiple environmental sources in rural China and associated risk to human health. Environ. Pollut. 251, 619–627. https://doi.org/10.1016/j.envpol.2019.05.002.

Jiang, B., Du, P.C., Jia, P.Y., Liu, E.B., Kudinha, T., Zhang, H., Yang, Q.W., 2020. Antimicrobial susceptibility and virulence ofmcr-1-PositiveEnterobacteriaceaein China, a multicenter longitudinal epidemiological study. Front. Microbiol. 11 https://doi.org/10.3389/fmicb.2020.01611.

Johnson, T.J., Wannemuehler, Y., Doetkott, C., Johnson, S.J., Rosenberger, S.C., Nolan, L.K., 2008. Identification of minimal predictors of avian pathogenic Escherichia coli virulence for use as a rapid diagnostic tool. J. Clin. Microbiol. 46 (12), 3987–3996. https://doi.org/10.1128/JCM.00816-08.

Ju, F., Beck, K., Yin, X., Maccagnan, A., McArdell, C.S., Singer, H.P., Burgmann, H., 2019. Wastewater treatment plant resistomes are shaped by bacterial composition, genetic exchange, and upregulated expression in the effluent microbiomes. ISME J. 13 (2), 346–360. https://doi.org/10.1038/s41396-018-0277-8.

Kalova, A., Gelbicova, T., Overballe-Petersen, S., Litrup, E., Karpiskova, R., 2021. Characterisation of colistin -resistant Enterobacterales and acinetobacter strains carrying mcr genes from asian aquaculture products. Antibiotics-Basel 10 (7). https://doi.org/10.3390/antibiotics10070838.

Kawahara, R., Yamaguchi, T., Yamamoto, Y., 2021. Comparative genome analysis of livestock and human colistin-resistant Escherichia coli isolates from the same household. Infect. Drug Resist. 14, 841–847. https://doi.org/10.2147/IDR.S298120.

Li, L.G., Xia, Y., Zhang, T., 2017. Co-occurrence of antibiotic and metal resistance genes revealed in complete genome collection. ISME J. 11 (3), 651–662. https://doi.org/10.1038/ismej.2016.155.

Li, X., Fu, Y., Shen, M.Y., Huang, D.Y., Du, X.X., Hu, Q.F., Yu, Y.S., 2018. Dissemination of bla(NDM-5) gene via an IncX3-type plasmid among non-clonal Escherichia coli in China. Antimicrob Resist 7. https://doi.org/10.1186/s13756-018-0349-6.

Li, X.P., Sun, R.Y., Song, J.Q., Fang, L.X., Zhang, R.M., Lian, X.L., Sun, J., 2020. Within-host heterogeneity and flexibility of mcr-1 transmission in chicken gut. Int. J. Antimicrob. Agents 55 (1). https://doi.org/10.1016/j.ijantimicag.2019.09.010.

Li, F., Ye, K., Li, X., Ye, L., Guo, L., Wang, L., Yang, J., 2021. Genetic characterization of carbapenem-resistant Escherichia coli from China, 2015-2017. BMC Microbiol. 21 (1), 248. https://doi.org/10.1186/s12866-021-02307-x.

Li, W., Yan, Y.F., Chen, J., Sun, R.W., Wang, Y.X., Wang, T.F., Yang, B.W., 2021. Genomic characterization of conjugative plasmids carrying the mcr-1 gene in foodborne and clinical strains of Salmonella and Escherichia coli. Food Control 125. https://doi.org/10.1016/j.foodcont.2021.108032.

Lin, Y., Yang, L., Lu, L., Wang, K., Li, J., Li, P., Song, H., 2020. Genomic features of an Escherichia coli ST156 strain harboring chromosome-located mcr-1 and plasmid-mediated blaNDM-5. Infect. Genet. Evol. 85, 104499 https://doi.org/10.1016/j.meegid.2020.104499.

Liu, B.T., Song, F.J., 2019. Emergence of two Escherichia coli strains co-harboring mcr-1 and bla NDM in fresh vegetables from China. Infect. Drug Resist. 12, 2627–2635. https://doi.org/10.2147/IDR.S211746.

Liu, Y.-Y., Wang, Y., Walsh, T.R., Yi, L.-X., Zhang, R., Spencer, J., Shen, J., 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect. Dis. 16 (2), 161–168. https://doi.org/10.1016/s1473-3099(15)00424-7.

Liu, X., Geng, S., Chan, E.W.C., Chen, S., 2019. Increased prevalence of Escherichia coli strains from food carrying blaNDM and mcr-1-bearing plasmids that structurally resemble those of clinical strains, China, 2015 to 2017. Euro Surveill. 24 (13), 1800113 https://doi.org/10.2807/1560-7917.

Liu, Z.Y., Xiao, X., Li, Y., Liu, Y., Li, R.C., Wang, Z.Q., 2019. Emergence of IncX3 plasmid-harboring bla(NDM)(-5) dominated by Escherichia coli ST48 in a goose farm in jiangsu, China. Front. Microbiol. 10 https://doi.org/10.3389/fmicb.2019.02002.

Ma, Z.B., Zeng, Z.L., Liu, J., Liu, C., Pan, Y., Zhang, Y.N., Li, Y.F., 2021. Emergence of IncHI2 plasmid-harboring blaNDM-5 from porcine Escherichia coli isolates in Guangdong, China. Pathogens 10 (8). https://doi.org/10.3390/pathogens10080954.

Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Monnet, D.L., 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18 (3), 268–281. https://doi.org/10.1111/j.1469-0691.2011.03570.x.

Nang, S.C., Li, J., Velkov, T., 2019. The rise and spread of mcr plasmid-mediated polymyxin resistance. Crit. Rev. Microbiol. 45 (2), 131–161. https://doi.org/10.1080/1040841X.2018.1492902.

Ngbede, E.O., Adekanmbi, F., Poudel, A., Kalalah, A., Kelly, P., Yang, Y., Wang, C., 2021. Concurrent resistance to carbapenem and colistin among Enterobacteriaceae recovered from human and animal sources in Nigeria is associated with multiple genetic mechanisms. Front. Microbiol. 12, 740348 https://doi.org/10.3389/fmicb.2021.740348.

Nguyen, A.Q., Vu, H.P., Nguyen, L.N., Wang, Q., Djordjevic, S.P., Donner, E., Nghiem, L. D., 2021. Monitoring antibiotic resistance genes in wastewater treatment: current strategies and future challenges. Sci. Total Environ. 783, 146964 https://doi.org/10.1016/j.scitotenv.2021.146964.

Nukui, Y., Ayibieke, A., Taniguchi, M., Aiso, Y., Shibuya, Y., Sonobe, K., Saito, R., 2019. Whole-genome analysis of EC129, an NDM-5-, CTX-M-14-, OXA-10- and MCR-1-co-producing Escherichia coli ST167 strain isolated from Japan. J Glob Antimicrob Resist 18, 148–150. https://doi.org/10.1016/j.jgar.2019.07.001.

Oliveira, M., Nunes, M., Barreto Crespo, M.T., Silva, A.F., 2020. The environmental contribution to the dissemination of carbapenem and (fluoro)quinolone resistance genes by discharged and reused wastewater effluents: the role of cellular and extracellular DNA. Water Res. 182. https://doi.org/10.1016/j.watres.2020.116011.

Pal, C., Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G., 2015. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. BMC Genom. 16, 964. https://doi.org/10.1186/s12864-015-2153-5.

Peyclit, L., Baron, S.A., Rolain, J.-M., 2019. Drug repurposing to fight colistin and carbapenem-resistant bacteria. Front. Cell. Infect. Microbiol. 9 https://doi.org/10.3389/fcimb.2019.00193.

Poirel, L., Walsh, T.R., Cuvillier, V., Nordmann, P., 2011. Multiplex PCR for detection of acquired carbapenemase genes. Diagn. Microbiol. Infect. Dis. 70 (1), 119–123. https://doi.org/10.1016/j.diagmicrobio.2010.12.002.

Rebelo, A.R., Bortolaia, V., Kjeldgaard, J.S., Pedersen, S.K., Leekitcharoenphon, P., Hansen, I.M., Hendriksen, R.S., 2018. Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5 for surveillance purposes. Euro Surveill. 23 (6) https://doi.org/10.2807/1560-7917.ES.2018.23.6.17-00672.

Sabri, N.A., van Holst, S., Schmitt, H., van der Zaan, B.M., Gerritsen, H.W., Rijnaarts, H. H.M., Langenhoff, A.A.M., 2020. Fate of antibiotics and antibiotic resistance genes during conventional and additional treatment technologies in wastewater treatment plants. Sci. Total Environ. 741, 140199 https://doi.org/10.1016/j.scitotenv.2020.140199.

Schurch, A.C., Arredondo-Alonso, S., Willems, R.J.L., Goering, R.V., 2018. Whole genome sequencing options for bacterial strain typing and epidemiologic analysis based on single nucleotide polymorphism versus gene-by-gene-based approaches. Clin. Microbiol. Infect. 24 (4), 350–354. https://doi.org/10.1016/j.cmi.2017.12.016.

Serwecińska, L., 2020. Antimicrobials and antibiotic-resistant bacteria: a risk to the environment and to public health. Water 12 (12). https://doi.org/10.3390/w12123313.

Shen, Z., Wang, Y., Shen, Y., Shen, J., Wu, C., 2016. Early emergence of mcr-1 in Escherichia coli from food-producing animals. Lancet Infect. Dis. 16 (3) https://doi.org/10.1016/s1473-3099(16)00061-x.

Shen, Z., Hu, Y., Sun, Q., Hu, F., Zhou, H., Shu, L., Zhang, R., Wang, S., 2018. Emerging carriage of NDM-5 and MCR-1 in Escherichia coli from healthy people in multiple regions in China: a cross sectional observational study. eClinicalMedicine 6, 11–20. https://doi.org/10.1016/j.eclinm.2018.11.003.

Shin, H., Kim, Y., Han, D., Hur, H.G., 2021. Emergence of high level carbapenem and extensively drug resistant Escherichia coli ST746 producing NDM-5 in influent of wastewater treatment plant, Seoul, South Korea. Front. Microbiol. 12 https://doi.org/10.3389/fmicb.2021.645411.

Snesrud, E., He, S., Chandler, M., Dekker, J.P., Hickman, A.B., McGann, P., Dyda, F., 2016. A model for transposition of the colistin resistance gene mcr-1 by ISApl1. Antimicrob. Agents Chemother. 60 (11), 6973–6976. https://doi.org/10.1128/AAC.01457-16.

Sun, P.F., Xia, W.Y., Liu, G.Y., Huang, X., Tang, C.J., Liu, C.C., Pan, S.Y., 2019. Characterization of bla(NDM-5)-positive Escherichia coli prevalent in A university hospital in eastern China. Infect. Drug Resist. 12, 3029–3038. https://doi.org/10.2147/IDR.S225546.

Walsh, T.R., Wu, Y.N., 2016. China bans colistin as a feed additive for animals. Lancet Infect. Dis. 16 (10), 1102–1103. https://doi.org/10.1016/S1473-3099(16)30329-2.

Wang, M.G., Yu, Y., Wang, D., Yang, R.S., Jia, L., Cai, D.T., Liao, X.P., 2021. The emergence and molecular characteristics of New Delhi metallo beta-lactamase-producing Escherichia coli from ducks in Guangdong, China. Front. Microbiol. 12, 677633 https://doi.org/10.3389/fmicb.2021.677633.

Who, 2019. Critically Important Antimicrobials for Human Medicine. World Health Organization, Geneva, 6th revision.

Wright, G.D., 2019. Environmental and clinical antibiotic resistomes, same only different. Curr. Opin. Microbiol. 51, 57–63. https://doi.org/10.1016/j.mib.2019.06.005.

Xie, S., Li, L., Zhan, B., Shen, X., Deng, X., Tan, W., Fang, T., 2022. Pogostone enhances the antibacterial activity of colistin against MCR-1-positive bacteria by inhibiting the biological function of MCR-1. Molecules 27 (9). https://doi.org/10.3390/molecules27092819.

Yamaguchi, T., Kawahara, R., Hamamoto, K., Hirai, I., Khong, D.T., Nguyen, T., Yamamoto, Y., 2020. High prevalence of colistin-resistant Escherichia coli with chromosomally carried mcr-1 in healthy residents in Vietnam. mSphere 5 (2). https://doi.org/10.1128/mSphere.00117-20.

Yan, W., Zhang, Q., Zhu, Y., Jing, N., Yuan, Y., Zhang, Y., Li, Y., 2021. C: a multicenter study. Infect. Drug Resist. 14, 2657–2666. https://doi.org/10.2147/IDR.S314490.

Zhang, F.F., Xie, L.Y., Wang, X.L., Han, L.Z., Guo, X.K., Ni, Y.X., Sun, J.Y., 2016. Further spread of bla(NDM-5) in Enterobacteriaceae via IncX3 plasmids in Shanghai, China. Front. Microbiol. 7 https://doi.org/10.3389/fmicb.2016.00424.

Zhang, S.H., Huang, Y.B., Yang, G.Z., Lei, T., Chen, M.T., Ye, Q.H., Wu, Q.P., 2021. High prevalence of multidrug-resistant Escherichia coli and first detection of IncHI2/IncX4-plasmid carrying mcr-1 E. coli in retail ready-to-eat foods in China. Int. J. Food Microbiol. 355 https://doi.org/10.1016/j.ijfoodmicro.2021.109349.

Zhao, Q., Berglund, B., Zou, H., Zhou, Z., Xia, H., Zhao, L., Li, X., 2021. Dissemination of blaNDM-5 via IncX3 plasmids in carbapenem-resistant Enterobacteriaceae among humans and in the environment in an intensive vegetable cultivation area in eastern China. Environ. Pollut. 273, 116370 https://doi.org/10.1016/j.envpol.2020.116370.

Zheng, B., Dong, H., Xu, H., Lv, J., Zhang, J., Jiang, X., Du, Y., Xiao, Y., Li, L., 2016. Coexistence of MCR-1 and NDM-1 in clinical Escherichia coli isolates. Clin. Infect. Dis. 63 (10), 1393–1395. https://doi.org/10.1093/cid/ciw553.

Zhou, Y.F., Liu, P., Zhang, C.J., Liao, X.P., Sun, J., Liu, Y.H., 2019. Colistin combined with tigecycline: a promising alternative strategy to combat Escherichia coli harboring bla ndm- 5 and mcr-1. Front. Microbiol. 10, 2957. https://doi.org/10.3389/fmicb.2019.02957.

Zou, H., Berglund, B., Wang, S., Zhou, Z., Gu, C., Zhao, L., Li, X., 2022. Emergence of blaNDM-1, blaNDM-5, blaKPC-2 and blaIMP-4 carrying plasmids in Raoultella spp. in the environment. Environ. Pollut. 306, 119437 https://doi.org/10.1016/j.envpol.2022.119437.

Garcia-Fernandez, A., Villa, L., Bibbolino, G., Bressan, A., Trancassini, M., Pietropaolo, V., Carattoli, A., 2020. Novel Insights and Features of the NDM-5-Producing Escherichia coli Sequence Type 167 High-Risk. Clone. mSphere. 5 (2), e00269–20. https://doi.org/10.1128/mSphere.00269-20.

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Interaction between the animal-based dietary pattern and green space on cognitive function among Chinese older adults: A prospective cohort study

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ABSTRACT

Green space is associated with better cognition, while the animal-based dietary pattern can be a risk factor. We aimed to verify the associations and explore their interaction among the elderly. The China Longitudinal Healthy Longevity Survey (CLHLS) cohort including 17,827 participants was used. The average green space coverage rate was used to measure green space exposure. The animal-based diet index (ADI) was scored based on the non-quantitative frequency questionnaire of ten types of food intake (three types of animal foods and seven types of plant foods). We used the Mini-Mental State Examination (MMSE) to assess cognitive function. The Cox proportional hazard regression was applied to explore the correlations and interactions. In the models, we gradually adjusted for the potential risk factors. Compared with participants living in the area with the lowest green space, those living with the highest were associated with a 20% decrease in the risk of cognitive impairment (hazard ratio (HR): 0.80, 95% CI: 0.73, 0.89). As for ADI, the highest group was related to a 64% increase in the risk of cognitive impairment (HR: 1.64, 95% CI: 1.38, 1.95). The protective effect of the highest green space group on cognitive impairment was more evident among participants with low ADI (HR = 0.72, 95% CI: 0.62, 0.83), compared to those with high ADI. Green space was positively associated with cognition, while the animal-based dietary pattern was a cognitive disadvantage. The animal-based dietary pattern may mitigate the beneficial effects of green space on cognition.

1. Introduction

Since the 21st century, the aging society had become increasingly apparent with the prolongation of life expectancy and the increase in the elderly population. Cognitive impairment was one of the major diseases associated with aging (Li et al., 2021), and older adults were more likely to develop cognitive impairment than younger people. Consequently, cognitive impairment emerged as a serious health problem (Marron et al., 2018). The worsening of cognitive impairment could eventually lead to dementia, which could burden healthcare systems around the world and increase healthcare costs for society as a whole (Prince et al., 2015; Marešová et al., 2015).

Some studies have shown that the plant-based dietary pattern is beneficial for health (Medawar et al., 2019; Rajaram et al., 2019; Wu et al., 2019; Lin et al., 2019). For example, the findings of Medawar supported the beneficial effects of a plant-based diet on health and disease metabolic markers. Meanwhile, their results showed that there was still no conclusive evidence for the cognitive and mental effects of a plant-based diet (Medawar et al., 2019). Rajaram pointed out that dietary patterns that focus on plant foods rich in polyphenols could have positive effects on cognitive health in older adults (Rajaram et al., 2019). Wu found that higher adherence to healthy dietary patterns in midlife was associated with a lower risk of cognitive impairment in late life in Chinese adults (Wu et al., 2019). However, the results were inconsistent when analyzing the relationship between the animal-based dietary pattern and cognitive function. Some researchers suggested that the animal-based dietary pattern might be associated with cognitive impairment. In the Newcastle (UK) Cohort Study, Granic found that a dietary pattern high in red meat was associated with poorer cognitive performance (Granic et al., 2016). Data from 194 cognitively healthy individuals participating in the Uppsala elderly cohort study confirmed that lower consumption of meat and meat products was associated with better cognitive scores (Titova et al., 2013). Some studies showed different results. The Maine-Syracuse longitudinal study showed that

Abbreviations: CLHLS, China Longitudinal Healthy Longevity Survey; ADI, The animal-based diet index; MMSE, Mini-Mental State Examination; HR, Hazard ratio; NDVI, Normalized difference vegetation index; CIs, confidence intervals; SD, Standard deviation.

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among 333 participants without dementia and stroke, higher cognitive scores were prospectively associated with a higher intake of healthy dietary choices. These healthy diets included vegetables, legumes, nuts, fish, and meat (Crichton et al., 2015). To determine the association between the animal-based dietary pattern and cognition, this study focused on their relationship based on a long-term longitudinal cohort study.

Evolving evidence suggested that limited green space had an enormously detrimental impact on cognitive function in older adults (Cassarino and Setti, 2015). Zhang and his colleagues examined the effect of several environmental factors on cognitive function. Their results added value to existing knowledge about the relationship between green space and cognition (Zhang et al., 2022). The indicators of green space measurement in different studies were not consistent. Some studies used normalized difference vegetation index (NDVI) (Dadvand et al., 2015; Browning and Locke, 2020; Jimenez et al., 2022; Hystad et al., 2019), some used tree canopy to measure green space (Kuo et al., 2018; Li et al., 2019), while others used the average green space coverage rate (Beere and Kingham, 2017; Hodson and Sander, 2017; Kweon et al., 2017, 2017d; Kweon et al., 2017; de Vries et al., 2016). As far as we know, the indicator NDVI had some limitations. First, NDVI did not assess tree canopy cover (Markevych et al., 2017). Second, the NDVI values could be inaccurate due to the values were mainly derived from satellite imagery and were affected by season and cloud cover. Finally, the numerical cutoff value of NDVI used to define healthy vegetation was uncertain. And small patches of fragmented green space might skew the NDVI mean (Markevych et al., 2017). As for the index of the tree canopy, it was too narrow and not representative enough to measure the green space. In addition, the tree canopy shape and benefit were influenced by seasonal changes (Massetti et al., 2019). Based on previous studies and the data available to us, we used the average green space coverage rate to measure the exposure to green space. It has been proven to be representative and accurate (Beere and Kingham, 2017; Hodson and Sander, 2017; Kweon et al., 2017).

Given that green space was a protective factor for cognitive function and the animal-based diet was a risk factor, we could hypothesize that the animal-based diet might modify the relationship between green space and cognition. Our study aimed to verify the associations of green space and the animal-based dietary pattern with cognitive function and test this hypothesis.

2. Methods

2.1. Study population

The Chinese Longitudinal Healthy Longevity Study (CLHLS) (Lv et al., 2019; Yu et al., 2021; Cao et al., 2020; Liu et al., 2019) was the first nationwide longitudinal survey in China. This study began in 1998 and was conducted every 2–3 years. Its details could be found elsewhere (Lv et al., 2018; Zeng et al., 2017; Vlosky, 2008). Some new participants were recruited at each follow-up to form a new cohort. The survey was conducted at the participants' homes by a trained investigator using a structured questionnaire. When respondents could not answer questions, spouses or other close relatives were interviewed as surrogate respondents. However, questions about cognition had to be answered by the participants themselves. All respondents signed written informed consent.

Since the dietary questionnaire of the CLHLS cohort was changed in 2008, we used four waves of the CLHLS data from 1998, 2000, 2002, and 2005 in this study to ensure consistency across dietary questionnaires. The total sample consisted of 32,673 participants. However, 14846 participants were excluded due to missing data on cognitive function information at baseline, missing data on diet information, and cognitive impairment at baseline. Ultimately, the sample used for analysis consisted of 17,827 individuals. The overall process of the inclusion and exclusion of participants in this study is shown in Fig. S1.

2.2. Cognitive function assessment

We used the Chinese version of the Mini-Mental State Examination (MMSE) to measure cognitive function. The Chinese version of the MMSE was an adaptation of the scale developed by Folstein (Folstein et al., 1975; Nguyen et al., 2003). The reliability and validity of the scale had been verified (An and Liu, 2016; Zhang et al., 2021; Ren et al., 2021). The information on MMSE was performed using a simplified questionnaire by trained investigators. The MMSE assessed five domains of cognitive function, including reaction, orientation, recall, language, attention, and calculation (Folstein et al., 1975). When participants were unable to answer to cognitive questions, we coded these responses as incorrect answers based on the literature (Folstein et al., 1975). Scores on the MMSE scale ranged from zero to thirty, with higher scores representing better cognitive function. Table S1 shows more details about the Chinese MMSE scale.

2.3. Green space exposure assessment

In this study, the average green space coverage rate was used to measure green space exposure. The green space coverage rate of a city was equal to the green space area of the city divided by the total area. The green space area changed every year, which could be collected from the City Statistical Yearbooks. The average green space coverage rate was equal to the sum of the green space coverage rates divided by the number of years. In this study, we calculated the average green space coverage rate for each participant based on exposure time and city.

We calculated long-term green space exposure and short-term green space exposure separately to reflect the exposure of each participant (Zhu et al., 2022). For long-term green space exposure, the exposure time was defined as the time from recruitment into the cohort to death, the diagnosis of cognitive impairment (MMSE <18), end of follow-up (2018), or loss to follow-up, whichever came first. For short-term green space exposure, the exposure time was determined to be three years before the outcome (Zhu et al., 2022). Long-term green space exposure was used as the primary analysis, and short-term green space exposure was used as the sensitivity analysis. The data on the green space area in the participants' residential cities was extracted from the City Statistical Yearbooks at http://www.stats.gov.cn/tjsj./ndsj/.

2.4. Dietary assessment

In the CLHLS cohort, the dietary data were collected using a non-quantitative frequency questionnaire for plant and animal foods by trained investigators. Using non-quantitative food frequency questionnaires to assess dietary patterns was reliable and valid in several studies (Mohammadifard et al., 2015; Saeedi et al., 2016; Wong et al., 2012). Ten types of foods (three types of animal foods and seven types of plant foods) that were most common in the Chinese diet were included in our study. The animal foods included eggs, fish, and meat. And the plant foods included fresh vegetables, salt-preserved vegetables, fresh fruit, garlic, bean, tea, and sugar.

The animal-based diet index (ADI) was constructed to evaluate the animal-based dietary pattern. The method for constructing the ADI referred to Satija's method of constructing the Plant-Based Dietary Index (Satija et al., 2016). Specifically, ADI was scored based on the frequency of intake. The frequency of intake in the CLHLS questionnaire was recorded as "rarely or never", "occasionally", or "almost every day" for most food groups, including eggs, fish, meat, salt-preserved vegetables, garlic, bean, tea, and sugar. However, the frequency of intake of fresh vegetables and fresh fruits was recorded as "rarely or never", "occasionally", "except winter", or "almost every day". To standardize the scores for all foods and avoid the undue influence of some foods, we excluded participants whose intake frequency of fresh vegetables and fresh fruits was "except winter". We scored 3 for the most frequent consumption, and 1 for the least frequent consumption of animal food

(positive scores). We scored 1 for the most frequent consumption, and 3 for the least frequent consumption of plant food (reverse scores). ADI was the total score of the above ten food scores, ranging from 11 to 30 points. A higher ADI score indicated a higher frequency of eating animal foods. Participants were divided into three groups according to ADI: low (11–17 points), intermediate (18–23 points), and high (24–30 points). When discussing the moderating role of ADI in the association between green space and cognitive function, we divided ADI into low (11–20 points) and high (21–30 points).

2.5. Covariates

We used the results of the questionnaire at baseline to assess potential confounders. Based on some studies (Gao et al., 2014; Liu et al., 1990; Shipley et al., 2007; van Gelder et al., 2007; Dewey and Saz, 2001; Langa et al., 2008), we selected the following covariates as potential confounders, including sociodemographic factors, lifestyle-related variables, and factors related to health status. The sociodemographic factors included age (continuous), sex ("males" or "female"), residence ("rural" or "urban"), ethnicity ("Han", "Hui", "Korea", "Man", "Mongolia", "Yao", "Zhuang", or "others"), living pattern ("alone", "in an institution", or "with household member"), current marital status ("married", "divorced", "widowed", "separated", or "never married"), and education level ("no schooling" or "some schooling"). The lifestyle-related variables included current smoking status ("yes" or "no"), drinking status ("yes" or "no"), and regular exercising ("yes" or "no"). The health status factors involved weight, self-reported history of heart disease ("yes" or "no"), hypertension ("yes" or "no"), and diabetes ("yes" or "no"). Multivariate imputation was used to impute the missing information for covariates.

2.6. Statistical analysis

According to the green space (low, intermediate, and high), the participants were divided into three groups. We summarized the baseline characteristics of each group using descriptive statistics. Continuous variables were presented as the mean (standard deviation (SD)), and categorical variables were presented as the number (percentages (%)). The distributions of baseline characteristics were compared by using ANOVA and χ^2 test across the groups for less than 2 groups. For variables with more than 2 groups, we used the chi-square trend test.

The Cox proportional hazard regression model was used to calculate the hazard ratio (HR) and 95% confidence interval (CI) between long-term green space exposure or ADI and cognitive impairment. The independent variable of the average green space coverage rate and the ADI were included in the model as a categorical variable and a continuous variable. In Model 1, the covariates were age and gender. In Model 2, residence, ethnicity, living pattern, current marital status, and education level were further supplemented. In Model 3, smoking status, drinking status, regular exercise, weight, and self-reported history of diseases (heart disease, hypertension, and diabetes) were additionally adjusted.

Cox proportional hazards regression model was used for stratified analysis to test the interaction of green space and ADI with the risk of cognitive impairment. We stratified the analysis by ADI scores (high and low) to see whether the associations differed by ADI. The group of participants with low ADI was used as the reference group. Furthermore, we plotted restricted cubic splines to explore the non-linear relationship between green space and cognitive impairment among participants whose ADI was low and high. In addition, we plotted restricted cubic splines to explore non-linearity associations of green space and the animal-based dietary pattern with risks of cognitive impairment. Furthermore, we explored their interaction. When we tested the interaction of green space and ADI with the risk of cognitive impairment, we adjusted for the same variables as the analysis between green space or ADI and cognition.

In addition, several sensitivity analyses were performed. First, we stratified the analysis by each animal-based food group to explore the effect on the cognition of various animal foods. Second, we investigated changing the variable long-term green area exposure to the short-term green area to verify the stability of the results. Third, we categorized participants into six groups according to different combinations of ADI (low or high) and green space (low, intermediate, and high) to test the interaction of ADI and green space with the risk of cognitive impairment. We considered participants whose ADI was low but whose green space exposure was high as the reference. Forth, the primary analysis was repeated among participants with complete data. Finally, participants who died within two years of follow-up were excluded to minimize potential reverse causal bias.

$P < 0.05$ in two-sided was considered statistically significant. All analyses were performed using R version 4.0.2 (R Foundation for Statistical Computing).

3. Results

3.1. Descriptive characteristics

The baseline characteristics of the 17,827 oldest old are shown in Table 1. Their average age was 86.35 years old (standard deviation (SD): 10.20 years), 53.24% were females, 51.72% lived in rural, and 12.74% lived alone. About 22.47% and 23.65% smoked and drank alcohol at baseline. Approximately 51.94% of participants did exercise regularly. The mean ADI was 20.09 (SD: 2.50). During the follow-up, we documented 2429 cognitive impairments.

For long-term green space exposure, the exposure time was defined as the time from recruitment into the cohort to death, the diagnosis of cognitive impairment (MMSE <18), end of follow-up (2018), or loss to follow-up, whichever came first.

3.2. Association of green space and cognitive function

The risks of cognitive impairment under green space are shown in Table 2, Fig. 1, and Fig. 2. We found that the results in the three models were similar. In Model 1, living in the areas with the highest green space was associated with a 25% decrease in the risk of cognitive impairment (HR: 0.75, 95% CI: 0.68,0.83), compared to those living in the areas with the lowest green space. In Model 2, the HR value was 0.78 (95% CI: 0.68, 0.83). In the fully adjusted model (Model 3), living in the areas with the highest green space was associated with a 20% decrease in the risk of cognitive impairment (HR: 0.81, 95% CI: 0.73, 0.90).

In Model 1, the covariates were age and gender. In Model 2, residence, ethnicity, living pattern, current marital status, and education level were further supplemented. In Model 3, smoking status, alcohol consumption, regular exercise, weight, and self-reported history of diseases (heart disease, hypertension, and diabetes) were additionally adjusted.

3.3. Association of the animal-based dietary pattern and cognitive function

Increased consumption of animal foods was significantly associated with an increased risk of developing poor cognitive function (Table 3, Figs. 1, and Fig. 2). The results of the three models are also consistent. In a fully adjusted model (Model 3), the intermediate group was associated with a 35% increase in the risk of cognitive impairment (HR: 1.35, 95% CI: 1.19,1.53), and the highest group was associated with a 64% increase (HR: 1.64, 95% CI: 1.38, 1.96), compared with the group with the lowest intake of animal food. Furthermore, each 1-point increase in ADI was associated with an increase in the risk of cognitive impairment (HR: 1.08, 95% CI: 1.06,1.09). Their associations were also non-linear.

In Model 1, the covariates were age and gender. In Model 2, residence, ethnicity, living pattern, current marital status, and education level were further supplemented. In Model 3, smoking status, alcohol

Table 1
Baseline characteristics of participants by the group of long-term green space coverage rate.

	Overall	Long-term green space group			p[b]
		Low	Intermediate	High	
N	17827	5924	6009	5894	
Cohort (%)					
1998	6005 (33.68)	2245 (37.90)	2100 (34.95)	1660 (28.16)	<0.0001
2000	4897 (27.47)	1929 (32.56)	1580 (26.29)	1388 (23.55)	
2002	4193 (23.52)	1269 (21.42)	1321 (21.98)	1603 (27.20)	
2005	2732 (15.33)	481 (8.12)	1008 (16.77)	1243 (21.09)	
Status (%)					
Cognitive impairment	2429 (13.63)	836 (14.11)	895 (14.89)	698 (11.84)	<0.0001
Normal	15398 (86.37)	5088 (85.89)	5114 (85.11)	5196 (88.16)	
Time[a] (mean (SD))	1729.06 (1301.54)	1570.27 (1109.11)	1728.43 (1377.88)	1889.29 (1378.86)	<0.0001
ADI (mean (SD))	20.09 (2.50)	18.69 (2.04)	20.11 (2.11)	21.49 (2.50)	<0.0001
ADI group (%)					
Low	2653 (14.88)	929 (15.68)	852 (14.18)	872 (14.79)	0.0009
Intermediate	13713 (76.92)	4549 (76.79)	4688 (78.02)	4476 (75.94)	
High	1461 (8.20)	446 (7.53)	469 (7.80)	546 (9.26)	
Residence (%)					
Rural	9221 (51.72)	3009 (50.79)	3246 (54.02)	2966 (50.32)	0.0001
Urban	8606 (48.28)	2915 (49.21)	2763 (45.98)	2928 (49.68)	
Age (mean (SD))	86.35 (10.20)	86.06 (10.07)	86.63 (10.20)	86.36 (10.31)	0.0101
Weight (mean (SD))	49.38 (14.84)	48.20 (10.50)	48.20 (16.00)	51.70 (16.90)	<0.0001
Sex (%)					
Female	9491 (53.24)	3183 (53.73)	3212 (53.45)	3096 (52.53)	0.3901
Male	8336 (46.76)	2741 (46.27)	2797 (46.55)	2798 (47.47)	
Ethnicity (%)					
Han	16763 (94.03)	5596 (94.46)	5574 (92.76)	5593 (94.89)	<0.0001
Hui	176 (0.99)	64 (1.08)	53 (0.88)	59 (1.00)	
Korea	13 (0.07)	8 (0.14)	2 (0.03)	3 (0.05)	
Man	88 (0.49)	35 (0.59)	19 (0.32)	34 (0.58)	
Mongolia	4 (0.02)	1 (0.02)	2 (0.03)	1 (0.02)	
Yao	61 (0.34)	10 (0.17)	36 (0.60)	15 (0.25)	
Zhuang	659 (3.70)	188 (3.17)	297 (4.94)	174 (2.95)	
Others	63 (0.35)	22 (0.37)	26 (0.43)	15 (0.25)	
Living pattern (%)					
Alone	2271 (12.74)	768 (12.96)	788 (13.11)	715 (12.13)	0.2783
In an institution	915 (5.13)	313 (5.28)	287 (4.78)	315 (5.34)	
With household member	14641 (82.13)	4843 (81.75)	4934 (82.11)	4864 (82.52)	
Current smoking status(%)					
No	13821 (77.53)	4545 (76.72)	4635 (77.13)	4641 (78.74)	0.0210
Yes	4006 (22.47)	1379 (23.28)	1374 (22.87)	1253 (21.26)	
Current drinking status(%)					
No	13611 (76.35)	4411 (74.46)	4628 (77.02)	4572 (77.57)	0.0001
Yes	4216 (23.65)	1513 (25.54)	1381 (22.98)	1322 (22.43)	
Regular exercise (%)					
No	8568 (48.06)	2821 (47.62)	2972 (49.46)	2775 (47.08)	0.0243
Yes	9259 (51.94)	3103 (52.38)	3037 (50.54)	3119 (52.92)	
Current marital status (%)					
Married	4849 (27.20)	1594 (26.91)	1590 (26.46)	1665 (28.25)	0.0808
Divorced	109 (0.61)	48 (0.81)	27 (0.45)	34 (0.58)	
Never married	241 (1.35)	81 (1.37)	77 (1.28)	83 (1.41)	
Separated	333 (1.87)	104 (1.76)	110 (1.83)	119 (2.02)	
Widowed	12295 (68.97)	4097 (69.16)	4205 (69.98)	3993 (67.75)	
Self-reported history of Hypertension (%)					
No	14944 (83.83)	5015 (84.66)	5078 (84.51)	4851 (82.30)	0.0005
Yes	2883 (16.17)	909 (15.34)	931 (15.49)	1043 (17.70)	
Self-reported history of diabetes (%)					
No	17504 (98.19)	5824 (98.31)	5927 (98.64)	5753 (97.61)	0.0001
Yes	323 (1.81)	100 (1.69)	82 (1.36)	141 (2.39)	
Self-reported history of heart disease (%)					
No	17150 (96.20)	5677 (95.83)	5803 (96.57)	5670 (96.20)	0.1061
Yes	677 (3.80)	247 (4.17)	206 (3.43)	224 (3.80)	
Education level (mean (SD))	2.11 (3.60)	2.16 (3.64)	1.94 (3.42)	2.23 (3.74)	<0.0001

Note: Data are represented as mean (standard deviation (SD)) for continuous variables and the number (percentages (%)) for categorical variables.
[a] It means the number of days from the baseline survey to the outcome. The unit of this variable is day.
[b] The P value is the value of testing the difference of baseline feature distribution in each group. $P < 0.05$ in two-sided was considered statistically significant. P-values were calculated by ANOVA and $\chi 2$ test.

consumption, regular exercise, weight, and self-reported history of diseases (heart disease, hypertension, and diabetes) were additionally adjusted.

3.4. Interaction between the animal-based dietary pattern and green space on cognitive function

In addition, according to the results of the Cox proportional hazard

Table 2
The association between long-term green space exposure and risks of cognitive impairment.

	The average green space coverage rate (continuous variable) HR (95% CI)	The average green space coverage rate (categorical variable)		
		Low HR (95% CI)	Intermediate HR (95% CI)	High HR (95% CI)
Model1	4.29e-14(7.59e-17,2.43e-11)	1 (Reference)	1.03 (0.93,1.13)	0.75 (0.68,0.83)
Model2	2.70e-12(4.54e-15,1.61e-09)	1 (Reference)	1.00 (0.91,1.10)	0.78 (0.71,0.87)
Model3	8.76e-12(1.56e-14,4.93e-09)	1 (Reference)	1.04 (0.94,1.14)	0.81 (0.73,0.90)

Note: These were the results of Cox proportional hazard regression model. Data presented hazard ratio (95% confidence interval).

regression model (Model 3), there was a significant interaction between green space and ADI. The protective effect of the intermediate green space group on cognitive impairment was much more apparent among participants with low ADI (HR = 1.01, 95% CI: 0.88, 1.15) than participants with high ADI (HR = 1.07, 95% CI: 0.94, 1.23). However, the relationships were not significant. Furthermore, the protective effect of the highest green space group on cognitive impairment was much more evident among participants with low ADI (HR = 0.73, 95% CI: 0.63, 0.85), compared to those with high ADI (HR = 0.88, 95% CI: 0.77, 1.02) (Table 4, Figs. 1 and 3).

Similar interactions were also observed for sensitivity analyses. The results of the stratified analysis by each animal-based food group are shown in Table S2. The risk estimates of green space exposure on cognitive function were similar among the food of meat. The main results were consistent for the 3-year average green space (Table S3 and Table S4). When we categorized participants into six groups according to different combinations of ADI (low or high) and green space (low, intermediate, and high) to test the interaction of ADI and green space with the risk of cognitive impairment, the result was stable (Table S5 and Fig. S2). Similar results were also found in participants with complete data (Table S6, Table S7, and Fig. S8). The primary results were consistent with a sensitivity analysis that excluded participants who died within two years of follow-up (Tables S9–S11).

In Model 1, the covariates were age and gender. In Model 2, residence, ethnicity, living pattern, current marital status, and education level were further supplemented. In Model 3, smoking status, alcohol consumption, regular exercise, weight, and self-reported history of diseases (heart disease, hypertension, and diabetes) were additionally adjusted.

4. Discussion

In this prospective cohort study, we verified the role of green space and the animal-based dietary pattern on cognition in Chinese elderly participants. We found that people living in areas with higher green space tended to have better cognitive abilities. The animal-based dietary pattern can significantly modify this association. The protective effect of green space on cognition was attenuated among people with higher ADI. However, among those people with lower ADI, the association increased. This study suggested that lower adherence to the animal-based dietary pattern might benefit cognitive impairment from limited green space exposure.

With the aging of the population, cognitive impairment had gradually become an urgent health problem globally (Marron et al., 2018). Studies had shown that one person was diagnosed with dementia approximately every 3 s in the world (Prince et al., 2015). The prevalence of mild cognitive impairment in older adults over 65 years old in Korea was 22.6% (Won and Kim, 2008). A meta-analysis indicated that the mild cognitive impairment of Chinese elderly people had a pooled prevalence of 15.4% (Deng et al., 2021). China had the largest population of patients with dementia in the world. At the end of 2013, the total number of patients in China with cognitive impairment reached 8.18 million. With an annual increase of more than 0.36 million, the total number of patients with cognitive impairment was expected to reach 48.68 million by 2060 (Prince et al., 2015; Wu et al., 2019). The United States had the second highest number of dementia patients in the world after China (4.2 million) (Prince et al., 2015). Cognitive impairment was the leading cause of disability in the elderly population, and it seriously affected their quality of life (Hussenoeder et al., 2020; Peng et al., 2020). As well, disorders associated with severe cognitive impairment contributed hugely to the social care burden and health care costs (Vossius et al., 2011). Thus, identifying any modifiable factors that promote cognitive health could have significant public health implications. Despite numerous studies over the past few decades, breakthroughs had not been made in experimental interventions for disorders associated with cognitive impairment, and alternative pharmacological interventions had not emerged (Lanni et al., 2019). Therefore, lifestyle interventions were the most feasible and appropriate approach to improving cognitive impairment currently (Kivipelto et al., 2018). To provide vital clues for potential preventive control measures, population-based longitudinal studies in this area were urgently needed.

In recent years, people had paid more attention to the influence of environmental factors on psychology, such as cognition (James et al., 2015). The environmental factor of green space was even more critical due to the continuous degradation of green space originating from the intensification of urbanization in today's world (Cotella et al., 2020). Numerous studies pointed to the benefits of green space for brain health. There might be several potential mechanisms as follows. First, green spaces could reduce air pollution, noise, and heat, thereby reducing cognitive damage (Dadvand et al., 2015; Kuo, 2015). Second, green spaces could provide an environment that promotes physical activity and reduces stress (Kahn and Kellert, 2003; Maas et al., 2009; Ward et al., 2016) and thus might be associated with better brain health by affecting cerebral blood flow, angiogenesis, and integrity, cell proliferation and survival, vascular dysregulation and inflammation (Wilker et al., 2015). Furthermore, green space could modulate biodiversity, especially environmental microbial communities (Dockx et al., 2021). The environmental microbial community not only regulated the human immune system but also participated in shaping the human microbiome. For example, the gut microbiome could leverage the innate immune system to influence the regulatory functions of the brain (Galland, 2014; Rook, 2013). Finally, exposure to green space could relieve people's mental fatigue, reduce stress, and improve people's concentration to prevent cognitive impairment. Stress and attention recovery theories are consistent with this (Besser, 2021).

In this study, we verified that the animal-based dietary pattern was a cognitive disadvantage in Chinese elderly participants. Some human studies had demonstrated that animal-based dietary pattern is bad for cognition (Granic et al., 2016; Titova et al., 2013). However, other human studies showed different results (Crichton et al., 2015). In one animal study, Bruce-Keller AJ (Bruce-Keller et al., 2015) transplanted microbiota isolated from donors fed a high-fat diet into other healthy mice, disrupting the gut barrier and causing cognitive decline in normal mice. As far as we know, animal-based diets contained less fiber than plant-based diets. However, dietary fiber could delay cognitive decline. Shi reported that a fiber-deprived diet leads to cognitive impairment by altering the gut microbiota-hippocampal axis. Their research confirmed that increasing dietary fiber intake reduced the risk of diet-related cognitive impairment and some neurodegenerative diseases (Shi et al., 2021). Liu's study indicated that high dietary fiber intake was an effective intervention for cognitive impairment caused by maternal obesity (Li et al., 2021). Furthermore, Cristina Andrés-Lacueva pointed out that plant foods such as fruits and vegetables were rich in polyphenols and other bioactive compounds that help reducing the risk of age-related cognitive decline (Andres-Lacueva et al., 2005).

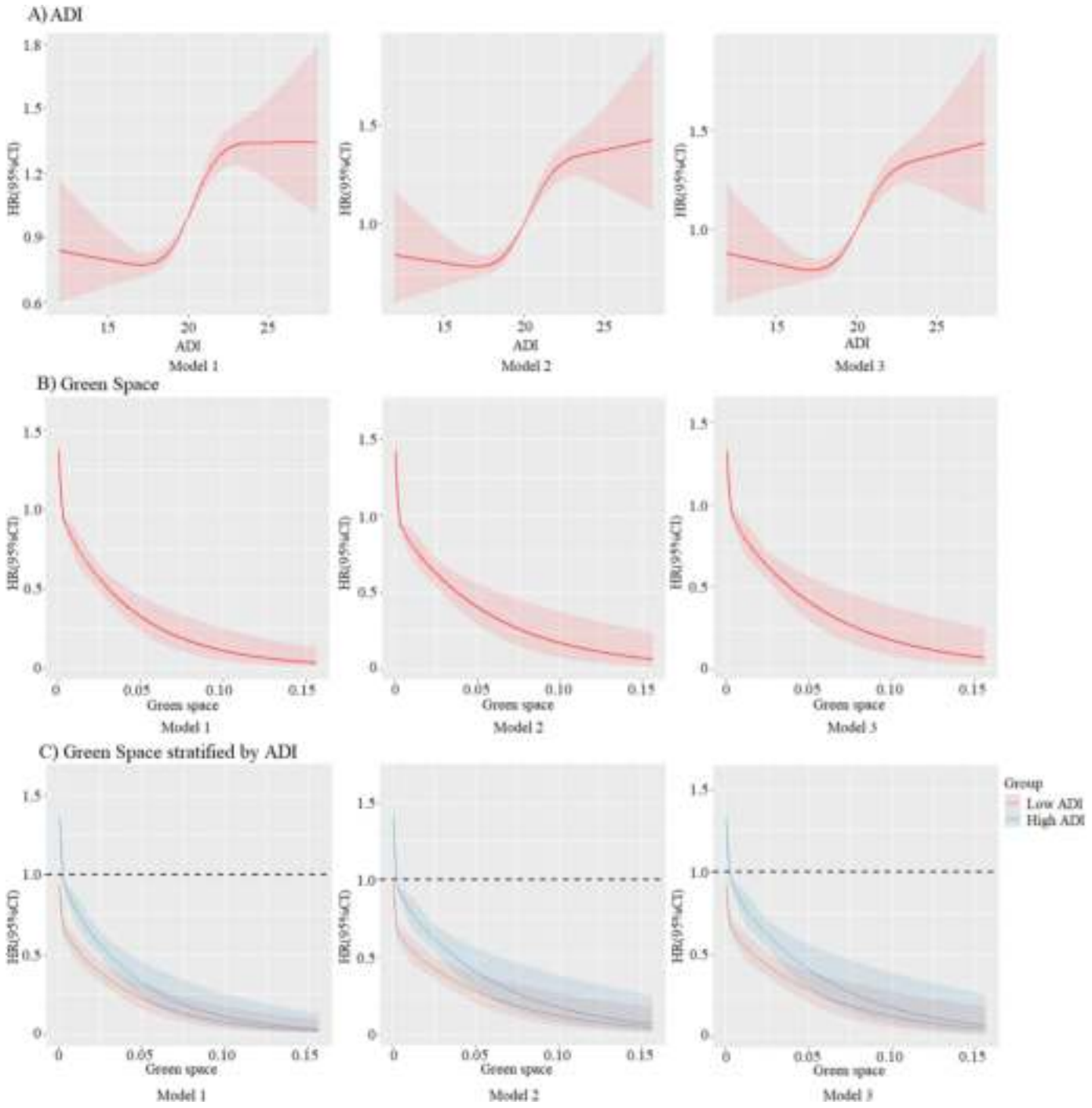

Fig. 1. Restricted cubic splines for animal-based diet index and risks of cognitive impairment (A). Restricted cubic splines for long-term green space exposure and risks of cognitive impairment (B). And restricted cubic splines for long-term green space exposure and risks of cognitive impairment, stratified by ADI (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Evidence on the interaction of green space and the animal-based dietary pattern with cognition was limited. However, studies had demonstrated that diet could interact with environmental factors to influence cognition and other health outcomes. For example, Zhu believed that eating plant-based foods and sticking to the plant-based dietary pattern could alleviate cognitive impairment caused by long-term exposure to PM2.5 (Zhu et al., 2022). Green space could significantly reduce air pollution. The development of urbanization was a trend in today's society, which was changing the way people interact with the environment (Nieuwenhuijsen et al., 2017; Zenghelis and Stern, 2016). This included reducing human contact with green space. Promoting shifts in dietary preferences and dietary patterns through health education might be a cost-effective strategy to help green space collectively reducing the associated disease burden caused by environmental factors.

The strengths of this study were as follows. First, no study had tested the interaction of green space and the animal-based dietary pattern with cognition in older adults to our knowledge. This study was the first to examine this question, and the new findings provided a new perspective on improving cognitive function. Second, the sample used in this study was representative and significant. Compared to other studies examining the risk factors related to cognitive function (Lv et al., 2019; Ren

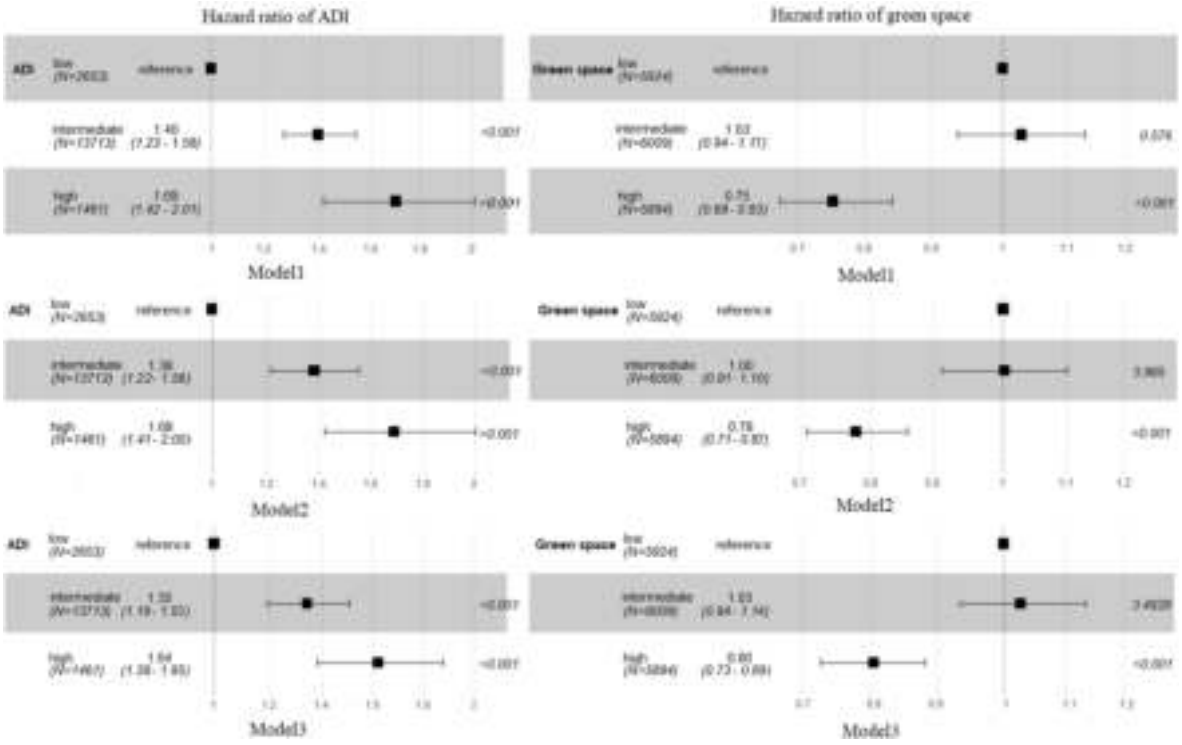

Fig. 2. HR (95% CI) of developing poor cognitive function by groups of animal-based diet index (left). And HR (95% CI) of developing poor cognitive function by groups of long-term green space exposure (right).

Table 3
The association between animal-based dietary index and risks of cognitive impairment.

	ADI (continuous variable) HR (95% CI)	ADI (categorical variable)		
		Low HR (95% CI)	Intermediate HR (95% CI)	High HR (95% CI)
Model1	1.08(1.06,1.10)	1(Reference)	1.40(1.23,1.58)	1.69 (1.42,2.01)
Model2	1.08(1.06,1.10)	1(Reference)	1.38(1.22,1.56)	1.68 (1.41,2.00)
Model3	1.08(1.06,1.09)	1(Reference)	1.35(1.19,1.53)	1.64 (1.38,1.96)

Note: These were the results of Cox proportional hazard regression model. Data presented hazard ratio (95% confidence interval).

Table 4
The association between long-term green space exposure and risks of cognitive impairment, stratified by ADI.

	Model1 HR (95% CI)	Model2 HR (95% CI)	Model3 HR (95% CI)
High ADI (continuous variable)	2.02e-12(3.90e-16,1.04e-08)	1.24e-10(2.15e-14,7.19e-07)	2.64e-10(4.94e-14,1.41e-06)
Low ADI (continuous variable)	1.73e-16(1.18e-20,2.54e-12)	1.01e-14(6.34e-19,1.62e-10)	1.21e-13(8.27e-18,1.78e-09)
High ADI (categorical variable)			
Low average green space coverage rate	1(Reference)	1(Reference)	1(Reference)
Intermediate average green space coverage rate	1.05(0.92,1.21)	1.03(0.90,1.18)	1.07(0.94,1.23)
High average green space coverage rate	0.83(0.72,0.96)	0.86(0.75,1.00)	0.88(0.77,1.02)
Low ADI (continuous variable)			
Low average green space coverage rate	1(Reference)	1(Reference)	1(Reference)
Intermediate average green space coverage rate	1.00(0.88,1.14)	0.98(0.85,1.11)	1.01(0.88,1.15)
High average green space coverage rate	0.67(0.58,0.77)	0.70(0.60,0.80)	0.73(0.63,0.85)

Note: These were the results of Cox proportional hazard regression model. Data presented hazard ratio (95% confidence interval).

et al., 2021; Zhang et al., 2019), our analysis adjusted for more covariates such as ethnicity and whether living alone. Finally, this study covered 22 provinces in China, which helped ensure the robustness of our findings.

The limitations of this study were as follows. First, there were still some variables that are not collected in the database that cannot be controlled. However, we adjusted for covariates selected from previous studies. Second, the participants in this study were Chinese elderly. Therefore, careful consideration should be given when generalizing the findings of this study to populations from other countries, other age groups, and other ethnic groups. Third, the metric we used to measure animal food intake was intake frequency, rather than the specific serving size, which made it impossible to investigate whether total energy intake had an effect. However, studies had shown that intake frequency was more important than portion size when distinguishing between high and low consumption of animal foods. Fourth, we used the MMSE scale rather than an accurate clinical diagnosis for the diagnosis of cognitive impairment. However, previous studies had demonstrated that the Chinese version of the adapted MMSE scale was reliable and valid (An and Liu, 2016; Zhang et al., 2021; Ren et al., 2021). Finally, we used the average green space coverage rate as the indicator to assess the green space. We didn't take into account the resolution for the green space coverage. And there were not defined grids. This was the direction for our future research.

5. Conclusions

In this study, we researched the beneficial roles of green space on cognition and the detrimental effects of the animal-based dietary pattern

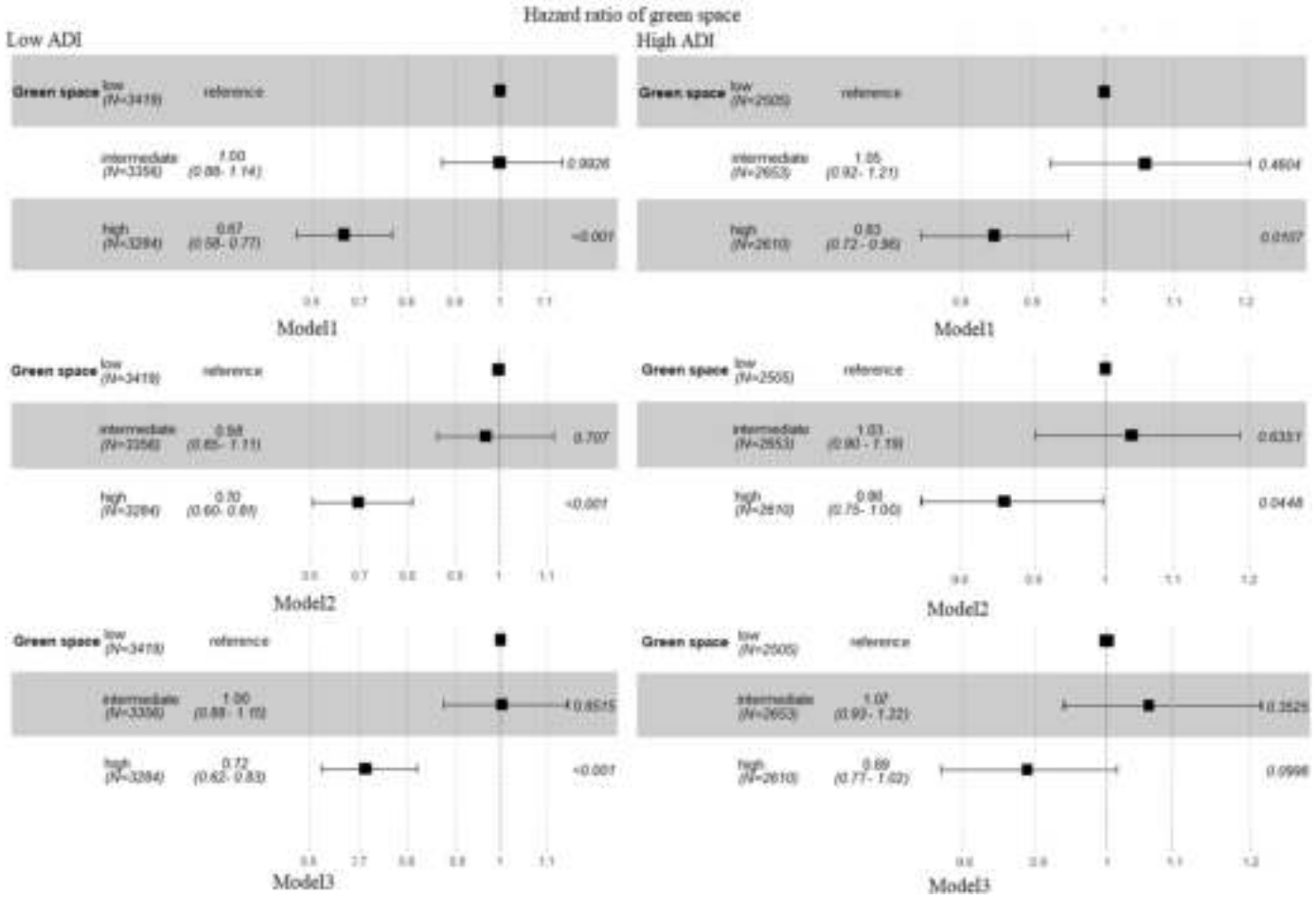

Fig. 3. HR (95% CI) of developing poor cognitive function by groups of long-term green space exposure, stratified by ADI.

on cognition. In addition, we found that the impact of green space exposure on the development of cognitive function was higher among the participants with lower ADI. Our findings pointed out that reducing the intake of animal-based diets as well as increasing the accessibility to green space could improve cognitive function. Changing the dietary pattern might be a strategy to increase the neurological health effects of green spaces.

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Ethics approval

This study was approved by the Peking University Biomedical Ethics Committee and the Duke University Health System Institutional Review Board (IRB00001052-13074). All participants or their legal representatives signed written consent forms to participate in the baseline and follow-up surveys.

Declaration of competing interest

All authors of this manuscript have directly participated in planning, execution, and analysis of this study.

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I am one author signing on behalf of all co-authors of this manuscript, and attesting the above.

Availability of data and materials

The CLHLS questionnaires are available at https://sites.duke.edu/centerforaging/programs/chinese-longitudinal-healthy-longevity-survey-clhls/survey-documentation/questionnaires/. The full datasets used in this analysis are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Wan Hu: Investigation, Data curation, Methodology, Software, Visualization, Writing – original draft. **Hengchuan Zhang:** Formal analysis. **Ruyu Ni:** Formal analysis. **Yawen Cao:** Validation. **Wenbin Fang:** Software. **Yingying Chen:** Data curation. **Guixia Pan:** Conceptualization, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare they have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114147.

References

An, R., Liu, G.G., 2016. Cognitive impairment and mortality among the oldest-old Chinese. Int. J. Geriatr. Psychiatr. 31, 1345–1353.
Andres-Lacueva, C., Shukitt-Hale, B., Galli, R.L., Jauregui, O., Lamuela-Raventos, R.M., Joseph, J.A., 2005. Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory. Nutr. Neurosci. 8, 111–120.
Beere, P., Kingham, S., 2017. Assessing the relationship between greenspace and academic achievement in urban New Zealand primary schools. N. Z. Geogr. 73.
Besser, L., 2021. Outdoor green space exposure and brain health measures related to Alzheimer's disease: a rapid review. BMJ Open 11, e043456.
Browning, M.H.E.M., Locke, D.H., 2020. The greenspace-academic performance link varies by remote sensing measure and urbanicity around Maryland public schools. Landsc. Urban Plann. 195, 103706.
Bruce-Keller, A.J., Salbaum, J.M., Luo, M., Blanchard, E.t., Taylor, C.M., Welsh, D.A., Berthoud, H.R., 2015. Obese-type gut microbiota induce neurobehavioral changes in the absence of obesity. Biol. Psychiatr. 77, 607–615.
Cao, J., Qian, D., Yang, F., 2020. Socioeconomic disparities in leisure activities over the life course of the oldest-old in China. Australas. J. Ageing 39, e416–e424.
Cassarino, M., Setti, A., 2015. Environment as 'Brain Training': a review of geographical and physical environmental influences on cognitive ageing. Ageing Res. Rev. 23, 167–182.
Cotella, G., Evers, D., Janin Rivolin, U., Solly, A., Berisha, E., 2020. Espon Super – Sustainable Urbanisation and Land-use Practices in European Regions. Guide Sustain. Urban. Land-Use.
Crichton, G.E., Elias, M.F., Davey, A., Alkerwi, A., Dore, G.A., 2015. Higher cognitive performance is prospectively associated with healthy dietary choices: the Maine syracuse longitudinal study. J. Prev. Alzheimers Dis. 2, 24–32.
Dadvand, P., Nieuwenhuijsen, M.J., Esnaola, M., Forns, J., Basagaña, X., Alvarez-Pedrerol, M., Rivas, I., López-Vicente, M., de Castro Pascual, M., Su, J., Jerrett, M., Querol, X., Sunyer, J., 2015. Green spaces and cognitive development in primary schoolchildren. Proc. Natl. Acad. Sci. U. S. A. 112, 7937–7942.
de Vries, S., Ten Have, M., van Dorsselaer, S., van Wezep, M., Hermans, T., de Graaf, R., 2016. Local availability of green and blue space and prevalence of common mental disorders in The Netherlands. BJPsych Open 2, 366–372.
Deng, Y., Zhao, S., Cheng, G., Yang, J., Li, B., Xu, K., Xiao, P., Li, W., Rong, S., 2021. The prevalence of mild cognitive impairment among Chinese People: A meta-analysis. Neuroepidemiology 55, 79–91.
Dewey, M.E., Saz, P., 2001. Dementia, cognitive impairment and mortality in persons aged 65 and over living in the community: a systematic review of the literature. Int. J. Geriatr. Psychiatr. 16, 751–761.
Dockx, Y., Täubel, M., Bijnens, E.M., Witters, K., Valkonen, M., Jayaprakash, B., Hogervorst, J., Nawrot, T.S., Casas, L., 2021. Residential green space can shape the indoor microbial environment. Environ. Res. 201, 111543.
Folstein, M.F., Folstein, S.E., McHugh, P.R., 1975. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. J. Psychiatr. Res. 12, 189–198.
Galland, L., 2014. The gut microbiome and the brain. J. Med. Food 17, 1261–1272.
Gao, S., Jin, Y., Unverzagt, F.W., Cheng, Y., Su, L., Wang, C., Ma, F., Hake, A.M., Kettler, C., Chen, C., Liu, J., Bian, J., Li, P., Murrell, J.R., Clark, D.O., Hendrie, H.C., 2014. Cognitive function, body mass index and mortality in a rural elderly Chinese cohort. Arch. Publ. Health 72, 9.
Granic, A., Davies, K., Adamson, A., Kirkwood, T., Hill, T.R., Siervo, M., Mathers, J.C., Jagger, C., 2016. Dietary patterns high in red meat, potato, gravy, and butter are associated with poor cognitive functioning but not with rate of cognitive decline in very old adults. J. Nutr. 146, 265–274.
Hodson, C., Sander, H., 2017. Green urban landscapes and school-level academic performance. Landsc. Urban Plann. 160, 16–27.
Hussenoeder, F.S., Conrad, I., Roehr, S., Fuchs, A., Pentzek, M., Bickel, H., Moesch, E., Weyerer, S., Werle, J., Wiese, B., Mamone, S., Brettschneider, C., Heser, K., Kleineidam, L., Kaduszkiewicz, H., Eisele, M., Maier, W., Wagner, M., Scherer, M., König, H.H., Riedel-Heller, S.G., 2020. Mild cognitive impairment and quality of life in the oldest old: a closer look. Qual. Life Res. 29, 1675–1683.
Hystad, P., Payette, Y., Noisel, N., Boileau, C., 2019. Green space associations with mental health and cognitive function: results from the Quebec CARTaGENE cohort. Environ. Epidemiol. 3, e040.
James, P., Banay, R.F., Hart, J.E., Laden, F., 2015. A review of the health benefits of greenness. Curr. Epidemiol. Rep. 2, 131–142.
Jimenez, M.P., Elliott, E.G., DeVille, N.V., Laden, F., Hart, J.E., Weuve, J., Grodstein, F., James, P., 2022. Residential green space and cognitive function in a large cohort of middle-aged women. JAMA Netw. Open 5, e229306.
Kahn, P.H., Kellert, S.R., 2003. Children and nature: psychological, sociocultural and evolutionary investigations. Contemp. Sociol. 32, 733.
Kivipelto, M., Mangialasche, F., Ngandu, T., 2018. Lifestyle interventions to prevent cognitive impairment, dementia and Alzheimer disease. Nat. Rev. Neurol. 14, 653–666.
Kuo, M., 2015. How might contact with nature promote human health? Promising mechanisms and a possible central pathway. Front. Psychol. 6, 1093.
Kuo, M., Browning, M., Sachdeva, S., Lee, K., Westphal, L., 2018. Might school performance grow on trees? Examining the link between "greenness" and academic achievement in urban, high-poverty schools. Front. Psychol. 9, 1669.
Kweon, B.-S., Ellis, C.D., Lee, J., Jacobs, K., 2017. The link between school environments and student academic performance. Urban For. Urban Green. 23, 35–43.
Langa, K.M., Larson, E.B., Karlawish, J.H., Cutler, D.M., Kabeto, M.U., Kim, S.Y., Rosen, A.B., 2008. Trends in the prevalence and mortality of cognitive impairment in the United States: is there evidence of a compression of cognitive morbidity? Alzheimers Dement 4, 134–144.
Lanni, C., Fagiani, F., Racchi, M., Preda, S., Pascale, A., Grilli, M., Allegri, N., Govoni, S., 2019. Beta-amyloid short- and long-term synaptic entanglement. Pharmacol. Res. 139, 243–260.
Li, D., Chiang, Y.-C., Sang, H., Sullivan, W.C., 2019. Beyond the school grounds: links between density of tree cover in school surroundings and high school academic performance. Urban For. Urban Green. 38, 42–53.
Li, Y., Jiang, H., Jin, X., Wang, H., Ji, J.S., Yan, L.L., 2021. Cognitive impairment and all-cause mortality among Chinese adults aged 80 years or older. Brain Behav. 11, e2325.
Lin, M.-N., Chiu, T.H., Chang, C.-E., Lin, M.-N., 2019. The inpact of a plant-based dietary pattern on dementia risk: a prospective cohort study. Innovat. Aging 3. S734-S734.
Liu, E., Feng, Y., Yue, Z., Zhang, Q., Han, T., 2019. Differences in the health behaviors of elderly individuals and influencing factors: evidence from the Chinese Longitudinal Healthy Longevity Survey. Int. J. Health Plann. Manag. 34, e1520–e1532.
Liu, I.Y., LaCroix, A.Z., White, L.R., Kittner, S.J., Wolf, P.A., 1990. Cognitive impairment and mortality: a study of possible confounders. Am. J. Epidemiol. 132, 136–143.
Lv, X., Li, W., Ma, Y., Chen, H., Zeng, Y., Yu, X., Hofman, A., Wang, H., 2019. Cognitive decline and mortality among community-dwelling Chinese older people. BMC Med. 17, 63.
Lv, Y.B., Gao, X., Yin, Z.X., Chen, H.S., Luo, J.S., Brasher, M.S., Kraus, V.B., Li, T.T., Zeng, Y., Shi, X.M., 2018. Revisiting the association of blood pressure with mortality in oldest old people in China: community based, longitudinal prospective study. BMJ 361, k2158.
Maas, J., van Dillen, S.M.E., Verheij, R.A., Groenewegen, P.P., 2009. Social contacts as a possible mechanism behind the relation between green space and health. Health Place 15, 586–595.
Marešová, P., Mohelská, H., Dolejš, J., Kuča, K., 2015. Socio-economic aspects of alzheimer's disease. Curr. Alzheimer Res. 12, 903–911.
Markevych, I., Schoierer, J., Hartig, T., Chudnovsky, A., Hystad, P., Dzhambov, A.M., de Vries, S., Triguero-Mas, M., Brauer, M., Nieuwenhuijsen, M.J., Lupp, G., Richardson, E.A., Astell-Burt, T., Dimitrova, D., Feng, X., Sadeh, M., Standl, M., Heinrich, J., Fuertes, E., 2017. Exploring pathways linking greenspace to health: theoretical and methodological guidance. Environ. Res. 158, 301–317.
Marron, E.M., Viejo-Sobera, R., Quintana, M., Redolar-Ripoll, D., Rodríguez, D., Garolera, M., 2018. Transcranial magnetic stimulation intervention in Alzheimer's disease: a research proposal for a randomized controlled trial. BMC Res. Notes 11, 648.
Massetti, L., Petralli, M., Napoli, M., Brandani, G., Orlandini, S., Pearlmutter, D., 2019. Effects of deciduous shade trees on surface temperature and pedestrian thermal stress during summer and autumn. Int. J. Biometeorol. 63, 467–479.
Medawar, E., Huhn, S., Villringer, A., Veronica Witte, A., 2019. The effects of plant-based diets on the body and the brain: a systematic review. Transl. Psychiatry 9, 226.
Mohammadifard, N., Sajjadi, F., Maghroun, M., Alikhasi, H., Nilforoushzadeh, F., Sarrafzadegan, N., 2015. Validation of a simplified food frequency questionnaire for the assessment of dietary habits in Iranian adults: isfahan Healthy Heart Program, Iran. ARYA Atheroscler 11, 139–146.
Nguyen, H.T., Black, S.A., Ray, L.A., Espino, D.V., Markides, K.S., 2003. Cognitive impairment and mortality in older mexican americans. J. Am. Geriatr. Soc. 51, 178–183.
Nieuwenhuijsen, M.J., Khreis, H., Triguero-Mas, M., Gascon, M., Dadvand, P., 2017. Fifty shades of green: pathway to healthy urban living. Epidemiology 28, 63–71.
Peng, T.C., Chen, W.L., Wu, L.W., Chang, Y.W., Kao, T.W., 2020. Sarcopenia and cognitive impairment: A systematic review and meta-analysis. Clin. Nutr. 39, 2695–2701.
Prince, M., Wimo, A., Guerchet, M., Ali, G.C., Wu, Y.T., Prina, M., 2015. World Alzheimer Report 2015. The Global Impact of Dementia. An Analysis of Prevalence, Incidence, Cost and Trends. Alzheimer's Disease International, London, England.
Rajaram, S., Jones, J., Lee, G.J., 2019. Plant-based dietary patterns, plant foods, and age-related cognitive decline. Adv. Nutr. 10, S422–s436.
Ren, Z., Li, Y., Li, X., Shi, H., Zhao, H., He, M., Zha, S., Qiao, S., Pu, Y., Liu, H., Zhang, X., 2021. Associations of body mass index, waist circumference and waist-to-height ratio with cognitive impairment among Chinese older adults: based on the CLHLS. J. Affect. Disord. 295, 463–470.

Rook, G.A., 2013. Regulation of the immune system by biodiversity from the natural environment: an ecosystem service essential to health. Proc. Natl. Acad. Sci. U. S. A. 110, 18360–18367.
Saeedi, P., Skeaff, S.A., Wong, J.E., Skidmore, P.M., 2016. Reproducibility and relative validity of a short food frequency questionnaire in 9-10 Year-old children. Nutrients 8.
Satija, A., Bhupathiraju, S.N., Rimm, E.B., Spiegelman, D., Chiuve, S.E., Borgi, L., Willett, W.C., Manson, J.E., Sun, Q., Hu, F.B., 2016. Plant-based dietary patterns and incidence of type 2 diabetes in US men and women: results from three prospective cohort studies. PLoS Med. 13, e1002039.
Shi, H., Ge, X., Ma, X., Zheng, M., Cui, X., Pan, W., Zheng, P., Yang, X., Zhang, P., Hu, M., Hu, T., Tang, R., Zheng, K., Huang, X.F., Yu, Y., 2021. A fiber-deprived diet causes cognitive impairment and hippocampal microglia-mediated synaptic loss through the gut microbiota and metabolites. Microbiome 9, 223.
Shipley, B.A., Der, G., Taylor, M.D., Deary, I.J., 2007. Association between mortality and cognitive change over 7 years in a large representative sample of UK residents. Psychosom. Med. 69, 640–650.
Titova, O.E., Ax, E., Brooks, S.J., Sjögren, P., Cederholm, T., Kilander, L., Kullberg, J., Larsson, E.M., Johansson, L., Ahlström, H., Lind, L., Schiöth, H.B., Benedict, C., 2013. Mediterranean diet habits in older individuals: associations with cognitive functioning and brain volumes. Exp. Gerontol. 48, 1443–1448.
van Gelder, B.M., Tijhuis, M.A., Kalmijn, S., Giampaoli, S., Kromhout, D., 2007. Decline in cognitive functioning is associated with a higher mortality risk. Neuroepidemiology 28, 93–100.
Vlosky, D.A., 2008. Healthy Longevity in China: Demographic, Socioeconomic, and Psychological Dimensions Series.
Vossius, C., Larsen, J.P., Janvin, C., Aarsland, D., 2011. The economic impact of cognitive impairment in Parkinson's disease. Mov. Disord. 26, 1541–1544.
Ward, J.S., Duncan, J.S., Jarden, A., Stewart, T., 2016. The impact of children's exposure to greenspace on physical activity, cognitive development, emotional wellbeing, and ability to appraise risk. Health Place 40, 44–50.
Wilker, E.H., Preis, S.R., Beiser, A.S., Wolf, P.A., Au, R., Kloog, I., Li, W., Schwartz, J., Koutrakis, P., DeCarli, C., Seshadri, S., Mittleman, M.A., 2015. Long-term exposure to fine particulate matter, residential proximity to major roads and measures of brain structure. Stroke 46, 1161–1166.
Won, J.S., Kim, K.H., 2008. [Evaluation of cognitive functions, depression, life satisfaction among the elderly receiving visiting nursing services]. Taehan Kanho Hakhoe Chi 38, 1–10.
Wong, J.E., Parnell, W.R., Black, K.E., Skidmore, P.M., 2012. Reliability and relative validity of a food frequency questionnaire to assess food group intakes in New Zealand adolescents. Nutr. J. 11, 65.
Wu, J., Song, X., Chen, G.C., Neelakantan, N., van Dam, R.M., Feng, L., Yuan, J.M., Pan, A., Koh, W.P., 2019. Dietary pattern in midlife and cognitive impairment in late life: a prospective study in Chinese adults. Am. J. Clin. Nutr. 110, 912–920.
Yu, Y., Yuan, C., Zhang, Q., Song, C., Cui, S., Ye, J., Zhang, X., Chen, C., 2021. Longitudinal association between home and community-based services provision and cognitive function in Chinese older adults: evidence from the Chinese Longitudinal Healthy Longevity Survey. Health Soc. Care Community 29, e288–e298.
Zeng, Y., Feng, Q., Hesketh, T., Christensen, K., Vaupel, J.W., 2017. Survival, disabilities in activities of daily living, and physical and cognitive functioning among the oldest-old in China: a cohort study. Lancet 389, 1619–1629.
Zenghelis, D., Stern, N., 2016. This Is Humankind's 'great Urbanisation' Era. We Must Do it Right, or the Planet Will Pay.
Zhang, L., Luo, Y., Zhang, Y., Pan, X., Zhao, D., Wang, Q., 2022. Green space, air pollution, weather, and cognitive function in middle and old age in China. Front. Public Health 10, 871104.
Zhang, M., Lv, X., Chen, Y., Tu, L., Fan, Z., Yao, Y., Yu, X., Guan, N., Wang, H., 2021. Excessive sleep increased the risk of incidence of cognitive impairment among older Chinese adults: a cohort study based on the Chinese Longitudinal Healthy Longevity Survey (CLHLS). Int. Psychogeriatr. 1–10.
Zhang, Q., Wu, Y., Han, T., Liu, E., 2019. Changes in cognitive function and risk factors for cognitive impairment of the elderly in China: 2005-2014. Int. J. Environ. Res. Publ. Health 16.
Zhu, A., Chen, H., Shen, J., Wang, X., Li, Z., Zhao, A., Shi, X., Yan, L., Zeng, Y., Yuan, C., Ji, J.S., 2022. Interaction between plant-based dietary pattern and air pollution on cognitive function: a prospective cohort analysis of Chinese older adults. Lancet Reg. Health West Pac. 20, 100372.

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Open defaecation by proxy: Tackling the increase of disposable diapers in waste piles in informal settlements

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ABSTRACT

Disposable diapers are becoming increasingly popular and present an emerging challenge for global waste management, particularly within LMICs. They offer a cheap and convenient way for caregivers to manage child excreta; however, insufficient understanding of safe disposal methods, combined with limited access to waste management services results in hazardous disposal. Used diapers are being increasingly found dumped in the open environment, including in water bodies and in open fields, leading to faecal contamination of the environment and an enhanced risk of transmission of faecal-oral diseases such as cholera and typhoid. United Nations SDG 6 aims to end open defaecation globally by 2030; however, improper disposal of used diapers will hamper progress towards reaching this goal. In this review, we identify current trends in use and subsequent disposal of single use disposable diapers in LMICs, and critically discuss the environmental and public health impacts of current practices, and potential solutions to address these challenges. Contemporary methods for managing the disposal of single use diapers for communities in LMICs tend to be cost prohibitive with few alternative options other than dumping in the environment. Modern cloth diapers offer a low waste alternative to disposable diapers but often carry an unaffordable high upfront cost. Here, in addition to advocating improved efforts by governments to upgrade access and quality of waste management services, we recommend the design and implementation of intervention schemes aimed to increase awareness of safe and hygienic disposal practices for disposable diapers.

1. Introduction

In 2020, it was estimated that 1.7 billion people lacked access to basic sanitation services, with 494 million people practicing open defaecation (WHO & UNICEF, 2021). Open defaecation is defined as the disposal of human faeces in the open environment, such as in fields, forests, water bodies, or with municipal solid waste (WHO & UNICEF, 2021). However, the concept of open defaecation also extends to the disposal of faeces contained within other materials, such as plastic bags or diapers, where there is potential for the faeces to become exposed to the open environment (WHO & UNICEF, 2021). Irrespective of the pathway, open defaecation poses a significant risk to public health and is widely associated with an increased prevalence of diarrhoeal diseases, particularly among children (Njuguna 2016; Ayalew et al., 2018; Anandan et al., 2021). In 2019, diarrhoeal disease was ranked as the fifth leading cause of disease worldwide, responsible for over 1.53 million deaths, with one third of these being children under 10 years of age (Abbafati et al., 2020; IHME 2020). In the last decade, there has

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been a marked increase in the global usage of disposable diapers, with sales in 2021 reaching 7.9 billion kilograms, a 36 % increase compared to 2013 (Brinckmann 2022). Growth is expected to continue to rise, increasing most rapidly in low- and middle-income countries (LMICs), with significant consequences for both environmental and human health (Tembo and Chazireni 2016; Ntekpe et al., 2020; Brinckmann 2022).

A concerted effort has been made towards improving global sanitation conditions, with the United Nations (UN) Sustainable Development Goal 6 (SDG 6) aiming to end open defaecation, and provide universal access to drinking water, sanitation, and hygiene by 2030 (WHO & UNICEF, 2021). There is a significant volume of data demonstrating that the elimination of open defaecation can have positive health effects, by reducing the prevalence of diarrhoeal disease and other associated morbidities such as active trachoma and childhood stunting (Njuguna 2016; Rahman et al., 2020; Delelegn et al., 2021). Moreover, the implementation of water, sanitation, and hygiene (WaSH) infrastructure and practices, such as piped water supplies, sewer connections, and regular hand washing with soap and water, can further reduce the impacts of open defaecation and reduce diarrhoea-related morbidity (Wolf et al., 2018). However, whilst it has been reported that the world is currently on track to end open defaecation by 2030 (WHO & UNICEF, 2021), this will require a fourfold increase in rates of progress to achieve universal access to basic sanitation services (WHO & UNICEF, 2021).

Research on open defaecation has mainly focused on direct excretion in the environment, and largely neglected the contribution of disposable diapers. Under the UNICEF and World Health Organisation (WHO) Joint Monitoring Program (JMP), a defining feature of improved sanitation is the hygienic separation of humans from their excreta (WHO & UNICEF, 2021). Although at the point of use disposable diapers readily achieve such a barrier, this is often transitory (Kubiak et al., 1993; Kamat and Malkani 2003), with improper disposal leading to barrier breakdown, and the increased likelihood of human contact with faecal material (Reese et al., 2015). Despite this risk, guidelines regarding the safe disposal of children's excreta and single use diapers are indistinct, with guidelines stating that children's faeces can only be safely disposed of in a toilet or latrine, or by burying; however, no mention is given to disposable diapers despite their increasing popularity (Brinckmann 2022).

Typically, diapers are disposed of in municipal solid waste (MSW) and sent to landfill, or directly dumped in the environment, particularly in LMICs where waste management options are often limited (Reese et al., 2015; Muia 2018). Diapers contain a mixture of materials, including organic compounds such as cellulose, and a range of plastic polymers such as polypropylene, polyethylene, and super absorbent polymers; consequently, once diapers are in the environment, they can take decades or even centuries to degrade (Shuker and Cadman 2018; Roman et al., 2020; Płotka-Wasylka et al., 2022). In many countries, it is common for dogs, pigs, birds, and other vermin to roam over landfill sites and open waste piles in search of food (Doron 2021). Faecal material in diaper waste is often targeted by dogs and pigs who tear open diapers and release the faeces into the environment (Fig. 1) (Remigios 2014; Mathe 2018; Ntekpe et al., 2020). Human faeces can potentially harbour a range of pathogenic viruses, bacteria, protozoa, and helminths (Jex et al., 2012); and without hygienic separation, can lead to the transmission of enteric diseases and helminthiasis (Anandan et al., 2021), which together are responsible for over 90 % of all diarrhoea-related deaths (Troeger et al., 2018).

To date, there has been minimal research into understanding the trends in, and challenges behind, the use of disposable diapers in LMICs. Given the increasing global usage of disposable diapers and their potential to act as a reservoir for pathogenic microorganisms, there is a pressing need to raise awareness of the problems associated with diapers in the environment. Here, we critically discuss the challenges associated with the use of disposable diapers in LMICs, drawing particular attention to common disposal practices employed by caregivers, and the resultant impacts on environmental and human health.

Fig. 1. Stray dog rummaging through disposable diaper waste on an open dump site in Ndirande, Blantyre, Malawi.

2. Current trends in the use of disposable diapers

There has been a transition away from the use of traditional cloth diapers towards single-use disposables over the course of the last century (Krafchik 2016). In 2021, global sales of disposable diapers reached nearly 8 billion kilograms, generating US$47.9 billion in revenue, and are predicted to rise to 9.2 billion kilograms by 2026, an annual increase of 3.7 % since 2013 (Brinckmann 2022). Sales have grown most rapidly in Asia, Africa, and the Americas, and the largest future increases are likely to occur in LMICs; for example, Nigeria is expected to see a 117 % rise by 2026 (Brinckmann 2022). Disposable diapers are considered a premium product, with greater availability to those with higher household incomes and higher levels of education (Eke and Opara 2013; Muia 2018). Income is predicted to rise in LMICs (IMF 2022), and partially explains the expected rapid increases in sales of disposable diapers in these regions. However, higher birth rates in LMICs compared to high income countries are also likely to contribute to the growth in sales (The World Bank 2019b; Brinckmann 2022).

Shifts in consumer preference are also evident, with many caregivers in LMIC settings now choosing to use disposable diapers over traditional cloth diapers (Table 1). In some areas, such as in Nakuru, Kenya, almost 100 % of caregivers now choose disposable single-use diapers (Wambui et al., 2015). The primary reason given for this preference is that disposable diapers are more convenient, particularly when water for washing cloth diapers is scarce or limited, or when during the rainy season cloth diapers are more difficult to dry (Jesca and Junior 2015; Wambui et al., 2015; Muia 2018). They also provide better containment of child excreta, particularly through the night, and can reduce the incidence of diaper dermatitis (Kubiak et al., 1993; Counts et al., 2014; Wambui et al., 2015). Disposable diapers are also more convenient for working mothers and those with less time for washing cloth diapers (Mathe 2018). Although disposable diapers are becoming more affordable, their use is still associated with middle- and higher-income households (Jesca and Junior 2015; Agestika et al., 2022).

In a survey across 21 villages in the Kampong Speu and Battambang provinces of Cambodia, only 13 % of caregivers used disposable diapers, despite recognising that they were convenient, clean, and timesaving (Miller-Petrie et al., 2016). Most respondents reported that their child defaecated in a latrine, a potty, or in the yard (Miller-Petrie et al., 2016). Fifteen of these villages were in rural locations where low usage likely reflects limited accessibility of disposable diapers as rural communities often experience reduced connectivity to transport networks, combined with lower household incomes compared to urban areas (Population Reference Bureau, 2015; World Bank Group 2019). The average cost of a disposable diaper (US$0.43) was found to be nearly three times the price of a traditional cloth diaper (US$0.11) in these regions (Miller-Petrie

Table 1
Percentage of caregivers in some example LMICs that use disposable versus cloth diapers.

Country	Area	*n*	Child's age (years)	Disposable diaper (%)	Cloth diaper (%)	Both (%)	Reference
Zimbabwe	Urban	60	<2	78.0	18.0	4.0	(Jesca and Junior 2015)
Kenya	Urban	87	–	86.2	13.8		(Muia 2018)
Kenya	Urban	148	–	94.6	5.4		(Wambui et al., 2015)
Zimbabwe	Urban	380	–	60.7	24.6	12.9	(Nyamayedenga and Tsvere 2020)
Nigeria	Urban	141	<2	44.7	10.6	44.7	(Eke and Opara 2013)
Indonesia	Urban	184	<5	33.2	16.3		(Agestika et al., 2022)
Cambodia	Rural	129	<5	13.0	4.0		(Miller-Petrie et al., 2016)

et al., 2016). In contrast, the use of disposable diapers in urban areas can be high: in two densely populated suburbs of Nairobi, Kenya, over 86 % of caregivers used disposable diapers, with a significant positive association with both household income and level of education (Muia 2018).

As a single use product, sales are directly translated into waste, with billions of kilograms of diaper waste generated each year (Brinckmann 2022). Most diapers are disposed of indiscriminately, being either incinerated, or combined with municipal solid waste (MSW) and subsequently transferred into the open environment (Ellen MacArthur Foundation 2020; Ntekpe et al., 2020). Data collected from 19,000 sites across 82 countries between 2011 and 2018 identified disposable diapers as being among the top 25 most common items found on the sea floor and in the top 40 most common items found in terrestrial settings (Roman et al., 2020); diaperscan also comprise 21 % of waste found in waterways (e.g., in Indonesia; Shuker and Cadman 2018). Once in the environment, disposable diapers pose a risk to the environment, wildlife, and human health (Kordecki et al., 2022), as it can take centuries for the plastic components to decompose (Płotka-Wasylka et al., 2022).

3. Environmental hazards of disposable diapers

In many LMICs, there is often a lack of awareness of the appropriate methods for disposing of used diapers, and of the health and environmental implications of improper disposal (Remigios 2014; Mathe 2018; Muia 2018). Whilst product packaging encourages caregivers to dispose of diapers with MSW (Remigios 2014), they are also commonly discarded in latrines, or by burning or dumping in the environment (Fig. 2). Recommended practice also states that faecal material should be cleaned from diapers before disposal (UNICEF 2006; Remigios 2014); however, many caregivers believe this to be unnecessary or find it challenging without a reliable water supply or access to a working latrine.

The most common method of disposal is with household MSW, in compliance with manufacturer guidelines; however, this is only practiced by 35 % of caregivers (Fig. 2) and has limited relevance to those living within informal settlements where the provision of MSW services is minimal or absent (Kaza et al., 2018; Turpie et al., 2019). In LMICs the management of MSW is typically poor due to a lack of financial resources, infrastructure, and expertise (Muia 2018). The fraction of MSW that is mismanaged, i.e., unaccounted for, negatively correlates with GDP per capita (Lebreton and Andrady 2019). In sub–Saharan Africa and South Asia, only 44 % of MSW is collected, versus 100 % in North America, and 90 % in Europe and Central Asia (Kaza et al., 2018; Turpie et al., 2019). The mismanaged portion is most often dumped alongside roads, in waterways, or on open land; or burned in close proximity to communities, polluting the air, water, and soil. Even when collected, the majority of MSW in LMICs is disposed of in landfill, most of which operate as open dumpsites (Gutberlet and Uddin 2017; Kaza et al., 2018). Without proper management, hazardous leachates such as heavy metals and hydrocarbons can contaminate groundwater or be transported into surface waters (Akinola et al., 2018; Aralu et al., 2021). Harmful volatile and greenhouse gases are also generated from the decomposing waste (Chiemchaisri et al., 2019; Ngwabie et al., 2019) and the heat generated during biological and chemical decomposition of waste can lead to spontaneous fires (Chavan et al., 2022).

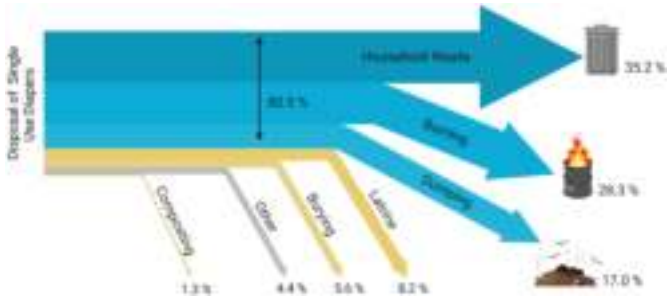

Fig. 2. Methods of discarding used disposable baby diapers by caregivers in LMICs. Values are weighted averages based on data collected from peer reviewed studies (Jesca and Junior 2015; Wambui et al., 2015; Miller-Petrie et al., 2016; Tembo and Chazireni 2016; Muia 2018; Nyamayedenga and Tsvere 2020; Agestika et al., 2022). Figure created with BioRender.com.

Besides anecdotal evidence, there is limited data discerning the exact quantity of diaper waste among MSW. Small-scale studies are highly variable and indicate that diapers make up between 0.2 and 22.2 % of total MSW (Reese et al., 2015; Perez et al., 2021). Countries with higher fertility rates and a more youthful population tend to have a higher percentage of disposable diapers in MSW. For example, in Bolivia, where 11.3 % of the population is under the age of 5 years and the fertility rate is 2.8 (births per woman), 12.1 % of MSW is made up of disposable diapers. In contrast, in Japan, where 4.0 % of the population is under the age of five and the fertility rate is 1.4, only 2.8 % of MSW consists of diapers (The World Bank 2019a; Perez et al., 2021). However, sampling methods may explain some of this variability, depending on whether waste is characterised when it arrives at dump sites or whether waste is directly surveyed at the household level (Thanh et al., 2010; Taboada-González et al., 2014).

Unreliable, insufficient, or non-existent household waste collection by local councils drives many people to manage their waste independently, with little choice but to burn or dump waste in their local environment (Remigios 2014; Jesca and Junior 2015; Nyamayedenga and Tsvere 2020). Over 28 % of caregivers choose to burn used disposable diapers (Fig. 2), believing it to be a quick and easy way to reduce or eliminate waste (Velis and Cook 2021). However, diapers are not readily combustible and often require catalysts such as paraffin (Remigios 2014; Nyamayedenga and Tsvere 2020). Disposable diapers can also contain a cocktail of harmful chemicals including polycyclic aromatic hydrocarbons (PAHs), dioxins, furans, formaldehyde, and volatile organic compounds such as naphthalene and toluene (ANESES 2019). When openly burned, diapers and other MSW release noxious gases and small particulate matter ($PM_{2.5}$) into the environment, which can pose a serious risk to human health (Kumari et al., 2017; Velis and Cook 2021). Air pollution is a leading contributor towards the global burden of disease, particularly within LMICs. Globally, in 2019, air pollution was estimated to have been responsible for more than 6.6 million deaths and over 213 million disability-adjusted life years (DALYs) (Cohen et al., 2017; IHME 2020; Velis and Cook 2021). In particular, $PM_{2.5}$ is linked to infection of the lower respiratory tract, lung cancer, ischaemic heart disease, cerebrovascular disease (stroke), and chronic obstructive pulmonary disease (Cohen et al., 2017; IHME 2020).

Therefore, whilst burning used diapers is a quick and easy solution to waste management, it leaves unsightly residues, is damaging to human health, and is clearly not an environmentally sustainable strategy.

Open dumping is another popular and widespread form of independent waste management and is routinely practised by 93 % and 66 % of people on low and low-middle incomes respectively, compared to just 2 % of people in high income groups (Kaza et al., 2018). In the absence of adequate bins, 17 % of caregivers favour dumping used disposable diapers at illegal dumpsites (Fig. 2). These are typically small to medium sized open sites located within or at the periphery of communities (Remigios 2014; Kordecki et al., 2022); however, the use of smaller more discreet sites, recently coined "Jay-dumping", is becoming more common (Nyamayedenga and Tsvere 2020). Jay-dumping sites tend to be located further away from communities and are used by single households (Nyamayedenga and Tsvere 2020). Regardless, open dumping of disposable diaper waste is unsightly, attracts vermin, and enhances the risk of disease transmission (Remigios 2014; Jesca and Junior 2015; Mathe 2018).

In informal settlements, diapers are often disposed of in pit latrines; however, this reduces the fill time, requiring more frequent emptying which can be costly for low-income households, who often delay emptying and continue to use latrines when no longer hygienically safe (Jenkins et al., 2015; Gudda et al., 2019). Diapers and other solid waste, such as cloth and sanitary items, also make emptying pits more challenging as they need to be removed by hand, increasing workers' contact with faecal material (Chipeta et al., 2017; Portiolli et al., 2021). Conversely, in areas with modern sewerage systems, diapers are frequently disposed of in flushable latrines. Sewer systems are not designed to manage disposable diapers and when flushed, the absorbent cellulose fluff pulp and super absorbent polymer (SAP) components absorb water and swell leading to permanent blockages and sewerage overflows (Chinyama and Toma 2013; Remigios 2014).

Some caregivers in LMICs choose to bury disposable diapers (Fig. 2); and although deemed a safe and hygienic practice (UNICEF 2006), this only applies to the organic component. Plastics are not readily biodegradable and can persist in the environment for hundreds, if not thousands of years, depending on the plastic type and environmental conditions (Chamas et al., 2020). Furthermore, buried plastics can release leachates, such as plasticisers and flame retardants, which lead to environmental toxicity (Zimmermann et al., 2021). Disposal through composting is much the same as only the cellulose component and faecal material can be readily biodegraded, leaving the plastic components to persist (Ferronato et al., 2020).

4. Public health risks of unregulated diaper disposal

When improperly disposed of, human faeces pose a risk for the transmission of enteric diseases and helminthiasis via the faecal-oral route (Anandan et al., 2021). Globally, more than 1.6 million people die per annum from diarrhoeal-related diseases and approximately 1.5 billion people are infected with helminths (including hookworm, ascariasis and trichuriasis), with a disproportionately high burden in LMICs (Pullan et al., 2014; Troeger et al., 2018). Cholera (caused by *Vibrio cholerae*) is endemic in many LMICs and widely associated with faecal contamination of the environment (Oguttu et al., 2017; Muzembo et al., 2022; WHO 2022). Outbreaks lead to the loss of approximately 95,000 lives each year and have been intensifying in recent years, with a rise in reported case numbers and the geographical range of outbreaks (WHO 2022). Furthermore, there is evidence that faecal contamination in the environment can contribute towards the emergence and spread of antimicrobial resistance (Thongsamer et al., 2021).

In LMICs, the recycling industry is largely run by the informal sector (Kaza et al., 2018). Informal waste pickers (IWPs) often operate at dumpsites (formal and informal), scavenging for materials of worth, such as plastic or metal, which can be sold on to the recycling industry. Most operate with minimal PPE, using their bare hands to rummage through waste, and exposing themselves to physical, chemical, and biological hazards (Kasinja and Tilley 2018; Zolnikov et al., 2021). Cuts from broken glass, cans, and needles are common, as well as exposure to chemical solvents and pesticides (Cruvinel et al., 2019; Zolnikov et al., 2021). Working conditions lead to poor health, with many IWPs suffering from episodic diarrhoea, bronchitis, eye infections, and osteo-muscular disorders (Chokhandre et al., 2017; Cruvinel et al., 2019). The presence of disposable diapers amongst MSW presents an increasingly common biological hazard, exposing IWPs to human faecal material and increasing the risk of contracting communicable diarrhoeal diseases such as cholera and typhoid (Remigios 2014).

Used diapers also create obnoxious odours, attracting flies and animals such as dogs and rodents (Remigios 2014; Jesca and Junior 2015; Mathe 2018). Flies are notorious mechanical vectors of disease, able to transport bacteria and viruses from faeces to food via surface contact (Pace et al., 2017; Thomson et al., 2021; Asada et al., 2022). Furthermore, human faeces provide a preferential medium for oviposition by *Musca sorbens* (the eye-seeking fly), a vector for the bacterium *Chlamydia trachomatis* (Emerson et al., 2001). Infection with *C. trachomatis* is a predominant cause of the eye disease active trachoma, a major cause of child blindness worldwide (Bourne et al., 2013; MacLeod et al., 2019; Delelegn et al., 2021). Dogs are also a particular nuisance, frequently documented to scatter and tear open used diapers (Fig. 1); even when disposed of with MSW, dogs are known to break into bins and seek out diapers (Remigios 2014; Mathe 2018). Such scavenging behaviour can re-expose faecal material to the environment and increase the risk of enteric disease spread.

The attraction of animals to diapers at dumpsites also presents an additional risk of zoonotic disease transmission (Krystosik et al., 2020; Doron 2021). Dogs, chickens, and birds are well known to feed on human faeces, and it can constitute one fifth of the diet (by mass) of free roaming dogs and provides a valuable dietary resource (Butler et al., 2018). However, these coprophagic habits create a pathway for human-animal disease transmission. Whilst the consumption of contaminated faeces may not lead to infection, animals can become a reservoir, transmitting disease via their faeces (Finley et al., 2007; Nijsse et al., 2014). Animal faeces have been identified as a significant reservoir for viruses and enteric pathogens including *Campylobacter*, non-typhoidal *Salmonella*, and *Cryptosporidium* (Delahoy et al., 2018).

5. Design and implementation of intervention schemes

Despite heavy criticism of current methods of disposal, very few alternatives have been offered. The mismanagement of disposable diaper waste in LMICs is multifaceted and influenced by factors at numerous levels, including the caregiver, the local council, and the national government. As such, solutions need to be targeted at different scales. In LMICs, governments typically lack the financial resources to improve waste management systems and struggle to cover operational costs, leading to lapses in services (Kaza et al., 2018; Muia 2018). Thus, initial efforts may be better focussed at the level of the caregiver, identifying feasible alternatives to disposable diapers and promoting behavioural change regarding waste disposal.

5.1. Diaper recycling systems

Recycling of disposable diapers is complex and expensive; and consequently, predominantly only available in high income countries (HIC) (Khoo et al., 2019). In LMICs, initial thought has gone into the introduction of community level diaper collection schemes, whereby households separate disposable diapers from general MSW and deliver them to a collection point for specialist disposal. However, these schemes rely on the willingness of caregivers to participate. In a survey of caregivers in Nairobi, Kenya, only 19.6 % ($n = 148$) would be openly willing to take their used diapers to a collection point, while others would only consider it if given a financial incentive. Willingness was

positively correlated with caregivers' level of education, indicating that implementation of educational campaigns alongside collection schemes may help boost participation and increase their viability (Wambui et al., 2015). If the challenge of separating out the plastic components of diapers can be overcome, low-cost composting methods that breakdown the organic fractions of disposable diapers, i.e., human excreta and cellulose, into compost suitable for use as agricultural fertiliser may offer a solution and add value to this challenging waste stream (Ferronato et al., 2020).

5.2. Modern reusable cloth diapers

Arguments are regularly made for reversion to cloth reusable diapers (Tembo and Chazireni 2016). In Indonesia, Agestika et al. (2022) found that disposable diaper usage increased unhygienic practices regarding the disposal of child's faeces, irrespective of the household sanitation level; indicating that with the current level of waste management, together with the environmental awareness of caregivers, cloth diapers can be more hygienic. Cloth diapers increase the likelihood of safe disposal of child's faeces since the faeces must be removed before washing (Miller-Petrie et al., 2016), e.g., through disposal in a latrine or by burying (Agestika et al., 2022). Cloth diapers can be cheaper and have a lower environmental impact compared to disposable diapers, although this is often context dependent (Miller-Petrie et al., 2016; UNEP 2021). It is estimated that for a single child over a two- and half-year diapering period, the manufacture and use of reusable cloth diapers requires between 1221 and 1854 m^3 of water, which is up to 14-fold higher than for disposable diapers (Aumônier et al., 2008). Modern reusable cloth diapers also carry a higher upfront cost (Tumulango et al., 2021). Life cycle assessments for cloth diapers have revealed that their primary environmental impacts stem from the energy requirements of laundering whereas for disposable diapers, this arises from their manufacture (Aumônier et al., 2008; UNEP, 2021). When washed and dried in a water and energy-efficient manner, such as on a cold cycle in a fully loaded modern washing machine followed by air drying, cloth diapers have a lower environmental impact than disposable diapers (UNEP 2021). However, this has less relevance in LMICs where access to electric washing facilities can be limited.

Hand washing laundry is laborious, time consuming, and water intensive, with respondents spending an average of 9.5 h per week carrying out routine washing duties and using 59 L of water per 7.5 kg wash (The Washing Machine Project 2022). Furthermore, a high proportion of households in LMICs must travel off-premises to collect water (WHO & UNICEF, 2021), transporting it using buckets and exposing themselves to risks of physical injury from carrying heavy loads and navigating treacherous routes; a burden that falls disproportionately on women (Adams et al., 2022). In addition, the availability of soap for washing is often limited, which combined with a lack of water and drying facilities, is a major obstacle to the uptake of reusable cloth menstrual products, which share similar laundry requirements to those of reusable diapers (Kambala et al., 2020; Rossouw and Ross 2021; Roxburgh et al., 2022). There is also the associated cost and environmental footprint of fuel if heating water for washing, which typically arises from non-renewable sources. In Sub-Saharan Africa, oil and natural gas are becoming the primary fuels used for this purpose; however, charcoal is still dominant in some areas as it is cheap, readily available and has a high energy content (Makonese et al., 2016; IEA 2019). Switching from disposable diapers to modern cloth diapers would likely exacerbate these challenges.

There have been some positive results in trials investigating the feasibility of introducing modern reusable diapers to communities in LMICs that are struggling to manage their disposable diaper waste. In the Pacific island nation of Vanuatu, 96 % of trial participants ($n = 59$) across three rural communities expressed positive views on modern reusable diapers. However, there were concerns regarding the associated extra labour, access to water for washing, and their size and comfort (Tumulango et al., 2021). Furthermore, a starter pack of modern reusable diapers carries a high purchase price, retailing at US$166 in the current trial. Whilst these costs can be offset by the reuse of diapers over the duration of a child's diapering period, many caregivers cannot afford this initial outlay (Aumônier et al., 2008; Tumulango et al., 2021). Financial literacy was also identified as a barrier to uptake as not all caregivers had a complete understanding of the long-term cost-benefit of reusable diapers. Conclusions of the trial recommended that governments and reusable diaper manufacturers explore arrangements for structural financial support schemes (Tumulango et al., 2021), as unless costs can be reduced or spread over time, it is unlikely that modern reusable diapers will be an accessible solution to the escalating challenge of disposable diaper waste.

5.3. Raising awareness and behavioural change

Irrespective of the alternatives, disposable diaper usage within LMICs remains high (Fig. 2) and raising awareness of the implications of current practices surrounding their use is key. Many caregivers do not understand the risks associated with improper disposal and lack the knowledge to make informed choices about alternative practices (Remigios 2014; Mathe 2018; Muia 2018). Although dissemination of information in LMICs can be challenging, there have been many successful WaSH interventions that serve as good models for encouraging behavioural change in LMIC communities (Malolo et al., 2021; Panulo et al., 2022; Simiyu et al., 2022).

The 'Hygienic Family' intervention in rural Malawi used a combination of group meetings, workshops, and household visits, alongside rewards and messaging campaigns, to successfully implement behavioural change surrounding household WaSH practices and food hygiene (Morse et al., 2019; Panulo et al., 2022). The study resulted in health and social benefits for both study participants and the local community, with a 13 % reduction in reported cases of diarrhoea (Morse et al., 2020; Malolo et al., 2021). Meetings, workshops, and household visits acted as points of contact for learning and provided participants with ongoing support, whilst messaging solidified the learning, with posters, leaflets, and other prompts acting as visual reminders of key points (Garofano and Webster 2019; Malolo et al., 2021). Rewards included high-value household items, such as plastic buckets or soap, and provided participants with the means and motivation to progress. Participants also became role models to non-participating community members, inspiring other households to implement the new hygiene practices and increasing dissemination of the desired behavioural change within the community (Malolo et al., 2021).

Similarly, positive results have been achieved using artistic and participatory based approaches to encourage improved hand hygiene in Latin American communities (Zisa et al., 2022). The Lazos de Agua programme targeted 1680 households across Colombia, Mexico, Nicaragua, and Paraguay using short films, theatre productions, songs, murals, and puppet shows to convey messages in a culturally relevant context, resulting in a 15 % increase in the proportion of the population practising proper hand hygiene after a 22-month intervention period (Zisa et al., 2022). If translated well, similar frameworks could be effective in implementing behavioural change regarding the management of disposable diaper waste in LMIC communities. Interventions could be designed around promoting safer disposal habits and to encourage the use of cloth diapers instead of disposables, and by rewarding improved practice with free hygiene consumables as in the Hygienic Family intervention (Malolo et al., 2021).

Manufacturers also have a responsibility to provide information on best practice for the disposal of used diapers; typically, this information is printed on external packaging. However, in LMICs, many everyday household items are sold singularly or in small volumes, which makes them more accessible to those with low purchasing power, such as those living in informal settlements and piece-rate workers; consequently, consumers will not have access to the external packaging and so may not

receive this information (Remigios 2014; Donovan and Park 2022). Printing disposal instructions on individual diapers or on posters and leaflets situated around communities may help overcome this packaging limitation and disseminate this important information to those consumers who are only able to make small affordable daily purchases (Donovan and Park 2022).

Any scheme directed at changing the behaviour of caregivers must also be complemented by infrastructural development. In LMICs, an increase in waste management services is often recognised as an important factor in reducing open dumping and littering; for example, the provision of more bins and more frequent waste collections (Kaza et al., 2018; Garofano and Webster 2019). However, this can be challenging to implement as governments are often financially restricted and waste management is often regarded as low priority (Kaza et al., 2018). Furthermore, formal or informal waste collection in informal settlements can be impaired by road inaccessibility, potential violence and crime, social stigma, and difficulties associated with collecting payment (Kaza et al., 2018). Local bylaws may assist in discouraging open dumping, but these would require significant enforcement which is also limited by resources and capacity (Garofano and Webster 2019).

6. Conclusions and future perspectives

Disposable diapers present an important resource for caregivers in LMICs, providing a convenient and affordable method for managing child excreta. Yet, the current lack of infrastructure and guidance on the disposal of used diapers, together with a general lack of community awareness of the environmental and human health consequences of improper disposal, is having considerable negative impacts on LMIC communities and could hamper progress towards achieving SDG6. Current indicators as defined by the UNICEF and WHO JMP ladder for sanitation, do not account for the contribution of disposable diapers towards open defaecation. Under these criteria, countries could feasibly conclude that they have eliminated open defaecation without ensuring the safe and hygienic disposal of diapers, leading to the misconception that this aspect of SDG6 has been met. Given the predicted rise in usage of disposable diapers, particularly within LMICs, it is imperative that their contribution is formally acknowledged.

Little progress has been made on the development of feasible, low-cost solutions for the disposal of single-use diapers in LMICs; and moving forward this must become a priority for manufacturers and the research community. Behavioural change is also key, and intervention schemes aimed to educate caregivers on safe disposal practices for disposable diapers will be invaluable in achieving this. Higher priority must be given to the waste management sector with more funding allocated towards waste collection services and the development of sanitary waste disposal. Moreover, manufacturers of disposable diapers must take greater responsibility for the disposal of their products, putting more resources into educating consumers of best practice. However, these changes will be limited in effectiveness if not supported by the appropriate infrastructure. Governments hold ultimate responsibility for national waste management strategies, and integrated top-down changes will be essential for addressing the emerging challenge of disposable diaper waste.

CRediT authorship contribution statement

HW, RQ: Conceptualization. **RQ:** Funding acquisition, Supervision. **HW:** Investigation, Visualization, Writing - original draft. **HW, TM, MM, PK, NF, DO, MJ, TM, KC, RQ:** Writing - review & editing.

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References

Abbafati, C., et al., 2020. Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet 396 (10258), 1204–1222.

Adams, E.A., Byrns, S., Kumwenda, S., Quilliam, R., Mkandawire, T., Price, H., 2022. Water journeys: household water insecurity, health risks, and embodiment in slums and informal settlements. Soc. Sci. Med. 313, 115394 https://doi.org/10.1016/J.SOCSCIMED.2022.115394.

Agestika, L., Sintawardani, N., Hamidah, U., Nyambe, S., Yamauchi, T., 2022. Pattern of child faeces management and disposable diaper usage among under-fives in an Urban Slum of Bandung, Indonesia. J. Water, Sanit. Hyg. Dev. 12 (1), 32–40.

Akinola, B.S., Awoyemi, M.O., Matthew, O.J., Adebayo, A.S., 2018. Geophysical and hydro-chemical investigation of contamination plume in a basement complex formation around Sunmoye dumpsite in Ikire, Southwestern Nigeria. Model. Earth Syst. Environ. 4 (2), 753–764.

Anandan, M., Vs, S., Rubeshkumar, P., Ponnaiah, M., Jesudoss, P., Karumanagounder, K., Murhekar, M., 2021. Outbreak of acute diarrhoeal disease attributed to consumption of faecal contaminated water supplied through damaged pipelines in Thiruper, Tiruvallur district, Tamil Nadu, India, 2016. Clin. Epidemiol. Glob. Health 10, 100701. https://doi.org/10.1016/J.CEGH.2021.100701.

ANESES, 2019. Revised OPINION of the French Agency for Food, Environmental and Occupational Health & Safety on the Safety of Baby Diapers 2017-SA-0019 [Patent].

Aralu, C.C., Okoye, P.A.C., Akpomie, K.G., Eboagu, N.C., 2021. Levels of polycyclic aromatic hydrocarbons in leachates from unlined dumpsite of Agu-Awka Anambra state. https://doi.org/10.1080/03067319.2021.1993842.

Asada, Y., Chua, M., Tsurumi, M., Nyambe, I., Narada, H., 2022. Detection of Escherichia coli, rotavirus, and Cryptosporidium spp. from drinking water, kitchenware, and flies in a periurban community of Lusaka, Zambia. J. Water Health 20 (7), 1027–1037. https://doi.org/10.2166/wh.2022.276.

Aumônier, S., Collins, M., Garrett, P., 2008. An Updated Lifecycle Assessment Study for Disposable and Reusable Nappies. Science Report – SC010018/SR2, Bristol.

Ayalew, A.M., Mekonnen, W.T., Abaya, S.W., Mekonnen, Z.A., 2018. Assessment of diarrhea and its associated factors in under-five children among open defecation and open defecation-free rural settings of Dangla District, Northwest Ethiopia. J. Environ. Publ. Health 2018. https://doi.org/10.1155/2018/4271915.

Bourne, R., Stevens, G., White, R., Smith, J., Flaxman, S., Price, H., Jonas, J., Keeffe, J., Leasher, J., Naidoo, K., Pesudovs, K., Resnikoff, S., Taylor, H., 2013. Causes of vision loss worldwide, 1990–2010: a systematic analysis. The Lancet Global Health 1 (6), e339–e349. https://doi.org/10.1016/S2214-109X(13)70113-X.

Brinckmann, M., 2022. Baby Diapers Report 2022: Statista Consumer Market Outlook - Segment Report. Article No: did-48854-1.

Butler, J.R.A., Brown, W.Y., du Toit, J.T., 2018. Anthropogenic food subsidy to a commensal carnivore: the value and supply of human faeces in the diet of free-ranging dogs. Animals 2018 8, 67, 67 8(5).

Chamas, A., et al., 2020. Degradation rates of plastics in the environment. ACS Sustain. Chem. Eng. 8 (9), 3494–3511.

Chavan, D., Manjunatha, G.S., Singh, D., Periyaswami, L., Kumar, S., Kumar, R., 2022. Estimation of spontaneous waste ignition time for prevention and control of landfill fire. Waste Manag. 139, 258–268. https://doi.org/10.1016/J.WASMAN.2021.11.044.

Chiemchaisri, C., Chiemchaisri, W., Boocha, M., 2019. Emissions of volatile organic compounds from solid wastes and leachate at a municipal solid waste dumpsite in Thailand. Energy, Environ. Sustain. 357–367.

Chinyama, A., Toma, T., 2013. Understanding the poor performance of urban sewerage systems: a case of coldstream high density suburbs, chinhoyi, Zimbabwe. Urban Plan. Des. Res. 1 (3).

Chipeta, W.C., Holm, R.H., Kamanula, J.F., Mtonga, W.E., de los Reyes, F.L., 2017. Designing local solutions for emptying pit latrines in low-income urban settlements (Malawi). Phys. Chem. Earth 336–342. https://doi.org/10.1016/J.PCE.2017.02.012. Parts A/B/C 100.

Chokhandre, P., Singh, S., Kashyap, G.C., 2017. Prevalence, predictors and economic burden of morbidities among waste-pickers of Mumbai, India: a cross-sectional study. J. Occup. Med. Toxicol. 12 (1), 1–8.

Cohen, A.J., et al., 2017. Estimates and 25-year trends of the global burden of disease attributable to ambient air pollution: an analysis of data from the Global Burden of Diseases Study 2015. Lancet 389 (10082), 1907–1918.

Counts, J.L., Helmes, C.T., Kenneally, D., Otts, D.R., 2014. Modern disposable diaper construction: innovations in performance help maintain healthy diapered skin. Clin. Pediatr. 53 (9 Suppl. l), 10S–13S.

Cruvinel, V.R.N., et al., 2019. Health conditions and occupational risks in a novel group: waste pickers in the largest open garbage dump in Latin America. BMC Publ. Health 19 (1), 1–15.

Delahoy, M.J., Wodnik, B., McAliley, L., Penakalapati, G., Swarthout, J., Freeman, M.C., Levy, K., 2018. Pathogens transmitted in animal feces in low- and middle-income countries. Int. J. Hyg Environ. Health 221 (4), 661–676. https://doi.org/10.1016/J.IJHEH.2018.03.005.

Delelegn, D., Tolcha, A., Beyene, H., Tsegaye, B., 2021. Status of active trachoma infection among school children who live in villages of open field defecation: a comparative cross-sectional study. BMC Publ. Health 21 (1), 1–10.

Donovan, K.P., Park, E., 2022. Knowledge/seizure: debt and data in Kenya's zero balance economy. Antipode 54 (4), 1063–1085.

Doron, A., 2021. Stench and sensibilities: on living with waste, animals and microbes in India. Aust. J. Anthropol. 32 (S1), 23–41.

Eke, G.K., Opara, P. lbo, 2013. Mothers knowledge and home management of nappy rash in port harcourt, Nigeria. Nigerian Health J. 13 (4), 152–157.

Ellen MacArthur Foundation, 2020. A circular economy for nappies and how to implement it locally. Isle of Wight, United Kingdom. https://bbia.org.uk/wp-content/uploads/2020/11/A-Circular-Economy-for-Nappies-final-oct-2020.pdf. (Accessed 11 August 2022). Available at:

Emerson, P., Bailey, R., Walraven, G., Lindsay, S., 2001. Human and other faeces as breeding media of the trachoma vector Musca sorbens. Med. Vet. Entomol. 15 (3), 314–320. https://doi.org/10.1046/J.0269-283X.2001.00318.X.

Ferronato, N., Pinedo, M.L.N., Torretta, V., 2020. Assessment of used baby diapers composting in Bolivia. Sustainability 12 (12), 5055.

Finley, R., Ribble, C., Aramini, J., Vandermeer, M., Popa, M., Litman, M., Reid-Smith, R., 2007. The risk of salmonellae shedding by dogs fed Salmonella-contaminated commercial raw food diets. Can. Vet. J. 48 (1), 69.

Garofano, N.T., Webster, M., 2019. The Commonwealth Litter Programme: Final Report – Best Practices for Vanuatu (Suffolk).

Gudda, F.O., Moturi, W.N., Oduor, O.S., Muchiri, E.W., Ensink, J., 2019. Pit latrine fill-up rates: variation determinants and public health implications in informal settlements, Nakuru-Kenya. BMC Publ. Health 19 (1), 1–13.

Gutberlet, J., Uddin, S.M.N., 2017. Household waste and health risks affecting waste pickers and the environment in low- and middle-income countries. Int. J. Occup. Environ. Health 23 (4), 299–310. https://doi.org/10.1080/10773525.2018.1484996.

IEA, 2019. Africa energy outlook 2019. https://www.iea.org/reports/africa-energy-outlook-2019. (Accessed 6 December 2022). Available at:

IHME, 2020. Global burden of disease study 2019 (GBD 2019). https://vizhub.healthdata.org/gbd-results/. (Accessed 9 August 2022). Seattle. Available at:

IMF (2022). International Monetary Fund, 2022. World economic outlook: countering the cost-of-living crisis. https://www.imf.org/en/Publications/WEO/Issues/2022/10/11/world-economic-outlook-october-2022#Projections. (Accessed 29 November 2022). Washington, DC. Available at:

Jenkins, M.W., Cumming, O., Cairncross, S., 2015. Pit latrine emptying behavior and demand for sanitation services in dar Es salaam, Tanzania. Int. J. Environ. Res. Publ. Health 2015 12, 2588–2611, 12(3), pp. 2588–2611.

Jesca, M., Junior, M., 2015. Practices regarding disposal of soiled diapers among women of child bearing age in poor resource urban setting. J. Nurs. Health Sci. 4 (4), 63–67.

Jex, A.R., et al., 2012. Detection of diarrhoeal pathogens in human faeces using an automated, robotic platform. Mol. Cell. Probes 26 (1), 11–15. https://doi.org/10.1016/J.MCP.2011.10.004.

Kamat, M., Malkani, R., 2003. Disposable diapers : a hygienic alternative. Indian J. Pediatr. 70 (11), 879–881.

Kambala, C., Chinangwa, A., Chipeta, E., Torondel, B., Morse, T., 2020. Acceptability of menstrual products interventions for menstrual hygiene management among women and girls in Malawi. Reprod. Health 17 (1), 1–12.

Kasinja, C., Tilley, E., 2018. Formalization of informal waste pickers' cooperatives in Blantyre, Malawi: a feasibility assessment. Sustain. 2018 10 (4), 1149, 1149 10.

Kaza, S., Yao, L.C., Bhada-Tata, P., van Woerden, F., 2018. What a Waste 2.0: A Global Snapshot of Solid Waste Management to 2050. World Bank, Washington, DC. © World Bank. License: CC BY 3.0 IGO. Available at: https://openknowledge.worldbank.org/handle/10986/30317. (Accessed 11 August 2022). Accessed.

Khoo, S.C., Phang, X.Y., Ng, C.M., Lim, K.L., Lam, S.S., Ma, N.L., 2019. Recent technologies for treatment and recycling of used disposable baby diapers. Process Saf. Environ. Protect. 123, 116–129. https://doi.org/10.1016/J.PSEP.2018.12.016.

Kordecki, H., Antrobus-Wuth, R., Uys, M.-T., van Wyk, I., Root, E.D., Berrian, A.M., 2022. Disposable diaper waste accumulation at the human-livestock-wildlife interface: a one health approach. Environ. Challen. 8, 100589.

Krafchik, B., 2016. History of diapers and diapering. Int. J. Dermatol. 55, 4–6.

Krystosik, A., Njoroge, G., Odhiambo, L., Forsyth, J.E., Mutuku, F., LaBeaud, A.D., 2020. Solid wastes provide breeding sites, burrows, and food for biological disease vectors, and urban zoonotic reservoirs: a call to action for solutions-based research. Front. Public Health 7, 405. https://doi.org/10.3389/FPUBH.2019.00405/BIBTEX.

Kubiak, M., Kressner, B., Raynor, W., Davis, J., Syverson, R.E., Laabs, J., 1993. Comparison of stool containment in cloth and single-use diapers using a simulated infant feces. Pediatrics 91 (3), 632–636.

Kumari, K., Kumar, S., Rajagopal, V., Khare, A., Kumar, R., 2017. Emission from open burning of municipal solid waste in India. Environ. Technol. 40 (17), 2201–2214. https://doi.org/10.1080/09593330.2017.1351489.

Lebreton, L., Andrady, A., 2019. Future scenarios of global plastic waste generation and disposal. Palgrave Communications 5 (1), 1–11.

MacLeod, C., Binnawi, K., Elshafie, B., Sadig, H., Hassan, A., Cocks, N., Willis, R., Chu, B., Solomon, A., 2019. Unimproved water sources and open defecation are associated with active trachoma in children in internally displaced persons camps in the Darfur States of Sudan. Trans. Royal Soc. Trop. Med. Hygiene 113 (10), 599–609. https://doi.org/10.1093/TRSTMH/TRZ042.

Makonese, T., Masekameni, D.M., Annegarn, H.J., 2016. Energy use scenarios in an informal urban settlement in Johannesburg, South Africa. In: Proceedings of the 24th Conference on the Domestic Use of Energy. DUE 2016. https://doi.org/10.1109/DUE.2016.7466703.

Malolo, R., Kumwenda, S., Chidziwisano, K., Kambala, C., Morse, T., 2021. Social outcomes of a community-based water, sanitation and hygiene intervention. J. Water, Sanit. Hyg. Dev. 11 (3), 483–493.

Mathe, M., 2018. Environmental pollution-perceptions and views on usage and disposal of diapers: a case study of gwanda urban. Int. J. Innov. Sci. Res. Technol. 3 (5).

Miller-Petrie, M.K., Voigt, L., McLennan, L., Cairncross, S., Jenkins, M.W., 2016. Infant and young child feces management and enabling products for their hygienic collection, transport, and disposal in Cambodia. Am. J. Trop. Med. Hyg. 94 (2), 456–465.

Morse, T., Chidziwisano, K., Tilley, E., Malolo, R., Kumwenda, S., Musaya, J., Cairncross, S., 2019. Developing a contextually appropriate integrated hygiene intervention to achieve sustained reductions in diarrheal diseases. Sustainability 11 (17), 4656, 4656 11.

Morse, T., Tilley, E., Chidziwisano, K., Malolo, R., Musaya, J., 2020. Health outcomes of an integrated behaviour-centred water, sanitation, hygiene and food safety intervention–A randomised before and after trial. Int. J. Environ. Res. Publ. Health 17 (8), 2648, 2648 17.

Muia, V.K., 2018. Disposal methods of soiled diapers in low-income households of Nairobi county in Kenya. IJRDO - J. Appl. Sci. 4 (7), 11–20.

Muzembo, B.A., Kitahara, K., Debnath, A., Ohno, A., Okamoto, K., Miyoshi, S.I., 2022. Cholera outbreaks in India, 2011–2020: a systematic review. Int. J. Environ. Res. Publ. Health 19 (9), 5738.

Ngwabie, N.M., Wirlen, Y.L., Yinda, G.S., VanderZaag, A.C., 2019. Quantifying greenhouse gas emissions from municipal solid waste dumpsites in Cameroon. Waste Manag. 87, 947–953. https://doi.org/10.1016/J.WASMAN.2018.02.048.

Nijsse, R., Mughini-Gras, L., Wagenaar, J.A., Ploeger, H.W., 2014. Coprophagy in dogs interferes in the diagnosis of parasitic infections by faecal examination. Vet. Parasitol. 204 (3–4), 304–309. https://doi.org/10.1016/J.VETPAR.2014.05.019.

Njuguna, J., 2016. Effect of eliminating open defecation on diarrhoeal morbidity: an ecological study of Nyando and Nambale sub-counties, Kenya. BMC Publ. Health 16 (1), 1–6.

Ntekpe, M., Mbong, E., Edem, E., Hussain, S., 2020. Disposable diapers: impact of disposal methods on public health and the environment. Am. J. Med. Publ. Health 1 (2), 1009.

Nyamayedenga, V.K., Tsvere, P.M., 2020. Real time data capture: a response to unsustainable dumping of disposable diapers and sanitary pads in gweru city, Zimbabwe. East Afr. J. Educ. Soc. Sci. 1 (2), 54–64.

Oguttu, D.W., Okullo, A., Bwire, G., Nsubuga, P., Ario, A.R., 2017. Cholera outbreak caused by drinking lake water contaminated with human faeces in Kaiso Village, Hoima District, Western Uganda. October 2015 Infect. Dis. Poverty 6 (1), 1–7.

Pace, R.C., Talley, J.L., Crippen, T.L., Wayadande, A.C., 2017. Filth fly transmission of Escherichia coli O157:H7 and Salmonella enterica to lettuce, Lactuca sativa. Ann. Entomol. Soc. Am. 110 (1), 83–89.

Panulo, M., Chidziwisano, K., Beattie, T.K., Tilley, E., Kambala, C., Morse, T., 2022. Process evaluation of the hygienic family intervention: a community-based water, sanitation, and hygiene project in rural Malawi. Int. J. Environ. Res. Publ. Health 2022 19 (11), 6771. Page 6771 19.

Perez, M., Navarro, P., Morillas, A., Valdemar, R., Araiza, J., 2021. Waste management and environmental impact of absorbent hygiene products: a review. Waste Manag. Res. 39 (6), 767–783.

Płotka-Wasylka, J., Makoś-Chełstowska, P., Kurowska-Susdorf, A., Treviño, M.J.S., Guzmán, S.Z., Mostafa, H., Cordella, M., 2022. End-of-life management of single-use baby diapers: analysis of technical, health and environment aspects. Sci. Total Environ. 836, 155339 https://doi.org/10.1016/J.SCITOTENV.2022.155339.

Portiolli, G.F., Rogers, T.W., Beckwith, W., Tsai, J., Ole-Moiyoi, P., Wilson, N., de Los Reyes, F.L., 2021. Development of trash exclusion for mechanized pit latrine emptying. Environ. Sci. J. Integr. Environ. Res.: Water Research & Technology 7 (10), 1714–1722.

Pullan, R.L., Smith, J.L., Jasrasaria, R., Brooker, S.J., 2014. Global numbers of infection and disease burden of soil transmitted helminth infections in 2010. Parasites Vectors 7 (1), 1–19.

Rahman, M.H.U., Malik, M.A., Chauhan, S., Patel, R., Singh, A., Mittal, A., 2020. Examining the linkage between open defecation and child malnutrition in India. Child. Youth Serv. Rev. 117, 105345 https://doi.org/10.1016/J.CHILDYOUTH.2020.105345.

Reese, H., Alman, B., Null, C., 2015. Disposing of children's diapers with solid waste: a global concern? Waterlines 34 (3), 255–268. https://doi.org/10.3362/1756-3488.2015.024.

Reference Bureau, Population, 2015. The urban-rural divide in health and development data sheet. Washington, DC. Available at: https://www.prb.org/wp-content/uploads/2015/05/urban-rural-datasheet.pdf. (Accessed 11 October 2022). Accessed.

Remigios, M., 2014. The environmental health implications of the use and disposal of disposable child diapers in senga/nehosho suburb in Gweru city, Zimbabwe. Glob. J. Biol. Aquacult. Health Sci. 3 (2), 122–127.

Roman, L., Hardesty, B.D., Leonard, G.H., Pragnell-Raasch, H., Mallos, N., Campbell, I., Wilcox, C., 2020. A global assessment of the relationship between anthropogenic debris on land and the seafloor. Environ. Pollut. 264, 114663 https://doi.org/10.1016/J.ENVPOL.2020.114663.

Rossouw, L., Ross, H., 2021. Understanding Period poverty: socio-economic inequalities in menstrual hygiene management in eight low- and middle-income countries. Int. J. Environ. Res. Publ. Health 18 (5), 2571, 2571 18.

Roxburgh, H., Magombo, C., Kaliwo, T., Tilley, E.A., Hampshire, K., Oliver, D.M., Quilliam, R.S., 2022. Blood flows: mapping journeys of menstrual waste in Blantyre, Malawi. Cities and Health 6 (4), 738–751.

Shuker, I.G., Cadman, C.A., 2018. Indonesia - Marine Debris Hotspot: Rapid Assessment. Report No. 126686 Synthesis Report. Washington, D.C. Available at: https://documents.worldbank.org/en/publication/documents-reports/documentdetail/983771527663689822/indonesia-marine-debris-hotspot-rapid-assessment-synthesis-report. (Accessed 16 August 2022).

Simiyu, S., Aseyo, E., Anderson, J., Cumming, O., Baker, K.K., Dreibelbis, R., Mumma, J. A.O., 2022. A mixed methods process evaluation of a food hygiene intervention in low-income informal neighbourhoods of kisumu, Kenya. Matern. Child Health J. 1–13.

Taboada-González, P., Armijo-de-Vega, C., Aguilar-Virgen, Q., Ojeda-Benítez, S., 2014. Household solid waste characteristics and management in rural communities. Open Waste Manag. J. 3 (1), 167–173. https://doi.org/10.2174/1875934301003010167.

Tembo, E., Chazireni, E., 2016. The negative environmental impact of disposable diapers: the case of mberengwa district, Zimbabwe. Int. J. Health Sci. 4 (2), 2158–2161.

Thanh, N.P., Matsui, Y., Fujiwara, T., 2010. Household solid waste generation and characteristic in a Mekong Delta city, Vietnam. J. Environ. Manag. 91 (11), 2307–2321. https://doi.org/10.1016/J.JENVMAN.2010.06.016.

The Washing Machine Project, 2022. Investigating the feasibility of distributing the divya 1.5 manual washing machine in Kenya. A study on laundry habits and preferences in homa-bay county, Kenya. https://www.thewashingmachineproject.org/publications. (Accessed 5 December 2022). Available at:

The World Bank, 2019a. DataBank. Health nutrition and population statistics. https://databank.worldbank.org/source/health-nutrition-and-population-statistics. (Accessed 13 September 2022). Available at:

The World Bank, 2019b. World Development Indicators [Fertility rate, total (births per woman)]. https://data.worldbank.org/indicator/SP.DYN.TFRT. (Accessed 16 August 2022). Available at:

Thomson, J.L., Cernicchiaro, N., Zurek, L., Nayduch, D., 2021. Cantaloupe facilitates Salmonella typhimurium survival within and transmission among adult house flies (Musca domestica L.). Foodbor. Pathogens and Dis. 18 (1), 49–55.

Thongsamer, T., et al., 2021. Environmental antimicrobial resistance is associated with faecal pollution in Central Thailand's coastal aquaculture region. J. Hazard Mater. 416, 125718 https://doi.org/10.1016/J.JHAZMAT.2021.125718.

Troeger, C., et al., 2018. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of diarrhoea in 195 countries: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Infect. Dis. 18 (11), 1211–1228.

Tumulango, F., Macalister, L., Whitebread, E., 2021. Introducing modern reusable nappies into Vanuatu – a trial study. https://www.mammaslaef.com/wp content/uploads/sites/105/2021/02/FINAL-PilotStudy-FullReport.pdf. (Accessed 7 December 2022). Available at:

Turpie, J., Letley, G., Ng'oma, Y., Moore, K., 2019. The case for banning single-use plastics in Malawi. https://www.lilongwewildlife.org/reports/. (Accessed 11 August 2022). Available at:

UNEP, 2021. Single-use Nappies and Their Alternatives: Recommendations from Life Cycle Assessments.

UNICEF, 2006. Core questions on drinking-water and sanitation for household surveys. https://apps.who.int/iris/handle/10665/43489. (Accessed 20 October 2022). Available at:

Velis, C.A., Cook, E., 2021. Mismanagement of plastic waste through open burning with emphasis on the global South: a systematic review of risks to occupational and public health. Environ. Sci. Technol. 55 (11), 7186–7207.

Wambui, K.E., Joseph, M., Makindi, S., 2015. Soiled diapers disposal practices among caregivers in poor and middle income urban settings. Int. J. Sci. Res. Publ. 5 (10).

WHO & UNICEF, 2021. Progress on Household Drinking Water, Sanitation and Hygiene 2000-2020: Five Years into the SDGs (Geneva).

WHO, 2022. Disease outbreak news; cholera – global situation. https://www.who.int/emergencies/disease-outbreak-news/item/2022-DON426. (Accessed 24 January 2023). Available at: Available at:

Wolf, J., et al., 2018. Impact of drinking water, sanitation and handwashing with soap on childhood diarrhoeal disease: updated meta-analysis and meta-regression. Trop. Med. Int. Health 23 (5), 508–525.

World Bank Group, 2019. World - measuring rural access: update 2017/18. http://documents.worldbank.org/curated/en/543621569435525309/World-Measuring-Rural-Access-Update-2017-18. (Accessed 7 October 2022). Washington, D.C. Available at:

Zimmermann, L., Bartosova, Z., Braun, K., Oehlmann, J., Völker, C., Wagner, M., 2021. Plastic products leach chemicals that induce in vitro toxicity under realistic use conditions. Environ. Sci. Technol. 55 (17), 11814–11823.

Zisa, A., Nilsson, K., Mirza, R., Vachon, T., 2022. Achieving handwashing with social art for behaviour change: the experience of the lazos de Agua programme in Latin America. H2Open J. 5 (2), 323–332.

Zolnikov, T.R., Furio, F., Cruvinel, V., Richards, J., 2021. A systematic review on informal waste picking: occupational hazards and health outcomes. Waste Manag. 126, 291–308. https://doi.org/10.1016/J.WASMAN.2021.03.006.

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Persistent contamination of a hospital hot water network by *Legionella pneumophila*

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ABSTRACT

Objectives: We assessed the contamination with *Legionella pneumophila* (*Lp*) of the hot water network (HWN) of a hospital, mapped the risk of contamination, and evaluated the relatedness of isolates. We further validated phenotypically the biological features that could account for the contamination of the network.
Methods: We collected 360 water samples from October 2017 to September 2018 in 36 sampling points of a HWN of a building from a hospital in France. *Lp* were quantified and identified with culture-based methods and serotyping. *Lp* concentrations were correlated with water temperature, date and location of isolation. *Lp* isolates were genotyped by pulsed-field gel electrophoresis and compared to a collection of isolates retrieved in the same HWN two years later, or in other HWN from the same hospital.
Results: 207/360 (57.5%) samples were positive with *Lp*. In the hot water production system, *Lp* concentration was negatively associated with water temperature. In the distribution system, the risk of recovering *Lp* decreased when temperature was >55 °C ($p < 10^{-3}$), the proportion of samples with *Lp* increased with distance from the production network ($p < 10^{-3}$), and the risk of finding high loads of *Lp* increased 7.96 times in summer ($p = 0.001$). All *Lp* isolates (n = 135) were of serotype 3, and 134 (99.3%) shared the same pulsotype which is found two years later (Lp G). *In vitro* competition experiments showed that a 3-day culture of Lp G on agar inhibited the growth of a different pulsotype of *Lp* (Lp O) contaminating another HWN of the same hospital ($p = 0.050$). We also found that only Lp G survived to a 24h-incubation in water at 55 °C ($p = 0.014$).
Conclusion: We report here a persistent contamination with *Lp* of a hospital HWN. *Lp* concentrations were correlated with water temperature, season, and distance from the production system. Such persistent contamination could be due to biotic parameters such as intra-*Legionella* inhibition and tolerance to high temperature, but also to the non-optimal configuration of the HWN that prevented the maintenance of high temperature and optimal water circulation.

1. Introduction

Legionellae are bacteria found in natural aquatic and soil habitats. They are ubiquitous in many types of water sources and also contaminate man-made water systems, such as sanitary hospital hot-water networks (Emmerson, 2001). *Legionellae* are also opportunistic pathogens responsible for Legionnaire's disease (LD), a severe and life-threatening pneumonia, especially in patients with impaired host defenses (Miyashita et al., 2020). Humans are contaminated through the inhalation or aspiration of water aerosols containing *Legionellae*. In Europe, *Legionella pneumophila* (*Lp*) serogroup 1 is involved in the vast majority of LD cases (Campese et al., 2011; European Centre for Disease Prevention and Control, 2020). Most LD cases are community-acquired but LD is also a significant cause of hospital-acquired pneumonia (European Centre for Disease Prevention and Control, 2020; Lin et al., 2011). Although the number of nosocomial cases is low, the mortality rate of hospital-acquired LD is higher than community-acquired LD (Vincenti et al., 2019).

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Lp frequently colonizes the hot water networks (HWNs) of healthcare facilities, making it mandatory to monitor water colonization to reduce the risk of patient contamination (Arvand et al., 2011; Bargellini et al., 2011; Bédard et al., 2016; Martinelli et al., 2000; Serrano-Suárez et al., 2013; Stout et al., 2007). Although no infectious dose has yet been established, European countries have defined alert (100-1000 CFU/L) and action thresholds (1000–10,000 CFU/L) that trigger corrective measures (Bédard et al., 2016; Legifrance, 2010).

Legionellae contamination of hot water systems correlates with low water temperatures (Bédard et al., 2015, 2016; Dennis, 1990; Gavaldà et al., 2019). Hence, the prevention of *Legionellae* contamination in healthcare facilities relies, among other measures, on the maintenance of an elevated water temperature(Centre scientifique et technique du bâtiment, 2012). At the University Hospital of Besançon (UHB, France), *Lp* has contaminated the HWN of two buildings for more than 15 years. Persistent contamination by *Lp* has already been described in healthcare facilities due to the complexity and ageing of HWNs, which limit the efficiency of decontamination measures (Oberdorfer et al., 2008; Pancer et al., 2013; Perola et al., 2005).

However, the comprehensive and long-term mapping of the *Lp* contamination of a HWN and the genotypic and phenotypic characterization of the clone have rarely been carried out. Here, we assessed the *Lp* contamination of the UHB hot water system, mapped the risk of patient contamination, and evaluated the relatedness of the isolates that contaminated the different buildings. We further identified the risk factors that could have favored contamination of the UHB HWN by *Lp* and phenotypically validated the biological features (resistance to interbacterial competition and high temperatures) that could account for the long-term contamination of the network.

2. Materials and methods

Study setting. At the 1400-bed UHB, we sampled the HWNs of the grey and orange buildings, built in 1982 and 1999, respectively. Annual controls showed the grey building to be heavily and regularly contaminated by *Lp*. In the grey building, three stations produce hot water: the GB-NEA (Grey Building Northeast Area) production station, the GB-SWA (Grey Building Southwest Area) production station, and the GB-Basement production station. The GB-NEA consists of a semi-instantaneous looped system, which supplies patient rooms through two distribution networks (GB-EA, Grey Building East Area, and GB-NA, Grey Building North Area, Table 1). The GB-EA distribution network is composed of 17 distribution columns, 11 of which (GE01 to GE11) serve patient rooms on the second, third, and fourth floors (Table 1). Renovation work in the GB-EA distribution network led us to pay particular attention to its contamination by *Lp*. In the orange building, the HWN is a non-looped system and consists of a single semi-instantaneous hot water production station (Table 1).

Water sampling and temperature monitoring. For the GB-NEA HWN, we collected 1-L water samples monthly over one year, from October 2017 to September 2018, from 36 sampling points: three in the GB-NEA production network and 33 in the GB-EA distribution network. Water from the GB-NEA production network was sampled from the bottom of the water storage cylinder and from the loop departure and return points (n = 33). For the GB-EA distribution network, we drew 327 samples from the 11 columns serving the patient rooms on the three floors. Water samples were taken from points of use in patient rooms, such as showers or faucets, and in common showers when the latter were present and used in the care units. In total, we collected 360 samples from the GB-NEA water network and included five annual control samples in 2018 and 2020 for the NA distribution network (Table 1). Water samples from the orange building and other HWNs of the grey building (GB-SWA and GB-Basement) were also included in this study (n = 21) and were part of the annual control in 2018 and 2020 (Table 1).

Water was sampled according to French guidelines (*i.e.* after faucet accessory removal and a water pre-flush until the temperature has stabilized and within 2–3 min) ("PR FD T90-522", 2006). The maximum water temperature (T_{max}) was measured. The target temperature was >60 °C at the loop departure and >50 °C for all other points of the distribution network (Centre scientifique et technique du bâtiment, 2012).

Microbiological detection of *Legionellae*. We detected and quantified *Legionellae* and *Lp* according to French technical guidelines ("NF T90-431", 2017). Briefly, water was analyzed within 24 h after sampling. Two hundred microliters was plated on GVPC agar (Thermo Fisher Oxoid, Dardilly, France). In addition, 10 ml and 100 ml were filtered through 0.45-µm membranes (Merck, Darmstadt, Germany), treated 5 min with a pH 2.0 KCl–HCl buffer, placed on GVPC agar, and incubated at 36 °C for 8 to 11 days. We subcultured colonies for which the aspect was suggestive of *Legionellae* on BCYE agar, with or without L-cysteine (Thermofisher Oxoid, Dardilly, France). Colonies growing only on BCYE supplemented with L-cysteine agar were confirmed to be *Legionellae*, among which *Lp* was identified and serotyped by latex agglutination (Thermofisher Oxoid, Dardilly, France). We stored each *Lp* isolate in brain heart infusion broth supplemented with 30% glycerol at −80 °C until further analysis in the Centre de Ressources Biologiques-Filière Microbiologique of Besançon (Biobank number BB-0033-00090).

Genotyping. The clonal relatedness of *Lp* isolates was investigated by pulsed-field gel electrophoresis (PFGE) following *Sfi*I digestion, as

Table 1
Description of the hot water networks of the grey and orange buildings of the University Hospital of Besançon (France) and localization of the water samples.

Building	Hot water production network			Hot water distribution network (distribution columns)			
	Denomination	Collected samples (n)	Temperature (°C) (2018 and 2020)	Denomination	Floors supplied by distribution columns	Collected samples (n)	Temperature (°C) (2018 and 2020)
Grey building (GB)	Northeast Area (GB-NEA)	33	52.1 and 47.2	East Area (GB-EA)[a]	+1 to + 4	327	57.5 and 49.8
				North Area (GB-NA)[b]	+1 to + 8	5	NA and 48.2
	Southwest Area (GB-SWA)	2	NA and 52.2	West Area (GB-WA)[a]	+1 to + 8	1	NA and 48.2
				South Area (GB-SA)[b]	+1 to + 8	1	NA and 43.9
	Basement (GB-Basement)	3	53.4 and 51.0	Basement distribution	- 3 to 0	0	/
Orange building	Orange production	0	/	Orange distribution	- 2 to + 2	14	54.9 and 58.8

NA = Not Available.
[a] The GB-EA and GB-WA distribution networks are each composed of 17 distribution columns that supplied four and eight floors respectively.
[b] The GB-NA and GB-SA distribution networks are each composed of 16 distribution columns that both supplied eight floors.

previously described (Oberdorfer et al., 2008). We compared the restriction profiles using GelCompar software (Applied Maths, Kortrijk, Belgium) and defined pulsotypes and clusters according to international recommendations (Tenover et al., 1995).

Inter-*Legionella* inhibition assays on agar plates. We tested the inhibition between *Legionellae* isolates using competitive assays between a 'resident *Legionellae*' and a 'challenger *Legionellae*' as previously described (Fig. 3A) (Levin et al., 2019). We streaked 10^6 CFU of each tested isolate on BCYE plates containing 0.2 g/L of cysteine followed by incubation at 37 °C for 72 h (hereafter called 'resident *Legionellae*'). Then, 3-μL spots of a 10^8 CFU/ml suspension of *Legionellae* were plated 1 cm (near spot) and 2 cm (far spot) from the streak (hereafter called 'challenger *Legionellae'*). Once the spots were dry, the plates were incubated for an additional 72 h before scoring for inhibition. We quantified living bacteria by taking agar plugs from around each spot. The plugs were transferred into a saline solution, vortexed, and plated to quantify the CFUs per spot. The inhibitory power of each 'resident isolates' over the 'challenger isolates' was calculated as the ratio of CFUs in the far spots to that in the near spots. We tested the inhibition between the dominant pulsotypes of *Lp* that contaminated the water systems of the hospital orange building (Lp O) and grey building (Lp G) and used the strains *Lp* ATCC33152 and *L. anisa* (*La*) ATCC35292 as controls. All experiments were performed in triplicate with one isolate per pulsotype (*i.e.* Lp G and Lp O). Lp G representative isolate came from water sample collected in the GB-EA distribution network (at point of use of the third floor on the distribution column 05) in July 2018. Lp O representative isolate came from water sample collected in orange building distribution network during the annual control in July 2017.

Temperature-dependent survival assays. Local pulsotypes Lp O and Lp G and the control strains, *Lp* ATCC33152 and *La* ATCC35292, were suspended at a concentration of ~10^8 CFU/ml in tap water previously sterilized by 0.22-μm filtration. Suspensions were incubated at 50 °C, 55 °C, and 60 °C, which were the targeted temperatures of the HWN. The concentrations of the bacterial suspensions were determined at inoculation and after 24 h of incubation by plating on standard BCYE agar with a Spiral plater (Interscience, Saint-Nom la Bretèche). All experiments were performed in triplicate.

Statistical analysis. Stata software (version 14.1, Texas, USA) was used for statistical analysis. We tested the differences in the percentage of positive samples and the percentage of samples with *Lp* concentration $> 10^3$ CFU/L for the sampling points in the GB-NEA and GB-EA distribution networks using Fisher and $\chi 2$ tests. For the GB-NEA production network, *Lp* concentrations at the bottom of the storage water cylinder and the loop departure and return points were compared using the Kruskal-Wallis test. The correlations between the *Lp* concentrations and T_{max} measured for the three sampling points of the GB-NEA production network were studied using Spearman's coefficient. Multivariate analysis was used to evaluate the role of the location (floors and columns), temperature (<55 °C and ≥55 °C), and seasonality (autumn, winter, spring, and summer) for the EA distribution columns. Multivariate analysis using logistic regression models to estimate the adjusted odds ratios and 95% confidence intervals (95% CI) was performed using two variables: *Lp* positive results (presence or absence) and concentrations ($<10^3$ CFU/L or $> 10^3$ CFU/L).

We compared the inhibition ratios for the inhibition assays between *Legionellae* using the Kruskal-Wallis test coupled with a *post-hoc* Dunn test. These tests were also used to compare the proportion of bacteria surviving after incubation at various temperatures. Concentrations below the limit of detection were set to the limit of detection (*i.e.,* 1 CFU/ml) for statistical analysis. The α value was set to 0.05 for all tests.

3. Results and discussion

***L. pneumophila* serogroup 3 contaminates the GB-NEA hot water network.** We collected 360 samples from October 2017 to September 2018. Among them, 207 (57.5%) were positive for *Lp*: 28 (84.8%) for the water production network and 179 (54.7%) for the distribution network (Table 2). All sampling points were positive at least once during the sampling period.

The proportion of positive samples in the GB-NEA production network differed between the three sampling points ($p = 0.041$), with a higher percentage at the bottom of the water storage cylinder (100%) than in the loop departure (63.6%, $p = 0.037$, Table 2). The percentage of samples with an *Lp* concentration $> 10^3$ UFC/L also differed between the three sampling points ($p = 0.003$) and was lowest at the loop departure ($p = 0.003$, Table 2). In the distribution columns, the percentage of positive samples ranged from 43.0% (on the second floor) to 77.0% (on the fourth floor), where it was the highest ($p < 0.001$, Table 2). The percentage of samples with an *Lp* concentration $> 10^3$ UFC/L was lowest on the second floor ($p = 0.033$, Table 2). All isolates belonged to serogroup 3.

The L. *pneumophila* concentration correlates with water temperature. In the GB-NEA production network, the median *Lp* concentration ranged from 130 CFU/L (loop departure) to 37,500 CFU/L (bottom of the water storage cylinder). The median concentration was 5450 CFU/L and, as already mentioned, was highest at the bottom of the water storage cylinder ($p = 0.002$, Table 2). The targeted temperatures (*i.e.,* > 50 °C and >60 °C) were reached for two months during the study period for the loop departure, four months for the bottom of the water storage cylinder, and eight months for the loop return (Fig. 1). For the production sampling points, the *Lp* concentration was negatively associated with the T_{max} (Fig. 1). Hence, the highest concentration observed in March for the loop departure (35,000 CFU/L) and at the bottom of the water storage cylinder (750,000 CFU/L) were concomitant with the lowest temperatures, 55.9 °C and 27.4 °C, respectively (Fig. 1A and C). Similarly, there was a negative correlation for the loop return ($p = 0.009$), which was highly colonized from January to April (from 5600 CFU/L in March to 55,000 CFU/L in January), with T_{max} below or equal to the threshold (from 48.0 to 50.2 °C, Fig. 1B).

Minimizing *Lp* contamination in HWNs can be achieved by maintaining elevated water temperatures. Hence, many studies have reported a negative correlation between *Lp* concentration and temperature (Bédard et al., 2015, 2016; Gavaldà et al., 2019; Groothuis et al., 1985; Rhoads et al., 2015).

In the GB-EA distribution network, the median *Lp* concentration ranged from 0 CFU/L (second and third floors) to 175 UFC/L (fourth floor) (Table 2), with the concentration being highest on the fourth floor ($p = 0.001$, Table 2). The results of multivariate analysis indicated that the risk of recovering *Lp* was 6.49 times higher on this floor (95 CI = 2.89–14.58, Table 3). Both the third ($p = 0.008$) and fourth floors ($p = 0.045$, Table 3) were associated with *Lp* concentrations $> 10^3$ CFU/L. The risk of recovering *Lp* from the GB-EA distribution network decreased when the temperature was >55 °C ($p < 10^{-3}$, Table 3). Moreover, it has been shown that temperatures >55 °C in distribution networks more efficiently control *Legionellae* contamination than 50 °C (Gavaldà et al., 2019). Columns GE01 and GE02, which were the furthest away from the site of production, were also *Lp* positive ($p < 0.001$ for the two columns) and showed *Lp* concentrations $> 10^3$ CFU/L ($p < 0.001$ for the two columns, Table 3). This was presumably due to oversizing of the return sections of these two columns, leading to a decrease in circulation speed and stagnation of the water in the ring pipe. Hence, water circulation speed was lower than the minimum recommended 0.15 m s^{-1} (data not shown) (Centre scientifique et technique du bâtiment, 2012).

Concerning seasonality, the risk of *Lp* contamination and finding a concentration $> 10^3$ CFU/L was 2.90 times (95 CI = 1.27–6.63) and 4.50 times (95 CI = 1.60–12.64) higher in the spring, respectively (Table 3). Summer was associated with a higher risk of recovering an *Lp* concentration $> 10^3$ CFU/L (odds ratio = 7.96, 95 CI = 2.47–25.71) (Table 3). Such seasonal water system contamination by *Lp* has already been reported (Perrin et al., 2019).

The greater contamination of the GB-NEA with *Lp* was probably due to difficulty in reaching the targeted temperatures. This was particularly

Table 2
***Legionella pneumophila* contamination rates for the GB-NEA production and GB-EA distribution network of the University Hospital of Besançon from October 2017 to September 2018.**

Network location	Sampled site	Water samples collected (n)[1]	Positive samples (n, %)[2]	Samples with *Lp* concentration > 10^3 CFU/L (n, %)[3]	*Lp* median concentration (CFU/L)
GB-NEA production		**33**	**28 (84.8)**	**22 (66.7)**	**5,450**
	Bottom of the water storage cylinder	12	12 (100)	11 (91.7)	37,500
	Loop departure	11	7 (63.6)	3 (27.3)	130
	Loop return	10	9 (90.0)	8 (80.0)	4,200
GB-EA distribution		**327**	**179 (54.7)**	**81 (24.7)**	**10**
	Second floor	121	52 (43.0)	21 (17.4)	0^4
	Third floor	106	50 (47.2)	31 (29.3)	0^4
	Fourth floor	100	77 (77.0)	29 (29.0)	175
Total		**360**	**207 (57.5)**	**103 (28.6)**	**-**

Bracketed comparisons: Bottom of the water storage cylinder vs Loop departure: $p = 0.037$; Bottom of the water storage cylinder vs Loop return: $p = 0.003$; Bottom of the water storage cylinder vs GB-EA distribution: $p = 0.002$; Loop departure vs Loop return: $p = 0.030$; Second floor vs Third floor: $p < 0.001$; Second floor vs Fourth floor: $p = 0.033$; Third floor vs Fourth floor: $p = 0.002$; GB-EA distribution vs Fourth floor: $p = 0.001$.

[1] Number of sampling points sampled monthly for the GB-NEA production and distribution columns of the GB-EA distribution network.

[2] Number of positives samples with *L. pneumophila* among all water samples collected.

[3] Number of samples with a *L. pneumophila* concentration > 10^3 CFU/L among all water samples collected.

[4] Concentrations < 10 CFU/L were considered as 0 CFU/L for calculation.

Significant differences ($p < 0.05$) in the percentage of positive samples and the percentage of samples with a *Lp* concentration > 10^3 CFU/L according to the location in the GB-NEA production and GB-EA distribution networks were assessed using Fisher's Exact test.

Significant differences ($p < 0.05$) between *Lp* median concentrations were assessed using the Kruskal-Wallis test.

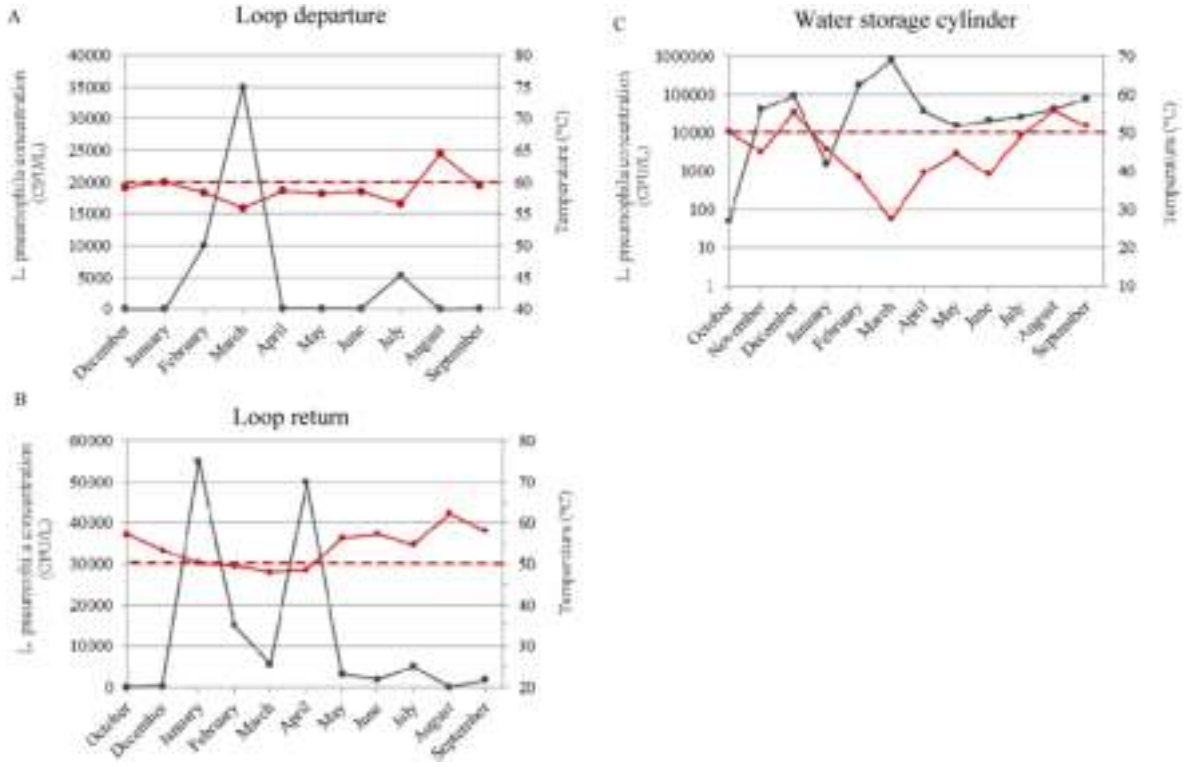

Fig. 1. Monthly concentration of *Legionella pneumophila* and maximum measured temperature (T_{max}) in the GB-NEA water production network of the University Hospital of Besançon from October 2017 to December 2018. (A) At the loop departure. (B) At the loop return. (C) At the bottom of water storage cylinder. Grey curves represent the monthly concentration of *Lp*, with a logarithmic scale for (C). Red curves represent the T_{max} measured during the sampling. Red dotted lines show the targeted temperature at the corresponding sampling points. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

true for the bottom of the water storage cylinder, which was always contaminated with a high load of *Lp* (Table 2). The difficulty in maintaining a continuously high temperature in this semi-instantaneous GB-NEA loop system may be due to an insufficiently heated water supply during periods of high water withdrawal (Centre scientifique et technique du bâtiment, 2012). The loop departure was less frequently contaminated with *Lp* than the bottom of the water storage cylinder (Table 2), although the targeted temperature (*i.e.,* > 60 °C) was rarely

Table 3
Logistic regression analysis for the association between the *Legionella* results and the location, maximum measured water temperature (T_{max}), and seasonality in the GB-EA distribution network of the University Hospital of Besançon.

	Positive L. *pneumophila* result			*L. pneumophila* concentration > 10^3 CFU/L		
	Adjusted OR[a]	95% CI[b]	*p*	Adjusted OR	95% CI	*P*
Floor						
Second floor	Reference	–	–	Reference	–	–
Third floor	1.68	0.81–3.48	0.17	2.86	1.32–6.20	0.008
Fourth floor	6.49	2.89–14.58	<0.001	2.28	1.02–5.12	0.045
Column						
GE03 to 11	Reference	–	–	Reference	–	–
GE02	21.68	5.66–83.08	<0.001	17.40	6.64–45.58	<0.001
GE01	32.80	6.80–158.26	<0.001	10.66	4.04–28.17	<0.001
T_{max}						
<55 °C	Reference	–	–	Reference	–	–
≥55 °C	0.033	0.012–0.088	<0.001	0.34	0.17–0.70	0.003
Seasonality						
Autumn	Reference	–	–	Reference	–	–
Winter	2.70	1.15–6.31	0.022	2.42	0.84–6.93	0.10
Spring	2.90	1.27–6.63	0.012	4.50	1.60–12.64	0.004
Summer	2.59	0.78–8.55	0.119	7.96	2.47–25.71	0.001

[a] OR, odds ratio.
[b] 95% CI, 95% confidence interval.

reached (Fig. 1A). However, temperatures were consistently >55 °C, contrary to the temperatures at the bottom of the water storage cylinder (Fig. 1C). Overall, the configuration of the hot water system prevented the maintenance of high temperatures and optimal water circulation, the two main preventive measures to reduce the risk of *Lp* contamination.

Specific pulsotypes persistently colonized the building hot water networks. We genotyped 187 isolates of *Lp* using PFGE, with 135 being retrieved from the GB-NEA HWN in 2017 and 2018. These were compared to isolates recovered from the GB-NEA in 2020 (n = 31) and from other HWNs of the UHB in 2018 and 2020 (GB-SWA network, n = 4; GB-Basement, n = 3; orange building HWN, n = 14). The vast majority of the isolates from the GB-NEA HWN (134 of 135 isolates) shared the same pulsotype, hereon called Lp G (Fig. 2). This pulsotypes was also shared by all isolates retrieved in 2018 and 2020 from other HWNs of the same building (*i.e.,* GB-SWA, GB-basement) and the GB-NEA two years later (in 2020) (Fig. 2).

In addition, most (12 out of 14) of the isolates cultured from the orange building clustered with another pulsotype (called Lp O) (Fig. 2). These data suggest that a single clone of *Lp* can persistently contaminate a HWN. Monoclonal contamination of HWNs with *Lp* has already been reported in six German hospitals (Oberdorfer et al., 2008). This suggests that co-colonization by multiple clones may be prevented by abiotic or biotic parameters, such as competition between subpopulations (Rangel-Frausto et al., 1999). Indeed, isolates with a type IV pilis and type II secretion systems would be able to colonize the biofilm and thus persist for a long time in water systems by competition with isolates without such systems (Lucas et al., 2006). Type IV pilis and type II secretion systems are very common amongst isolates; it is most likely that the *Lp* isolates tested here have genes coding for these systems.

Installed *Lp* isolates inhibit the growth of challenger *Lp*. The persistent contamination of a given HWN with a specific pulsotype of *Lp* led us to investigate how *Lp* isolates engage in inter-*Legionellae* competition. Using a previously described method (Levin et al., 2019), we found that the two pulsotypes resident in the grey and orange buildings (Lp G and Lp O, respectively) inhibited the growth of the challenger *Legionellae* plated 1 cm away on solid media (Fig. 3A). To quantify the observed inhibition, we recovered the challenger *Legionellae* grown at different distances from the resident *Legionellae* (Table S1). Although bacteria of the same pulsotype that were already growing on the plate slightly inhibited the challenger bacteria, the inhibition was not statistically significant (Fig. 3B, Table S1). On the contrary, we found a 16-fold difference in growth between Lp O antagonized by Lp G in the near spot *versus* Lp O plated outside of the zone of inhibition in the far spot (Fig. 3B). Similarly, Lp G growth was reduced 12-fold in the inhibition zone of Lp O (Kruskal-Wallis test, *p*-values = 0.050, followed by Dunn's test; Fig. 3B).

Interestingly, the observed inhibition did not target all *Legionella*e, as resident isolates of Lp G or Lp O did not inhibit the challengers *Lp* ATCC33152 and *La* ATCC35292 (Fig. 3B). Early studies suggested that *Lp* may compete with other *Legionella*e for similar biological niches (Wery et al., 2008) and it was recently proposed that established *Legionellae* communities may deploy molecules, such as homogentisic acid, that can protect against invasion by low-density competitors (Levin et al., 2019). Here, we validated that *Lp* installed in the grey building can inhibit the growth of *Lp* originating from the orange building, and *vice versa.*

Lp G and Lp O have a different tolerance to temperature. Environmental factors could be crucial for monoclonal contamination with *Lp* (David et al., 2017; Rodríguez-Martínez et al., 2015; Sharaby et al., 2017). Accordingly, water temperature influences the clonal diversity of *Legionellae,* which is lower in hot water than in cold water (Lesnik et al., 2016). Moreover, Sharaby et al. showed that different sites along a water network were dominated by three genotypes and that their location depended on the water temperature; these genotypes could behave as ecotypes, with distinct temperature ranges (Sharaby et al., 2017). As in other hospital buildings, the UHB HWN is composed of several ecological niches with different water temperatures, each favoring the implantation of a specific clone. Indeed, the median T_{max} in the HWN of the orange building varied from 55 °C to 60 °C between 2018 and 2020, whereas it varied from 44 °C to 57 °C between 2018 and 2020 in the hot water of the grey building (Table S3). We thus tested the ability of the Lp O and Lp G pulsotypes to survive in filter-sterilized sterile tap water at 50 °C, 55 °C, and 60 °C using *Lp* ATCC33152 and *La* ATCC35292 as controls. No strains remained culturable after 24h incubation at 60 °C (Fig. 4, Table S2). The proportion of *Legionellae* strains surviving at 50 °C varied from 4.13×10^{-5} to 2.46×10^{-4}, with no difference in the survival of Lp O and Lp G. At that temperature, we only found that Lp O was more persistent than *Lp* ATCC33152 (Kruskal-Wallis test, *p*-value = 0.016). This contrasted with the results for incubation at 55 °C for 24 h, after which Lp G persisted better than Lp O and *La* ATCC35292 (Kruskal-Wallis test, p-values = 0.014, Fig. 4, Table S2). Overall, we found that Lp G survived at a more stressful temperature (55 °C) than Lp O.

This result confirms that the resistance to high temperature varies between *Lp* clones (Sharaby et al., 2017). However, the incongruence between our results and the median T_{max} measured in the orange and

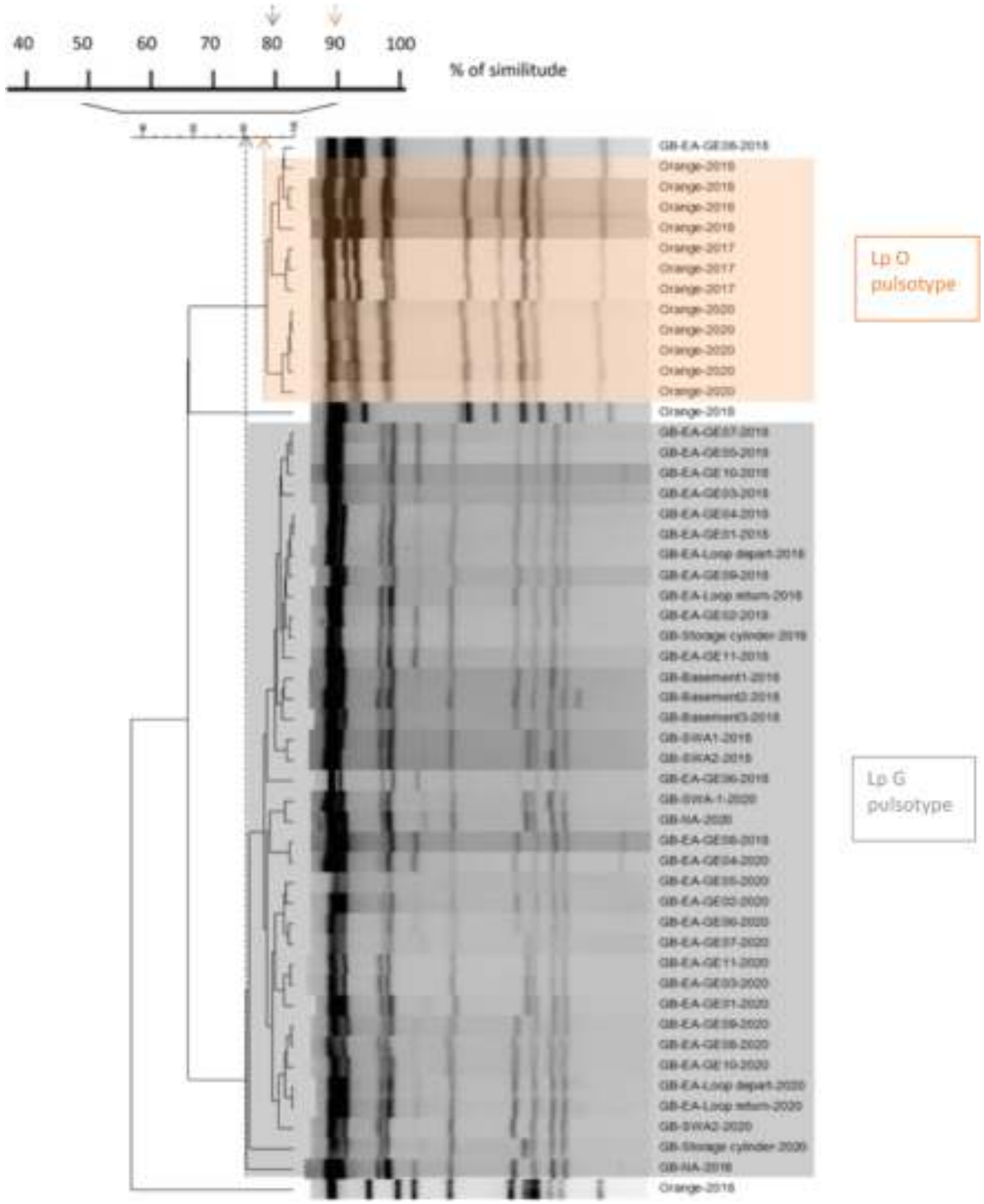

Fig. 2. Dendrogram representing the pulsotypes of *Legionella pneumophila* isolates from the grey and orange buildings of the University Hospital of Besançon between 2017 and 2020. For the GB-NEA HWN, 166 isolates were genotyped between 2018 and 2020 and among them, 165/166 shared the same genotype. For 2018 and 2020, one isolate per distribution column and per sampled site of the production network (water storage cylinder and loop departure and return points) were chosen to build the dendrogram. Thirty-one genotypes are represented and are named as follows: sample localization (GB-EA or GB-NA)-production or the number of the distribution column-year of collection. The genotypes of all isolates found at the sampled sites in 2017, 2018, and 2020 are represented in the dendrogram for the other HWNs of the grey (GB-SWA and GB-Basement) and orange buildings. Grey (Lp G pulsotype) and orange (Lp O pulsotype) boxes group the isolates from the HWNs of the orange and grey buildings, respectively, with the same pulsotype according to international recommendations. Orange and grey arrows show the percentage of similarity for the isolates from the orange and grey buildings, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

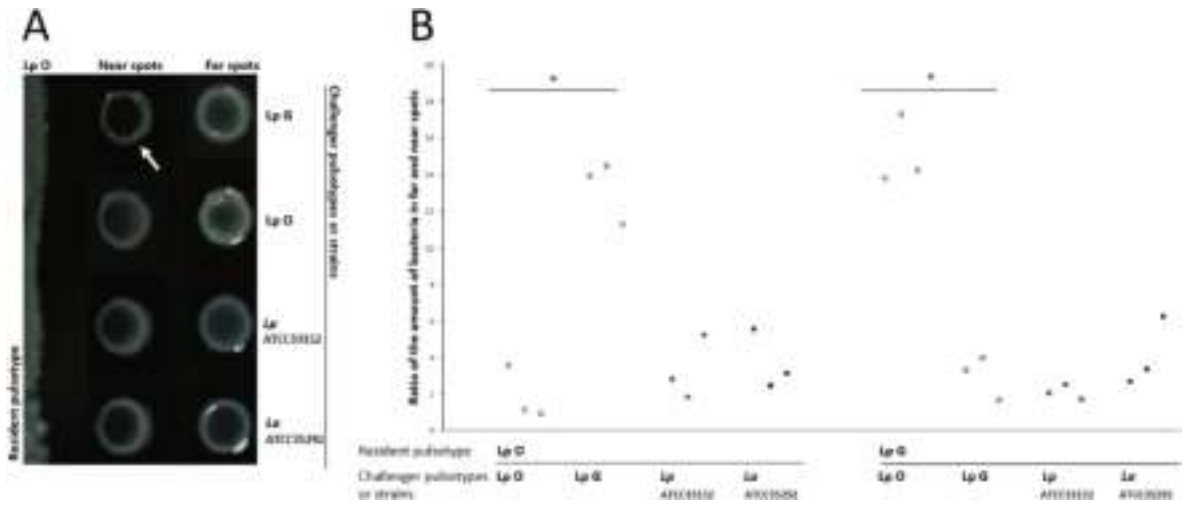

Fig. 3. *L. pneumophila* isolates of Lp G inhibit the growth of Lp O and *vice versa*. (A) When pre-incubated on low-cysteine BCYE charcoal agar plates, Lp O produces a zone of inhibition, affecting the growth of nearby *Legionellae*. Three-microliters of a 10^8 CFU/ml suspension of *Legionellae* were spotted in parallel columns three days after streaking Lp O. The white arrow indicates a zone of inhibition of the spot of Lp G 1 cm away the Lp O streak. (B) Quantification of the inhibition of challenger *Legionella* by the resident *Legionella*. Bacteria were removed from the plate in a plug of fixed area before plating for CFU counting. Data shown are the ratios of the CFU quantity in the far spots and that in the near spots. Each experiment was done in triplicate.

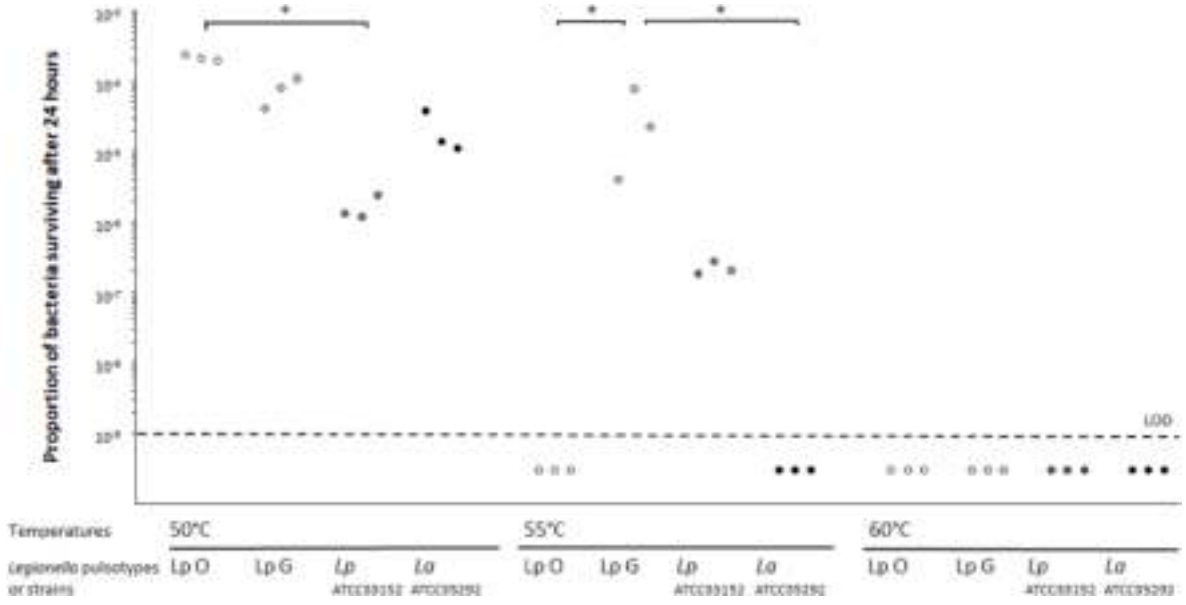

Fig. 4. The isolates of *Legionella pneumophila* Lp G and Lp O colonizing different buildings of the same hospital have different tolerance to temperature. We measured the ability to survive in filter-sterilized tap water at 50 °C, 55 °C, and 60 °C of Lp O (pulsotype colonizing the hot water system of orange building), Lp G (pulsotype colonizing the hot water system of grey building), and the reference strains *L. pneumophila* (*Lp*) ATCC33152 and *L. anisa* (*La*) ATCC35292. Each clone was suspended in water at 10^8 CFU/ml in triplicate and incubated 24 h. The results are shown as ratio of CFU/mL after 24 h incubation and to the CFU/mL before incubation. The limit of detection (LOD) was 1 CFU/ml and corresponded to a proportion of surviving bacteria of 1.00×10^{-9} (black dotted line).

grey buildings between 2018 and 2020 may be due to the evolution of Lp G in a system with drastic temperature changes. Hence, these conditions could have selected a pulsotype able to grow at low temperature and to survive in parts of the system with higher temperature. In the orange building, the more constant temperature will not allow this. Moreover, these results suggest also that other abiotic parameters may have also influenced the genotype distribution.

Limitations and strengths of the study. This study had several limitations. First, we only detailed the colonization by *Lp* in a portion of our hospital hot water system (the GB-NEA HWN). As sanitary controls of this portion had been strengthened due to hydraulic problems, this overestimated the percentage of samples positive for *Lp* for the whole hospital. Second, only one isolate of each positive sample was typed by PFGE, whereas it is known that up to three genetically different *Lp* genotypes can be found in one sample (Lück et al., 1998). Third, we explored the clonal relatedness of *Lp* isolates using PFGE. Despite its good performance and its wide use for epidemiological studies (Lück et al., 1998), this typing method is less discriminatory than genome-based typing methods (David et al., 2017). However, repeated sampling of all sites probably provided us with a complete picture of the genotypes contaminating the network. The long duration of the sampling period (2017–2020) is a strength of the study. We also paid particular attention to phenotypically explore the biological features that could account for the long-term association between a niche and a *Lp* clone.

Although no proven cases of nosocomial LD occurred during the

sampling period (2017–2020), corrective measures to control *Lp* contamination are needed. Measures implemented in the UHB (*i.e.,* thermal shocks, flushing of distribution columns, storage cylinder emptying) were insufficient to prevent long-term contamination of the HWN by *Lp*. These measures need be associated with optimization of the thermal and hydraulic performance of the HWN, such as the identification of dead ends and renovation of the networks themselves (Bédard et al., 2016).

4. Conclusion

We report persistent contamination of a hospital HWN with *Lp* serotype 3, with the distribution network being less contaminated than the production network. *Lp* concentrations were negatively associated with the T_{max} in the production network. We found that the risk of recovering *Lp* in the distribution network decreased when the temperature was >55 °C and increased in the spring and summer, as well as in the columns furthest away from the production network. The typing of isolates by PGFE showed each of the two sampled buildings to be persistently contaminated with a specific pulsotype of *Lp*. In-vitro experiments showed that *Lp* resident in one building inhibited the growth of the *Lp* originating from the other. Overall, we speculate that the suboptimal configuration of the HWN prevented the maintenance of sufficiently high temperatures and optimal water circulation, the two main preventive measures used to reduce the risk of *Lp* contamination.

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Author contributions

All authors contributed to the design or conduct of the study. DH, XB, and AJ developed the study protocol. SC, MR, and AJ collected the samples. SC, MR, AM, PC, DH, DH, and AJ performed or supervised the microbiological analyses. AM performed the statistical analyses. SC, AM, DH, MS, XB, and AJ drafted the manuscript and all authors reviewed and contributed to the manuscript. AJ coordinated the project.

Declaration of competing interest

None of the authors have anything to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114143.

References

Arvand, M., Jungkind, K., Hack, A., 2011. Contamination of the cold water distribution system of health care facilities by *Legionella pneumophila*: do we know the true dimension? Euro Surveill. 16, 19844.

Bargellini, A., Marchesi, I., Righi, E., Ferrari, A., Cencetti, S., Borella, P., Rovesti, S., 2011. Parameters predictive of *Legionella* contamination in hot water systems: association with trace elements and heterotrophic plate counts. Water Res. 45, 2315–2321.

Bédard, E., Fey, S., Charron, D., Lalancette, C., Cantin, P., Dolcé, P., Laferrière, C., Déziel, E., Prévost, M., 2015. Temperature diagnostic to identify high risk areas and optimize *Legionella pneumophila* surveillance in hot water distribution systems. Water Res. 71, 244–256.

Bédard, E., Boppe, I., Kouamé, S., Martin, P., Pinsonneault, L., Valiquette, L., Racine, J., Prévost, M., 2016. Combination of heat shock and enhanced thermal regime to control the growth of a persistent *Legionella pneumophila* strain. Pathogens 5, 5020035.

Campese, C., Bitar, D., Jarraud, S., Maine, C., Forey, F., Etienne, J., Desenclos, J.C., Saura, C., Che, D., 2011. Progress in the surveillance and control of *Legionella* infection in France, 1998–2008. Int. J. Infect. Dis. 15, e30–e37.

Centre scientifique et technique du bâtiment, 2012. Guide technique : Maîtrise du risque de développement des légionelles dans les réseaux d'eau chaude sanitaire. France.

David, S., Afshar, B., Mentasti, M., Ginevra, C., Podglajen, I., Harris, S.R., Chalker, V.J., Jarraud, S., Harrison, T.G., Parkhill, J., 2017. Seeding and establishment of *Legionella pneumophila* in hospitals: implications for genomic investigations of nosocomial legionnaires' disease. Clin. Infect. Dis. 64, 1251–1259.

Dennis, P.J.L., 1990. Reducing the risk of legionnaires' disease. Ann. Occup. Hyg. 34, 189–193.

Emmerson, A., 2001. Emerging waterborne infections in health-care settings. Emerg. Infect. Dis. 7, 272–276.

European Centre for Disease Prevention and Control, 2020. Legionnaires' Disease - Annual Epidemiological Report for 2018. https://www.ecdc.europa.eu/en/publications-data/legionnaires-disease-annual-epidemiological-report-2018, 22.11.22.

Gavaldà, L., Garcia-Nuñez, M., Quero, S., Gutierrez-Milla, C., Sabrià, M., 2019. Role of hot water temperature and water system use on *Legionella* control in a tertiary hospital: an 8-year longitudinal study. Water Res. 149, 460–466.

Groothuis, D.G., Veenendaal, H.R., Dijkstra, H.L., 1985. Influence of temperature on the number of *Legionella pneumophila* in hot water systems. J. Appl. Bacteriol. 59, 529–536.

Legifrance, 2010. Arrêté du 1er Février 2010 Relatif à la Surveillance des Légionelles dans les Installations de Production. de Stockage et de Distribution d'eau Chaude Sanitaire, Paris, France.

Lesnik, R., Brettar, I., Höfle, M.G., 2016. *Legionella* species diversity and dynamics from surface reservoir to tap water: from cold adaptation to thermophily. ISME J. 10, 1064–1080.

Levin, T.C., Goldspiel, B.P., Malik, H.S., 2019. Density-dependent resistance protects *Legionella pneumophila* from its own antimicrobial metabolite. HGA. Elife. 8, e46086.

Lin, Y.E., Stout, J.E., Yu, V.L., 2011. Prevention of hospital-acquired legionellosis. Curr. Opin. Infect. Dis. 24, 350–356.

Lucas, C.E., Brown, E., Fields, B.S., 2006. Type IV pili and type II secretion play a limited role in *Legionella pneumophila* biofilm colonization and retention. Microbiology 152, 3569–3573.

Lück, P.C., Wenchel, H.-M., Helbig, J.H., 1998. Nosocomial pneumonia caused by three genetically different strains of *Legionella pneumophila* and detection of these strains in the hospital water supply. J. Clin. Microbiol. 36, 1160–1163.

Martinelli, F., Caruso, A., Moschini, L., Turano, A., Scarcella, C., Speziani, F., 2000. A comparison of *Legionella pneumophila* occurrence in hot water tanks and instantaneous devices in domestic, nosocomial, and community environments. Curr. Microbiol. 41, 374–376.

Miyashita, N., Higa, F., Aoki, Y., Kikuchi, T., Seki, M., Tateda, K., Maki, N., Uchino, K., Ogasawara, K., Kiyota, H., Watanabe, A., 2020. Distribution of *Legionella* species and serogroups in patients with culture-confirmed Legionella pneumonia. J. Infect. Chemother. 26, 411–417.

NF T90-431, 2017. Qualité de l'eau — Recherche et dénombrement de *Legionella* spp et de *Legionella pneumophila* — Méthode par ensemencement direct et après concentration par filtration sur membrane ou centrifugation (n.d).

Oberdorfer, K., Müssigbrodt, G., Wendt, C., 2008. Genetic diversity of *Legionella pneumophila* in hospital water systems. Int. J. Hyg Environ. Health 211, 172–178.

Pancer, K., Matuszewska, R., Bartosik, M., Kacperski, K., Krogulska, B., 2013. Persistent colonization of 2 hospital water supplies by *L. pneumophila* strains through 7 years – sequence-based typing and serotyping as useful tools for complex risk analysis. Ann. Agric. Environ. Med. 20, 8.

Perola, O., Kauppinen, J., Kusnetsov, J., Karkkainen, U.M., Luck, P.C., Katila, M.L., 2005. Persistent *Legionella pneumophila* colonization of a hospital water supply: efficacy of control methods and a molecular epidemiological analysis. APMIS 113, 45–53.

Perrin, Y., Bouchon, D., Hechard, Y., Moulin, L., 2019. Spatio-temporal survey of opportunistic premise plumbing pathogens in the Paris drinking water distribution system. Int. J. Hyg Environ. Health 222, 687–694.

Rangel-Frausto, M.S., Rhomberg, P., Hollis, R.J., Pfaller, M.A., Wenzel, R.P., Helms, C. M., Herwaldt, L.A., 1999. Persistence of *Legionella pneumophila* in a hospital's water system: a 13-year survey. Infect. Control Hosp. Epidemiol. 20, 793–797.

Rhoads, W.J., Ji, P., Pruden, A., Edwards, M.A., 2015. Water heater temperature set point and water use patterns influence *Legionella pneumophila* and associated microorganisms at the tap. Microbiome 3.

Rodríguez-Martínez, S., Sharaby, Y., Pecellín, M., Brettar, I., Höfle, M., Halpern, M., 2015. Spatial distribution of *Legionella pneumophila* MLVA-genotypes in a drinking water system. Water Res. 77, 119–132.

Serrano-Suárez, A., Dellundé, J., Salvadó, H., Cervero-Aragó, S., Méndez, J., Canals, O., Blanco, S., Arcas, A., Araujo, R., 2013. Microbial and physicochemical parameters associated with *Legionella* contamination in hot water recirculation systems. Environ. Sci. Pollut. Res. 20, 5534–5544.

Sharaby, Y., Rodríguez-Martínez, S., Oks, O., Pecellin, M., Mizrahi, H., Peretz, A., Brettar, I., Höfle, M.G., Halpern, M., 2017. Temperature-dependent growth modeling of environmental and clinical *Legionella pneumophila* multilocus variable-number tandem-repeat analysis (MLVA) genotypes. Appl. Environ. Microbiol. 83 e03295-16.

Stout, J.E., Muder, R.R., Mietzner, S., Wagener, M.M., Perri, M.B., DeRoos, K., Goodrich, D., Arnold, W., Williamson, T., Ruark, O., Treadway, C., Eckstein, E.C., Marshall, D., Rafferty, M.E., Sarro, K., Page, J., Jenkins, R., Oda, G., Shimoda, K.J., Zervos, M.J., Bittner, M., Camhi, S.L., Panwalker, A.P., Donskey, C.J., Nguyen, M.-H., Holodniy, M., Yu, V.L., Legionella Study Group, 2007. Role of environmental surveillance in determining the risk of hospital-acquired legionellosis: a national

surveillance study with clinical correlations. Infect. Control Hosp. Epidemiol. 28, 818–824.
T90-522, F.D., 2006. Qualité de l'eau - Guide technique de prélèvement pour la recherche des *legionella* dans l'eau. France.
Vincenti, S., de Waure, C., Raponi, M., Teleman, A.A., Boninti, F., Bruno, S., Boccia, S., Damiani, G., Laurenti, P., 2019. Environmental surveillance of *Legionella* spp. colonization in the water system of a large academic hospital: analysis of the four–year results on the effectiveness of the chlorine dioxide disinfection method. Sci. Total Environ. 657, 248–253.
Wery, N., Bru-Adan, V., Minervini, C., Delgenes, J.P., Garelly, L., Godon, J.J., 2008. Dynamics of*Legionella* spp. and bacterial populations during the proliferation of *L. Pneumophila*in a cooling tower facility. Appl. Environ. Microbiol. 74, 3030–3037.

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PFASs: What can we learn from the European Human Biomonitoring Initiative HBM4EU

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ABSTRACT

Per- and polyfluoroalkyl substances (PFASs) were one of the priority substance groups selected which have been investigated under the ambitious European Joint programme HBM4EU (2017–2022). In order to answer policy relevant questions concerning exposure and health effects of PFASs in Europe several activities were developed under HBM4EU namely i) synthesis of HBM data generated in Europe prior to HBM4EU by developing new platforms, ii) development of a Quality Assurance/Quality Control Program covering 12 biomarkers of PFASs, iii) aligned and harmonized human biomonitoring studies of PFASs. In addition, some cohort studies (on mother-child exposure, occupational exposure to hexavalent chromium) were initiated, and literature researches on risk assessment of mixtures of PFAS, health effects and effect biomarkers were performed. The HBM4EU Aligned Studies have generated internal exposure reference levels for 12 PFASs in 1957 European teenagers aged 12–18 years. The results showed that serum levels of 14.3% of the teenagers exceeded 6.9 μg/L PFASs, which corresponds to the EFSA guideline value for a tolerable weekly intake (TWI) of 4.4 ng/kg for some of the investigated PFASs (PFOA, PFOS, PFNA and PFHxS). In Northern and Western Europe, 24% of teenagers exceeded this level. The most relevant sources of exposure identified were drinking water and some foods (fish, eggs, offal and locally produced foods). HBM4EU occupational studies also revealed very high levels of PFASs exposure in workers (P95: 192 μg/L in chrome plating facilities), highlighting the importance of monitoring PFASs exposure in specific workplaces. In addition, environmental contaminated hotspots causing high exposure to the population were identified.

In conclusion, the frequent and high PFASs exposure evidenced by HBM4EU strongly suggests the need to take all possible measures to prevent further contamination of the European population, in addition to adopting

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remediation measures in hotspot areas, to protect human health and the environment. HBM4EU findings also support the restriction of the whole group of PFASs. Further, research and definition for additional toxicological dose-effect relationship values for more PFASs compounds is needed.

1. Introduction

Per- and polyfluoroalkyl substances (PFASs) were one of the priority substance groups selected in the European Human Biomonitoring Initiative HBM4EU. Several policy-questions on exposure and health effects were developed and addressed under the Scoping Document at the beginning of the research program, including an attempt to answer whether existing regulations on PFASs were sufficient to protect human health. These policy questions were addressed in the Scoping Document for PFASs[1] at the beginning of the research program.

The HBM4EU project period coincided with growing awareness of the potentially harmful effects of PFAS exposures in the general population. As an example, in 2020 the European Food Safety Authority derived a new tolerable weekly intake (TWI) of 4.4 ng/kg bw per week, for the sum of PFOA, PFNA, PFHxS and PFOS (EFSA, 2020). The new TWI was derived on the basis of effects on the immune system, more specifically the reduction of antibody response after vaccination in children (Abraham et al., 2020). This TWI corresponds to a blood serum level of 6.9 μg/L in women of childbearing age, which would prevent the breastfed infant from exceeding 17.5 μg/L in serum; the reference point derived by EFSA. In this approach, EFSA assumed equal potency for the four substances as no studies were available to derive relative potency factors (RPFs) for human effects. The dietary exposure assessment performed by EFSA showed that large numbers of the EU-population exceeds the new TWI.

While most individuals are exposed to background-levels of PFASs, some populations living near contaminated sites are exposed at moderate, high or extremely high levels (Xu et al., 2020, 2021; Ingelido et al., 2018). The main sources of PFASs contamination at hotspots are (historical) PFASs production, which contaminated the soil and groundwater, or the use of PFASs containing products such as PFASs containing firefighting foams or PFASs contaminated biosolids applied as soil improving materials. The list of suspected PFASs contaminated sites in the EU is continuously growing. Occupational exposure to PFASs has only been investigated in a few studies (Olsen et al., 2007), such as in fluoropolymer manufacturing, firefighters when using aqueous film-forming foams (AFFF) or professional ski waxers (Langenbach and Wilson, 2021). However, PFASs have a wide range of applications; therefore, occupational exposure is expected to occur in other sectors as well (Langenbach and Wilson, 2021).

This publication synthesizes HBM4EU's activities, results and knowledge gained within the five years and half of HBM4EU and highlights how they may be used to inform science-based policy decisions on PFASs in Europe.

2. Results and discussion

2.1. Exposure to PFASs

2.1.1. General population: The HBM4EU teenagers aligned study

One of the overall objectives of HBM4EU was to assess exposure to PFASs among other priority chemicals in Europe in the best possible harmonized way.

A prioritized list of biomarkers, matrices and analytical methods was developed for a broad spectrum of PFASs, including both well-known PFASs (e.g. perfluoroalkyl carboxylic acids (PFCAs) and perfluoroalkane sulfonic acids (PFSAs) as well as novel PFASs (e.g. HFPO-DA (GenX) and ADONA) which were used as alternatives to the legacy ones (Vorkamp et al., 2021). Generally, the preferred matrix for targeted PFASs human biomonitoring is blood, specifically serum or plasma, but breast milk and urine may also be desired matrices in specific cases.

The HBM4EU QA/QC program covered analysis of 12 PFASs in serum. The laboratory proficiency tests included four rounds in collaboration with reference laboratories worldwide (Esteban López et al., 2021). Of the total of 26 eligible candidate laboratories that had initially been invited to participate in the HBM4EU QA/QC program for PFASs, 21 laboratories from 12 European countries qualified for the analysis of at least six PFASs and seven laboratories covered the selected 12 PFASs. This approach was followed within the HBM4EU aligned studies (Gilles et al., 2021, 2022; Govarts et al., 2023), in which exposure data to 12 PFASs in serum from 1957 teenagers aged 12–18 years, sampled between 2014 and 2021 in nine European countries geographically distributed across Europe, were obtained. The description of the approach of the HBM4EU aligned studies has been described elsewhere (Gilles et al., 2021, 2022; Govarts et al., 2023). These current serum PFASs measurement data indicate a geographical difference in exposure, with higher concentrations in Northern and Western Europe for the legacy PFASs (PFOA, PFNA, PFHxS, PFOS) (Fig. 1). Between 1 and 24% of the subjects across the nine data collections have levels above the serum level corresponding to the TWI for the sum of PFOA, PFNA, PFHxS and PFOS of 6.9 μg/L (EFSA, 2020), with an overall exceedance of 14%. For the PFASs which were detected at lower levels (PFPeA, PFHxA, PFHpA, PFDA, PFUnDA, PFDoDA, PFBS and PFHpS), the detection frequencies observed were strongly dependent on the limit of quantification (LOQ) reached in the laboratories, indicating it is crucial to lower LOQs for further interpretation of the data.

Higher serum levels of PFNA and PFOS were associated with higher consumption of fish and seafood and higher consumption of eggs (increase in serum levels by 20 and 21% for fish and seafood and by 14 and 11% for eggs respectively). Furthermore, higher PFASs was linked to higher consumption of offal (increase in serum levels by 14%) and local food (increase by 40%). (Richterová et al., 2022). Fig. 1 depicts the HBM4EU exposure indicator and represents the median (P50) serum concentrations in European teenagers.

2.1.2. Exposure in hotspots

In the framework of HBM4EU, a network of experts focusing on hotspots was established that calls for systematic identification of hotspots in the EU and the establishment of human biomonitoring studies aligned on these hotspots. This would offer the opportunity to obtain larger and more comparable datasets to better investigate the relationship between exposure levels and health effects. The expert HBM4EU hotspots group also developed a short guidance document on performing HBM at hotspots and how to communicate results and potential risks and also addressed the need to identify hotspots systematically (Brouwere et al., 2022).

2.1.3. Occupational exposure

Occupational exposure to PFASs may occur in the metal sector, especially electroplating activities. PFASs have been used as mist suppressants especially in chrome plating baths to prevent the evaporation of chromium (VI) vapours (Blepp et al., 2017; Glüge et al., 2020). PFOS was earlier the most important PFAS used in plating activities. Due to the restrictions of its manufacture and use, it has been largely replaced in the EU (EC, 2020).

[1] https://www.hbm4eu.eu/wp-content/uploads/2017/04/Scoping-document-on-per-and-poly-fluoralkyl-substances.pdf.

In HBM4EU an occupational study was conducted with the aim to study exposure to hexavalent chromium in various sectors (Santonen et al., 2019, 2022). This study also included biomonitoring of exposure to PFASs in a subset of workers performing chrome plating activities and some workers performing welding activities. Some of the chrome platers showed clearly elevated PFOS serum levels with the 95th percentile for PFOS among platers being 192 μg/L, which can be explained by the former application of PFOS in electroplating baths (Santonen et al., 2023). (Glüge et al., 2020) recently performed an analysis of the uses of PFASs, demonstrating that they are used in almost all industries. Considering this, it is highly likely that there are still various unrecognized sources of occupational exposure to PFASs which need to be identified, and their impact on workers' health needs to be assessed as well as exposure and risk reduction measures to be implemented.

2.2. Health effects of PFASs

2.2.1. General population: epidemiological studies

One of the first relatively large studies addressing health effects of PFASs in the general population examined the associations between concentrations of PFOS and PFOA in pregnant women with birth weight in their offspring (Apelberg et al., 2007; Fei et al., 2007). Both studies reported higher serum concentrations to be inversely associated with birth weight. Later studies from other birth cohorts largely supported these findings (Bach et al., 2014; Gao et al., 2021). Despite consistency of those reports, it has been suggested that they may reflect interindividual differences in uptake and excretion (Savitz, 2007), rather than true causal association. Very few studies have addressed the possible mechanism for lower birth weight with higher PFASs exposure. (Nielsen et al., 2020; Verner et al., 2015).

To identify mechanisms that could explain the inverse association between prenatal PFASs exposure and birth weight, a comprehensive review of existing data was conducted as part of HBM4EU (Gundacker et al., 2022). The results suggest that the thyroid-damaging effects of PFASs and their ROS-induced effects on adipocyte differentiation are possible mechanisms for body weight reduction. Other potential mechanisms related to decreased placental weight (Fei et al., 2008) include PFOS-induced detrimental effects on decidualisation (Yang et al., 2016), trophoblast cell viability and hormone release (Zhang et al., 2015), or angiogenesis (Forsthuber et al., 2022). The PFOS-induced inhibition of signaling through the vascular endothelial growth factor receptor 2 (VEGFR2) detected in the latter study suggests a mode of action related to an existing Adverse Outcome Pathway (AOP) addressing the adverse outcome "decreased birth weight". There is also some evidence that elevated PFOS concentrations are associated with decreased levels of insulin-like growth factor 1 (IGF1) in infant serum (Lopez-Espinosa et al., 2016) and in mouse liver and testis (Wan et al., 2011), and that PFOA decreases IGF2 methylation (Kobayashi et al., 2017). Growth factors may indeed play a central role, as decreased maternal IGF1 is associated with decreased fetal growth in humans and animals (Dimasuay et al., 2016).

Epidemiological studies have also shown relatively consistent findings between long-chain PFASs and increasing levels of serum cholesterol in both cross-sectional and prospective designs conducted in background and occupationally exposed subjects, which has been discussed in a HBM4EU review paper (Fragki et al., 2021). The molecular mechanism responsible for PFASs causing an increase in cholesterol levels is not fully understood, but may involve lipid uptake mechanisms including the bile acid surfactant system (Sinisalu et al., 2021), vesicle size, permeability of membranes or links to the immune system (Wit et al., 2022) as well as effects on PPAR receptors. Ecological comparison between people exposed to contaminated versus non-contaminated drinking water have also shown on average higher lipid concentrations, supporting a causal association (Li et al., 2020). The possible mechanism behind this association have also been addressed in the aforementioned HBM4EU publication (Fragki et al., 2021).

One of the outcomes that is currently considered more certain with respect to causality, is the effect of long-chain PFASs on the immune system, more specifically on antibody response following vaccination (Schrenk et al., 2020).

On the other hand, one weakness of reduced antibody response as the basis for setting a health-based guidance value (Schrenk et al., 2020) is that a link to more clinically adverse outcomes such as increased propensity of infections is not as well established in humans. Many published studies rely on retrospective parental reporting on their child's previous infections that can be considered at best as uncertain (Abraham et al., 2020; Granum et al., 2013). The two other studies relying on more accurate continuous prenatal reports (Fei et al., 2010) or registry-based outcomes for hospital admissions for infections in early childhood (Dalsager et al., 2016) have reported positive or no association, respectively. Lack of data using objective measures on propensity of infection was partly addressed within HBM4EU. One of those studies examined associations between pregnancy exposure to PFASs in relation to hospital admissions due to infections in children up to 4 years of age (Dalsager et al., 2021). That study, including ~1500 mother child pairs, found a relatively clear positive association between pregnancy concentrations of PFASs with higher propensity for hospital admission to infections in the offspring. Findings support the existing health-based guidance value for long-chain PFASs suggesting that reduced antibody titers may translate into increased propensity of infection, an outcome which is more clearly anchored in adversity compared to isolated observation on vaccination response.

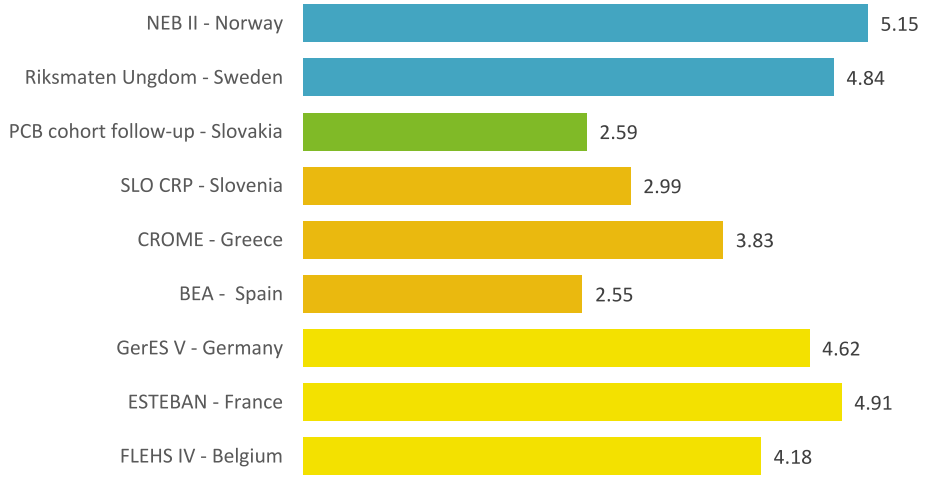

Fig. 1. Geographical differences in exposure to sum of PFOS, PFHxS, PFOA and PFNA in plasma/serum (μg/L) of teenagers in Europe by median value (P50).

The PFASs-induced pathways of immunotoxicity was also summarized and discussed in a review paper performed within HBM4EU, which strengthens the evidence that PFASs affect multiple aspects of the immune system and supports the overall conclusion that not only PFOA and PFOS, but also other members of the PFASs family alter immune functions in humans (Ehrlich et al., 2023).

2.2.2. Health effects in hotspots

HBM4EU has also conducted a review of the health effects of PFASs in hotspot regions. Health risks of PFASs have been studied in large populations which have suffered long term exposure to PFASs via contaminated drinking water. This has been facilitated as associations between groups with substantial exposure contrast offer the possibility of good statistical power to detect effects.

Although no HBM studies in hotspots have been performed within HBM4EU, the review on health effects in hotspot areas (Fletcher, 2022) has shown that hotspot studies enable identification of diseases associated with PFASs at clearly contrasting and relatively high exposures and body burdens. When the HBM4EU project was initiated very few hotspots had been identified in Europe, but this number increased considerably through the HBM4EU project period. There is an urgent need to map potentially PFASs contaminated sites and perform targeted HBM studies in hotspot areas, in future research programs such as the partnership for the assessment of risks from chemicals (PARC). It can be built on the HBM4EU network, which has been described above.

2.3. Biomarkers of effect

One of the aims of HBM4EU was the selection and implementation of effect biomarkers at large scales in future HBM studies in a systematic and standardized way, in order to complement exposure data with mechanistically-based biomarkers of early adverse effects. This study summarizes the prioritized existing biomarkers of effect for PFASs. The selection was made based on relevant mechanistic and/or AOP information, as well as on human data and health outcomes (Fernandez et al., 2021; Mustieles et al., 2018). Fig. 2 provides a graphic scheme for biomarkers of effect relevant for PFASs.

2.3.1. Omic and epigenetic effect biomarkers

Some molecular markers associated with PFASs exposure and related to reproduction, immunotoxicity, obesity and metabolic disorders were identified. For example, alterations in certain gene transcripts levels [NR1H2 (LXRB), ABCG1 & NPC1] involved in cholesterol metabolism and transport (Fletcher et al., 2013), as well as changes in the expression of genes linked to immune function (CYTIL1, IL27) (Pennings et al., 2016). PFASs have been investigated for endocrine disrupting properties in a broad range of *in vitro* assays, finding associations with the expression of certain nuclear receptors (e.g. AR, PXR, PPAR) (Caserta et al., 2013a, 2013b; La Rocca et al., 2012, 2014, 2015). Some studies have also involved epigenetic markers (DNA methylation, histone modification, microRNA expression), and oxidative stress markers as key mediators of some adverse health outcomes derived from early PFASs exposure (Kim et al., 2021).

Although molecular markers may constitute *per se* "biomarkers of effect"; however, their implementation in HBM studies should still be taken with precaution, given the limited epidemiological data on the molecular effects of PFASs. Incorporation of available information from experimental studies could help to construct more robust mechanistic pathways of PFASs toxicity (Fragki et al., 2021).

2.3.2. Immune biomarkers

Among the main effects linked to PFASs exposure are suppression of the antibody response to vaccination (i. e., reduced immune response), as well as lower levels of proteomic markers of inflammation) (Chang et al., 2016; Pennings et al., 2016; Salihovic et al., 2020). Among the biomarkers selected in HBM4EU for immunological effects are circulating antibody levels, basophil count, absolute eosinophil count (AEC), eosinophil cationic protein (ECP) concentrations, lymphocyte

Fig. 2. Biomarkers of effect relevant for PFASs.

subpopulations [e.g. immunoglobulin (IG)E markers and specific IGE antibodies], as well as CC16 protein levels. However, further research is needed to better characterise the pathways of PFASs on the developing immune system.

2.3.3. Cardiometabolic biomarkers

Among cardiometabolic markers, alterations of lipid metabolism markers and increased total serum cholesterol levels have been identified as one of the most critical effects of PFASs on human health (EFSA, 2020). The information on other cardiometabolic markers is much less (Zare Jeddi et al., 2021). The implementation of biomarkers of chronic inflammation (proinflammatory adipokine, leptin, and proinflammatory cytokines, IL-6 and IL-1β biomarkers), in addition to clinical biomarkers of metabolic function (adiponectin, leptin, adiponectin/leptin ratio and HOMA-IR) could help to elucidate the role of PFASs in the development of metabolic diseases, together with anthropometric biomarkers and cardiometabolic risk scores (Li et al. , 2021a; Papadopoulou et al., 2021).

2.3.4. Biochemical effect biomarkers (reproductive and thyroid hormones)

2.3.4.1. Reproductive hormones. Maternal or offspring sex hormone levels have been used as biomarkers of reproductive effect in some epidemiological studies (Bach et al., 2014; Petersen et al., 2020; Itoh et al., 2016; Maisonet et al., 2015). Some cross-sectional studies have also described negative associations between serum PFOA, PFOS and PFUA concentrations and serum SHBG, FSH and testosterone levels (Tsai et al., 2017; Zhou et al., 2016), with more significant associations among males (Joensen et al., 2013), as well as with lower estradiol and progesterone production in nulliparous women (Barrett et al., 2015).

2.3.4.2. Thyroid hormones. PFASs also interfere with the signaling pathways of the thyroid hormones, with negative repercussions on pregnancy outcome and fetal-infant development (Ballesteros et al., 2017; Coperchini et al., 2021; Tsai et al., 2017). Currently, the onset of hypothyroidism in the population exposed to PFASs represents the most frequent thyroid effect of these pollutants. To assess normal thyroid function, triiodothyronine (T3), thyroxine (T4), FT3, FT4 and TSH levels should be taken into account. In this regard, findings suggest that PFASs are associated in an age- and sex-specific manner (Blake et al., 2018; Jain, 2013; Rickard et al., 2022), in addition to probably varying according to coexistence with other environmental pollutants (Shrestha et al., 2015).

2.4. Risk assessments based on combined exposure to multiple PFASs detected in the human serum of teenagers in the HBM4EU aligned study effort

In HBM4EU, different risk assessment approaches have been used to determine whether the population of European teenagers where PFASs levels were measured (Govarts et al., 2023) would be at risk of developing adverse health effects taking into account combined exposure to multiple PFASs. Three approaches were used for comparison: the TWI established by (EFSA, 2020), the hazard index (HI) approach, and the relative potency factor (RPF) approach. The HI and RPF approach were adapted following the EFSA approach to allow using HBM data as primary input. For the HI approach, four epidemiological studies of two critical health end-points indicated by EFSA, immunotoxicity (Grandjean et al., 2012; Kielsen et al., 2016) and birth weight decrease (Meng et al., 2018; Wang et al., 2016) were selected. The geometric mean or median PFASs serum concentrations was included in the HI approach as effect level (i.e. Point of Departure) when there was a statistically significant association between exposure to PFASs and the health outcomes. This resulted in inclusion of PFOA, PFHxS, and PFDA (Grandjean et al., 2012), PFOS, PFNA, PFDA, PFUnDA and PFDoDA (Kielsen et al., 2016), PFNA, PFDA, PFUnDA, and PFDoDA (Wang et al., 2016), and PFOS, PFOA, PFNA, and PFHpS (Meng et al., 2018) in the HI approach, respectively (Bil et al., 2023). For the RPF approach, toxicokinetic models were generated for 10 PFASs to estimate the internal exposure in the male rat at the blood serum level over time. These internal exposures were then used to derive internal RPFs based on liver effects in male rats (Bil et al., 2022). This resulted in inclusion of PFHxA, PFOA, PFNA, PFDoDA, PFBS, PFHxS and PFOS in the RPF approach, respectively (Bil et al., 2023).

Whenever the approach allowed for this, exposure to multiple PFASs was considered at the individual level in order to arrive at precise mixture exposure values. Summed PFASs serum concentrations were calculated per individual, prior to retrieving the 50th and 95th percentile of the serum concentration distribution per study cohort. Based on any of the approaches considered, an increased risk to adverse health effects as specified also by EFSA 2020, was calculated in the highly exposed part of the HBM4EU study population. Even though these approaches differed in the number of PFASs included, their underlying hazard data, and their assumptions used to arrive at the outcomes, all three assessments point in the same direction. Overall, the results are in line with the risk characterization of (Schrenk et al., 2020) and it is clearly showed that adverse health effects may arise due to PFASs mixture exposure in the European population (Bil et al., 2023). Fig. 3 Fig. 3 shows the proportion of European teenagers whose combined exposure to PFOA + PFNA + PFHxS + PFOS, is above the EFSA health-based guidance value of 6.9 μg/L.

3. Science to policy

A key focus of the HBM4EU project was to generate science which can support decision-making and risk management of chemicals.

Within a workshop in the frame of HBM4EU the development and use of HBM based indicators have been discussed in order to learn from and ensure interoperability with other European indicators (Buekers et al., 2018). Further, HBM based indicators have been developed within HBM4EU to track progress, they present HBM data in an easy understandable and comparable way and they can be included e.g. in state-of-the-environment reporting at EU- and national level. Continued investment in monitoring, ideally with a time interval of two to three years between data points, would be needed for this purpose. Thus, to support the use of HBM4EU results for policy making related to PFASs, two workshops were organized (April 2021 and March 2022). Invitees for the workshops were HBM4EU researchers, representatives of national HBM studies and national authorities, representatives from various DGs of the European Commission and EU agencies. The workshops were organized in a confidential setting, to make it possible to discuss results that were not yet publicly available. Workshop participants concluded that HBM research plays an important role in raising awareness and putting the subject on the political agenda. In that regard, HBM data have played an important role in the development of the European Commission's PFASs strategy. Furthermore, results of HBM4EU, e.g. on health effects, mode of action and mixture risk assessment will support the forthcoming REACH restriction proposal to cover a wide range of PFASs uses (HBM4EU, 2021). Given the fact that many new policy actions on PFASs are on the table, HBM4EU results can become an important baseline to follow up effectiveness of policy measures.

A PFASs policy brief was prepared in HBM4EU (2022), which summarized the key findings, to inform future policy actions.

HBM4EU data and results have been reported within the public consultation on the EFSA scientific opinion on the risk to human health related to the presence of perfluoroalkyl substances in food, and have been shared with the member states supporting the call for evidence for a broad PFASs restriction. Further *ad hoc* support the EU-processes, e.g. in the development of HBM-indicators to support the Chemicals strategy for sustainability, the Zero pollution and the 8th Environmental action

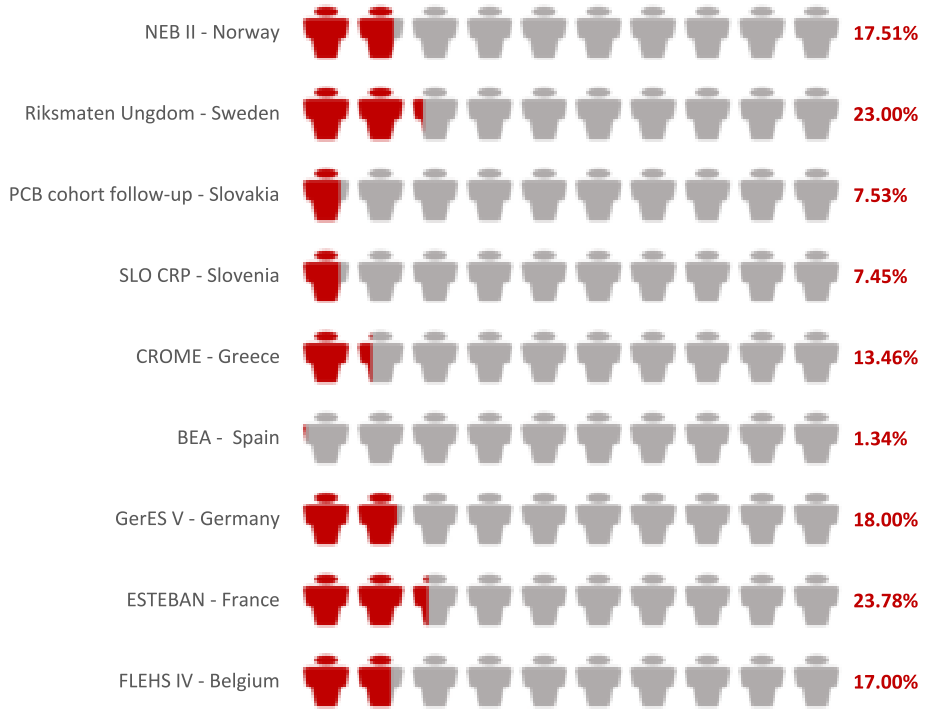

Fig. 3. Share of European teenagers with combined exposure levels to PFOA + PFNA + PFHxS + PFOS exceeding health-based guidance value of EFSA (6.9 μg/L).

programme indicators may be highly useful to track effectiveness of policies on PFASs (Vicente et al., 2023). HBM4EU will support countries facing PFASs contamination issues in hotspots, as a guidance has been developed and discussed with experts in the field.

The results of HBM4EU clearly support the need for far-reaching policy action on PFASs to reduce exposure for the general population, in PFASs hotspots and for occupationally exposed citizens.

4. Conclusions and recommendations

HBM4EU has implemented a broad range of activities and produced highly valuable results on PFASs exposure and health effects. A network of 21 highly qualified laboratories has been established ensuring that human biomonitoring of PFASs can be performed in a highly quality assured way in Europe. High quality human biomonitoring data have been generated for risk assessment. The large-scale aligned and harmonized studies under HBM4EU and research on health effects in HBM studies have revealed that the exposure of European teenagers exceeds health based guideline values, which is a concern for present and future generations. Exposures at specific occupational settings and in the increasingly emerging hotspots have been revealed to be even orders of magnitude higher and of utmost concern.

In order to prevent future pollution by PFASs, it is key to prevent further emissions and accumulation of PFASs in the environment and the use in products. The broad restriction of PFASs for all non-essential uses and activities on other levels are therefore critically needed. Cleaning up of existing hotspots is key to prevent human (and biotas) exposure from legacy uses of PFASs. Effective prediction of potential hot spot polluted areas will require mapping of sites where PFASs have been or currently are being produced, manufactured or used. It is recommended, that studies on PFASs hotspots also in the future will compile data from different cohorts to explore health effects. Further research is needed on the elimination of PFASs from the body, as well as a better understanding of the uptake, distribution (including into fatty tissues) and metabolism of PFASs precursors of PFAAs (Ng et al., 2021).

In following up from the aligned studies time trends will show the effectiveness of measures. Attention should be paid to the sampling scheme and accuracy in terms of harmonization, specifically on issues such as the representativeness of the population, inclusion of highly exposed and vulnerable citizens, including children (Trier et al., 2011). In order to take into account that the number of PFASs is constantly growing and, according to recent estimates, already covers millions of substances, it is necessary to further develop and harmonise methods that allow for quantifying and identifying emerging PFASs substances as well as the measurement of total PFASs content. In future HBM Studies also other PFASs compounds (as. For ex. FTOHs, etc.) should be integrated as also the research on additional toxicological doses-effect relationship values (TDI, Unit Risks, etc.).

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References

Abraham, K., Mielke, H., Fromme, H., Völkel, W., Menzel, J., Peiser, M., Zepp, F., Willich, S.N., Weikert, C., 2020. Internal exposure to perfluoroalkyl substances (PFASs) and biological markers in 101 healthy 1-year-old children: associations between levels of perfluorooctanoic acid (PFOA) and vaccine response. Arch. Toxicol. 94, 2131–2147. https://doi.org/10.1007/s00204-020-02715-4.

Apelberg, B.J., Witter, F.R., Herbstman, J.B., Calafat, A.M., Halden, R.U., Needham, L.L., Goldman, L.R., 2007. Cord serum concentrations of perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) in relation to weight and size at birth. Environ. Health Perspect. 115, 1670–1676. https://doi.org/10.1289/ehp.10334.

Bach, C.C., Bech, B.H., Brix, N., Nohr, E.A., Bonde, J.P.E., Henriksen, T.B., 2014. Perfluoroalkyl and polyfluoroalkyl substances and human fetal growth: a systematic review. Crit. Rev. Toxicol. 45, 53–67. https://doi.org/10.3109/10408444.2014.952400.

Ballesteros, V., Costa, O., Iñiguez, C., Fletcher, T., Ballester, F., Lopez-Espinosa, M.-J., 2017. Exposure to perfluoroalkyl substances and thyroid function in pregnant women and children: a systematic review of epidemiologic studies. Environ. Int. 99, 15–28. https://doi.org/10.1016/j.envint.2016.10.015.

Barrett, E.S., Chen, C., Thurston, S.W., Haug, L.S., Sabaredzovic, A., Fjeldheim, F.N., Frydenberg, H., Lipson, S.F., Ellison, P.T., Thune, I., 2015. Perfluoroalkyl substances and ovarian hormone concentrations in naturally cycling women. Fertil. Sterility 103, 1261–70.e3.

Bil, W, Govarts, E, Zeilmaker, MJ, Woutersen, M, Bessems, J, Ma, Y, et al., 2023. Approaches to mixture risk assessment of PFASs in the European population based on human hazard and biomonitoring data. Int. J. Hygiene Environ. Health 247, 114071. https://doi.org/10.1016/j.ijheh.2022.114071.

Bil, W, Zeilmaker, MJ, Bokkers, BGH, 2022. Internal Relative Potency Factors for the Risk Assessment of Mixtures of Per- and Polyfluoroalkyl Substances (PFAS) in Human Biomonitoring. Environ. Health Perspect. 130 (7), 77005. https://doi.org/10.1289/EHP10009.

Blake, B.E., Pinney, S.M., Hines, E.P., Fenton, S.E., Ferguson, K.K., 2018. Associations between longitudinal serum perfluoroalkyl substance (PFAS) levels and measures of thyroid hormone, kidney function, and body mass index in the Fernald Community Cohort. Environ. Pollut. 242, 894–904. https://doi.org/10.1016/j.envpol.2018.07.042.

Blepp, M., Willand, W., Weber, R., 2017. Use of PFOS in Chromium Plating – Characterisation of Closed-Loop Systems, Use of Alternative Substances. https://www.umweltbundesamt.de/publikationen/use-of-pfos-in-chromium-plating-characterisation-of.

Brouwere, K. de, Colles, A., Knudsen, L.E., Hond, E. den, Uhl, M., 2022. Setting up a Network of Experts for Developing a Guidance Document on How to Deal with Human Biomonitoring in PFAS Hotspots: Activity Report - WP 5 Science to Policy.

Buekers, J., David, M., Koppen, G., Bessems, J., Scheringer, M., Lebret, E., Sarigiannis, D., Kolossa-Gehring, M., Berglund, M., Schoeters, G., Trier, X., 2018. Development of policy relevant human biomonitoring indicators for chemical exposure in the European population. Int. J. Environ. Res. Publ. Health 15. https://doi.org/10.3390/ijerph15102085.

Caserta, D., Bordi, G., Ciardo, F., Marci, R., La Rocca, C., Tait, S., Bergamasco, B., Stecca, L., Mantovani, A., Guerranti, C., Fanello, E.L., Perra, G., Borghini, F., Focardi, S.E., Moscarini, M., 2013a. The influence of endocrine disruptors in a selected population of infertile women. Gynecol. Endocrinol. : the official journal of the International Society of Gynecological Endocrinology 29, 444–447. https://doi.org/10.3109/09513590.2012.758702.

Caserta, D., Ciardo, F., Bordi, G., Guerranti, C., Fanello, E., Perra, G., Borghini, F., La Rocca, C., Tait, S., Bergamasco, B., Stecca, L., Marci, R., Lo Monte, G., Soave, I., Focardi, S., Mantovani, A., Moscarini, M., 2013b. Correlation of endocrine disrupting chemicals serum levels and white blood cells gene expression of nuclear receptors in a population of infertile women. International Journal of Endocrinology, 510703. https://doi.org/10.1155/2013/510703, 2013.

Chang, E.T., Adami, H.-O., Boffetta, P., Wedner, H.J., Mandel, J.S., 2016. A critical review of perfluorooctanoate and perfluorooctanesulfonate exposure and immunological health conditions in humans. Crit. Rev. Toxicol. 46, 279–331. https://doi.org/10.3109/10408444.2015.1122573.

Coperchini, F., Croce, L., Ricci, G., Magri, F., Rotondi, M., Imbriani, M., Chiovato, L., 2021. Thyroid disrupting effects of old and new generation PFAS. Front. Endocrinol. 11, 612320 https://doi.org/10.3389/fendo.2020.612320.

Dalsager, L., Christensen, N., Halekoh, U., Timmermann, C.A.G., Nielsen, F., Kyhl, H.B., Husby, S., Grandjean, P., Jensen, T.K., Andersen, H.R., 2021. Exposure to perfluoroalkyl substances during fetal life and hospitalization for infectious disease in childhood: a study among 1,503 children from the Odense Child Cohort. Environ. Int. 149, 106395 https://doi.org/10.1016/j.envint.2021.106395.

Dalsager, L., Christensen, N., Husby, S., Kyhl, H., Nielsen, F., Høst, A., Grandjean, P., Jensen, T.K., 2016. Association between prenatal exposure to perfluorinated compounds and symptoms of infections at age 1-4years among 359 children in the Odense Child Cohort. Environ. Int. 96, 58–64. https://doi.org/10.1016/j.envint.2016.08.026.

Dimasuay, K.G., Boeuf, P., Powell, T.L., Jansson, T., 2016. Placental responses to changes in the maternal environment determine fetal growth. Front. Physiol. 7, 12. https://doi.org/10.3389/fphys.2016.00012.

EC, 2020. European Commission. COMMISSION DELEGATED REGULATION (EU) 2020/1203 of 9 June 2020 Amending Annex I to Regulation (EU) 2019/1021.

EFSA, 2020. European food safety authority. Risk to human health related to the presence of perfluoroalkyl substances in food: EFSA panel on contaminants in the food chain (EFSA CONTAM panel). Scientific Opinion. EFSA Journal 18 (9).

Ehrlich, V., Bil, W., Vandebriel, R., Granum, B., Luijten, M., Lindeman, B., Grandjean, P., Kaiser, A.-M., Hauzenberger, I., Hartmann, C., Gundacker, C., Uhl, M., 2023. Consideration of pathways for immunotoxicity of per- and polyfluoroalkyl substances (PFAS). Environ. Health 22, 19. https://doi.org/10.1186/s12940-022-00958-5.

Esteban López, M., Göen, T., Mol, H., Nübler, S., Haji-Abbas-Zarrabi, K., Koch, H.M., Kasper-Sonnenberg, M., Dvorakova, D., Hajslova, J., Antignac, J.-P., Vaccher, V., Elbers, I., Thomsen, C., Vorkamp, K., Pedraza-Díaz, S., Kolossa-Gehring, M., Castaño, A., 2021. The European human biomonitoring platform - design and implementation of a laboratory quality assurance/quality control (QA/QC) programme for selected priority chemicals. Int. J. Hyg Environ. Health 234, 113740. https://doi.org/10.1016/j.ijheh.2021.113740.

Fei, C., McLaughlin, J.K., Lipworth, L., Olsen, J., 2008. Prenatal exposure to perfluorooctanoate (PFOA) and perfluorooctanesulfonate (PFOS) and maternally reported developmental milestones in infancy. Environ. Health Perspect. 116, 1391–1395. https://doi.org/10.1289/ehp.11277.

Fei, C., McLaughlin, J.K., Lipworth, L., Olsen, J., 2010. Prenatal exposure to PFOA and PFOS and risk of hospitalization for infectious diseases in early childhood. Environ. Res. 110, 773–777. https://doi.org/10.1016/j.envres.2010.08.004.

Fei, C., McLaughlin, J.K., Tarone, R.E., Olsen, J., 2007. Perfluorinated chemicals and fetal growth: a study within the Danish National Birth Cohort. Environ. Health Perspect. 115, 1677–1682. https://doi.org/10.1289/ehp.10506.

Fernandez, M.F., Mustieles, V., Olea, N., Rodríguez-Carrillo, A., Olivas, A., Suárez, B., Reina-Pérez, I., Cynthia, S., Legoff, L., Smagulova, F., David, A., Bláha, L., Blahova, L., 2021. Guidelines for the Validation of Biomarkers of Effect: Implementing Quality Assurance, Trueness, Precision, and Accuracy as Well as the Availability of Quality Control Measures by Qualified Laboratories: Intra-laboratory Quality Control Measures for Effect Biomarkers: Fine-Tuning, Precision (Intra- and Interassay Variability) and Accuracy. HBM4EU Deliverable Report D14.7: WP14 - Effect Biomarkers. HBM4EU - HORIZON2020 Programme, Ref. Ares(2021)1084133 - 08/02/2021. https://www.hbm4eu.eu/work-packages/deliverable-14-7-guidelines-for-the-validation-of-biomarkers-of-effect-implementing-quality-assurance-trueness-precision-and-accuracy-as-well-as-the-availability-of-quality-control-measures-by-qua/. (Accessed 21 February 2023).

Fletcher, T., Unpublished. Review on Health Effects in PFAS Hotspots (accessed 3 March 2023).

Fletcher, T., Galloway, T.S., Melzer, D., Holcroft, P., Cipelli, R., Pilling, L.C., Mondal, D., Luster, M., Harries, L.W., 2013. Associations between PFOA, PFOS and changes in the expression of genes involved in cholesterol metabolism in humans. Environ. Int. 57–58, 2–10. https://doi.org/10.1016/j.envint.2013.03.008.

Forsthuber, M., Widhalm, R., Granitzer, S., Kaiser, A.M., Moshammer, H., Hengstschläger, M., Dolznig, H., Gundacker, C., 2022. Perfluorooctane sulfonic acid (PFOS) inhibits vessel formation in a human 3D co-culture angiogenesis model (NCFs/HUVECs). Environ. Pollut. 293, 118543 https://doi.org/10.1016/j.envpol.2021.118543.

Fragki, S., Dirven, H., Fletcher, T., Grasl-Kraupp, B., Bjerve Gützkow, K., Hoogenboom, R., Kersten, S., Lindeman, B., Louisse, J., Peijnenburg, A., Piersma, A. H., Princen, H.M.G., Uhl, M., Westerhout, J., Zeilmaker, M.J., Luijten, M., 2021. Systemic PFOS and PFOA exposure and disturbed lipid homeostasis in humans: what do we know and what not? Crit. Rev. Toxicol. 51, 141–164. https://doi.org/10.1080/10408444.2021.1888073.

Gao, X., Ni, W., Zhu, S., Wu, Y., Cui, Y., Ma, J., Liu, Y., Qiao, J., Ye, Y., Yang, P., Liu, C., Zeng, F., 2021. Per- and polyfluoroalkyl substances exposure during pregnancy and adverse pregnancy and birth outcomes: a systematic review and meta-analysis. Environ. Res. 201, 111632 https://doi.org/10.1016/j.envres.2021.111632.

Gilles, L., Govarts, E., Rambaud, L., Vogel, N., Castaño, A., Esteban López, M., Rodriguez Martin, L., Koppen, G., Remy, S., Vrijheid, M., Montazeri, P., Birks, L., Sepai, O., Stewart, L., Fiddicke, U., Loots, I., Knudsen, L.E., Kolossa-Gehring, M., Schoeters, G., 2021. HBM4EU combines and harmonises human biomonitoring data across the EU, building on existing capacity - the HBM4EU survey. Int. J. Hyg Environ. Health 237, 113809. https://doi.org/10.1016/j.ijheh.2021.113809.

Gilles, L., Govarts, E., Rodriguez Martin, L., Andersson, A.-M., Appenzeller, B.M.R., Barbone, F., Castaño, A., Coertjens, D., Hond, E. den, Dzhedzheia, V., Eržen, I., López, M.E., Fábelová, L., Fillol, C., Franken, C., Frederiksen, H., Gabriel, C., Haug, L.S., Horvat, M., Halldórsson, T.I., Janasik, B., Holcer, N.J., Kakucs, R., Karakitsios, S., Katsonouri, A., Klánová, J., Kold-Jensen, T., Kolossa-Gehring, M., Konstantinou, C., Koponen, J., Lignell, S., Lindroos, A.K., Makris, K.C., Mazej, D., Morrens, B., Murínová, Ľ.P., Namorado, S., Pedraza-Diaz, S., Peisker, J., Probst-Hensch, N., Rambaud, L., Rosolen, V., Rucic, E., Rüther, M., Sarigiannis, D., Tratnik, J.S., Standaert, A., Stewart, L., Szigeti, T., Thomsen, C., Tolonen, H., Eiríksdóttir, Á., an van Nieuwenhuyse, Verheyen, V.J., Vlaanderen, J., Vogel, N., Wasowicz, W., Weber, T., Zock, J.-P., Sepai, O., Schoeters, G., 2022. Harmonization of human biomonitoring studies in Europe: characteristics of the hbm4eu-aligned studies participants. Int. J. Environ. Res. Publ. Health 19, 6787. https://doi.org/10.3390/ijerph19116787.

Glüge, J., Scheringer, M., Cousins, I.T., DeWitt, J.C., Goldenman, G., Herzke, D., Lohmann, R., Ng, C.A., Trier, X., Wang, Z., 2020. An overview of the uses of per- and polyfluoroalkyl substances (PFAS). Environ. Sci. J. Integr. Environ. Res.: Process. Impacts 22, 2345–2373. https://doi.org/10.1039/d0em00291g.

Govarts, E., Gilles, L., Rodriguez Martin, L., Santonen, T., Apel, P., Alvito, P., Anastasi, E., Andersen, H.R., Andersson, A.-M., Andryskova, L., Antignac, J.-P., Appenzeller, B., Barbone, F., Barnett-Itzhaki, Z., Barouki, R., Berman, T., Bil, W., Borges, T., Buekers, J., Cañas-Portilla, A., Covaci, A., Csako, Z., Hond, E. den, Dvorakova, D., Fabelova, L., Fletcher, T., Frederiksen, H., Gabriel, C., Ganzleben, C., Göen, T., Halldorsson, T.I., Haug, L.S., Horvat, M., Huuskonen, P., Imboden, M., Jagodic Hudobivnik, M., Janasik, B., Janev Holcer, N., Karakitsios, S., Katsonouri, A., Klanova, J., Kokaraki, V., Kold Jensen, T., Koponen, J., Laeremans, M., Laguzzi, F., Lange, R., Lemke, N., Lignell, S., Lindroos, A.K., Lobo Vicente, J., Luijten, M., Makris, K.C., Mazej, D., Melymuk, L., Meslin, M., Mol, H., Montazeri, P., Murawski, A., Namorado, S., Niemann, L., Nübler, S., Nunes, B., Olafsdottir, K., Palkovicova Murinova, L., Papaioannou, N., Pedraza-Diaz, S., Piler, P., Plichta, V., Poteser, M., Probst-Hensch, N., Rambaud, L., Rauscher-Gabernig, E., Rausova, K., Remy, S., Riou, M., Rosolen, V., Rousselle, C., Rüther, M., Sarigiannis, D., Silva, M.J., Šlejkovec, Z., Snoj Tratnik, J., Stajnko, A., Szigeti, T., Tarazona, J.V., Thomsen, C.,

Tkalec, Ž., Tolonen, H., Trnovec, T., Uhl, M., an van Nieuwenhuyse, Vasco, E., Verheyen, V.J., Viegas, S., Vinggaard, A.M., Vogel, N., Vorkamp, K., Wasowicz, W., Weber, T., Wimmerova, S., Woutersen, M., Zimmermann, P., Zvonar, M., Koch, H., Kolossa-Gehring, M., Esteban López, M., Castaño, A., Stewart, L., Sepai, O., Schoeters, G., 2023. Harmonized human biomonitoring in European children, teenagers and adults: EU-wide exposure data of 11 chemical substance groups from the HBM4EU Aligned Studies (2014-2021). Int. J. Hyg Environ. Health 249, 114119. https://doi.org/10.1016/j.ijheh.2023.114119.

Grandjean, P., Andersen, E.W., Budtz-Jørgensen, E., Nielsen, F., Mølbak, K., Weihe, P., Heilmann, C., 2012. Serum vaccine antibody concentrations in children exposed to perfluorinated compounds. JAMA 307, 391–397. https://doi.org/10.1001/jama.2011.2034.

Granum, B., Haug, L.S., Namork, E., Stølevik, S.B., Thomsen, C., Aaberge, I.S., van Loveren, H., Løvik, M., Nygaard, U.C., 2013. Pre-natal exposure to perfluoroalkyl substances may be associated with altered vaccine antibody levels and immune-related health outcomes in early childhood. J. Immunot. 10, 373–379. https://doi.org/10.3109/1547691X.2012.755580.

Gundacker, C., Audouze, K., Widhalm, R., Granitzer, S., Forsthuber, M., Jornod, F., Wielsøe, M., Long, M., Halldórsson, T.I., Uhl, M., Bonefeld-Jørgensen, E.C., 2022. Reduced birth weight and exposure to per- and polyfluoroalkyl substances: a review of possible underlying mechanisms using the AOP-HelpFinder. Toxics 10, 684. https://doi.org/10.3390/toxics10110684.

HBM4EU, 2021. 2nd Phased Action Plan for Policies, Measures and Suggestions for Further Research at Domestic Level, Case Studies: Deliverable Report D5.7 - WP5: Translation of Results into Policy. https://www.hbm4eu.eu/work-packages/deliverable-5-7-2nd-phased-action-plan-for-policies-measures-and-suggestions-for-further-research-at-domestic-level-case-studies/. (Accessed 13 June 2022).

HBM4EU, 2022. POLICY BRIEF. https://www.hbm4eu.eu/wp-content/uploads/2022/05/Policy-Brief-PFAS.pdf. (Accessed 1 July 2022).

Ingelido, A.M., Abballe, A., Gemma, S., Dellatte, E., Iacovella, N., Angelis, G. de, Zampaglioni, F., Marra, V., Miniero, R., Valentini, S., Russo, F., Vazzoler, M., Testai, E., Felip, E. de, 2018. Biomonitoring of perfluorinated compounds in adults exposed to contaminated drinking water in the Veneto Region. Italy. Environment International 110, 149–159. https://doi.org/10.1016/j.envint.2017.10.026.

Jain, R.B., 2013. Association between thyroid profile and perfluoroalkyl acids: data from NHNAES 2007-2008. Environ. Res. 126, 51–59. https://doi.org/10.1016/j.envres.2013.08.006.

Joensen, U.N., Veyrand, B., Antignac, J.-P., Blomberg Jensen, M., Petersen, J.H., Marchand, P., Skakkebæk, N.E., Andersson, A.-M., Le Bizec, B., Jørgensen, N., 2013. PFOS (perfluorooctanesulfonate) in serum is negatively associated with testosterone levels, but not with semen quality, in healthy men. Human Reprod. 28, 599–608. https://doi.org/10.1093/humrep/des425.

Kielsen, K., Shamim, Z., Ryder, L.P., Nielsen, F., Grandjean, P., Budtz-Jørgensen, E., Heilmann, C., 2016. Antibody response to booster vaccination with tetanus and diphtheria in adults exposed to perfluorinated alkylates. J. Immunot. 13, 270–273. https://doi.org/10.3109/1547691X.2015.1067259.

Kim, S., Thapar, I., Brooks, B.W., 2021. Epigenetic changes by per- and polyfluoroalkyl substances (PFAS). Environ. Pollut. 279, 116929 https://doi.org/10.1016/j.envpol.2021.116929.

Kobayashi, S., Azumi, K., Goudarzi, H., Araki, A., Miyashita, C., Kobayashi, S., Itoh, S., Sasaki, S., Ishizuka, M., Nakazawa, H., Ikeno, T., Kishi, R., 2017. Effects of prenatal perfluoroalkyl acid exposure on cord blood IGF2/H19 methylation and ponderal index: the Hokkaido Study. J. Expo. Sci. Environ. Epidemiol. 27, 251–259. https://doi.org/10.1038/jes.2016.50.

La Rocca, C., Alessi, E., Bergamasco, B., Caserta, D., Ciardo, F., Fanello, E., Focardi, S., Guerranti, C., Stecca, L., Moscarini, M., Perra, G., Tait, S., Zaghi, C., Mantovani, A., 2012. Exposure and effective dose biomarkers for perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) in infertile subjects: preliminary results of the PREVIENI project. Int. J. Hyg Environ. Health 215, 206–211. https://doi.org/10.1016/j.ijheh.2011.10.016.

La Rocca, C., Tait, S., Guerranti, C., Busani, L., Ciardo, F., Bergamasco, B., Perra, G., Mancini, F.R., Marci, R., Bordi, G., Caserta, D., Focardi, S., Moscarini, M., Mantovani, A., 2015. Exposure to endocrine disruptors and nuclear receptors gene expression in infertile and fertile men from Italian areas with different environmental features. Int. J. Environ. Res. Publ. Health 12, 12426–12445. https://doi.org/10.3390/ijerph121012426.

La Rocca, C., Tait, S., Guerranti, C., Busani, L., Ciardo, F., Bergamasco, B., Stecca, L., Perra, G., Mancini, F.R., Marci, R., Bordi, G., Caserta, D., Focardi, S., Moscarini, M., Mantovani, A., 2014. Exposure to endocrine disrupters and nuclear receptor gene expression in infertile and fertile women from different Italian areas. Int. J. Environ. Res. Publ. Health 11, 10146–10164. https://doi.org/10.3390/ijerph111010146.

Langenbach, B., Wilson, M., 2021. Per- and polyfluoroalkyl substances (PFAS): significance and considerations within the regulatory framework of the USA. Int. J. Environ. Res. Publ. Health 18. https://doi.org/10.3390/ijerph182111142.

Li, Y., Barregard, L., Xu, Y., Scott, K., Pineda, D., Lindh, C.H., Jakobsson, K., Fletcher, T., 2020. Associations between perfluoroalkyl substances and serum lipids in a Swedish adult population with contaminated drinking water. Environ. Health 19, 33. https://doi.org/10.1186/s12940-020-00588-9.

Lopez-Espinosa, M.-J., Mondal, D., Armstrong, B.G., Eskenazi, B., Fletcher, T., 2016. Perfluoroalkyl substances, sex hormones, and insulin-like growth factor-1 at 6-9 Years of age: a cross-sectional analysis within the C8 health project. Environ. Health Perspect. 124, 1269–1275. https://doi.org/10.1289/ehp.1509869.

Maisonet, M., Calafat, A.M., Marcus, M., Jaakkola, J.J.K., Lashen, H., 2015. Prenatal exposure to perfluoroalkyl acids and serum testosterone concentrations at 15 years of age in female ALSPAC study participants. Environ. Health Perspect. 123, 1325–1330. https://doi.org/10.1289/ehp.1408847.

Meng, Q., Inoue, K., Ritz, B., Olsen, J., Liew, Z., 2018. Prenatal exposure to perfluoroalkyl substances and birth outcomes; an updated analysis from the Danish national birth cohort. Int. J. Environ. Res. Publ. Health 15. https://doi.org/10.3390/ijerph15091832.

Mustieles, V., Rodríguez-Carrillo, A., Fernandez, M.F., Olea, N., Bláha, L., Bonefeld-Jørgensen, E.C., Nawrot, T., Schoeters, G., Lambrechts, N., Remy, S., David, A., Cynthia, S., Fini, J.-B., Couderq, S., Hofer, T., Steffensen, I.-L., Luijten, M., Hernández, A., Lacasaña, M., González-Alzaga, B., Vinggaard, A.M., Johansson, H., Saber, A.T., Gundacker, C., Neophytou, C., Novakova, Z., Fragki, S., Piersma, A., Lampen, A., 2018. List of Effect Biomarkers for the First Set of Prioritized Substances. HBM4EU - Deliverable Report 14.2, WP14 Biomarkers of Effect. HBM4EU - HORIZON2020 Programme. Ref. Ares(2018)4706393 - 13/09/2018. https://www.hbm4eu.eu/work-packages/deliverable-14-2-list-of-effect-biomarkers-for-the-first-set-of-prioritized-substances-september-2018/.

Ng, C., Cousins, I.T., DeWitt, J.C., Glüge, J., Goldenman, G., Herzke, D., Lohmann, R., Miller, M., Patton, S., Scheringer, M., Trier, X., Wang, Z., 2021. Addressing urgent questions for PFAS in the 21st century. Environ. Sci. Technol. 55, 12755–12765. https://doi.org/10.1021/acs.est.1c03386.

Nielsen, C., Andersson Hall, U., Lindh, C., Ekström, U., Xu, Y., Li, Y., Holmäng, A., Jakobsson, K., 2020. Pregnancy-induced changes in serum concentrations of perfluoroalkyl substances and the influence of kidney function. Environ. Health 19, 80. https://doi.org/10.1186/s12940-020-00626-6.

Olsen, G.W., Burris, J.M., Ehresman, D.J., Froehlich, J.W., Seacat, A.M., Butenhoff, J.L., Zobel, L.R., 2007. Half-life of serum elimination of perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. Environ. Health Perspect. 115, 1298–1305. https://doi.org/10.1289/ehp.10009.

Papadopoulou, E., Stratakis, N., Basagaña, X., Brantsæter, A.L., Casas, M., Fossati, S., Gražulevičienė, R., Småstuen Haug, L., Heude, B., Maitre, L., McEachan, R.R.C., Robinson, O., Roumeliotaki, T., Sabidó, E., Borràs, E., Urquiza, J., Vafeiadi, M., Zhao, Y., Slama, R., Wright, J., Conti, D.V., Vrijheid, M., Chatzi, L., 2021. Prenatal and postnatal exposure to PFAS and cardiometabolic factors and inflammation status in children from six European cohorts. Environ. Int. 157, 106853. https://doi.org/10.1016/j.envint.2021.106853.

Pennings, J.L.A., Jennen, D.G.J., Nygaard, U.C., Namork, E., Haug, L.S., van Loveren, H., Granum, B., 2016. Cord blood gene expression supports that prenatal exposure to perfluoroalkyl substances causes depressed immune functionality in early childhood. J. Immunot. 13, 173–180. https://doi.org/10.3109/1547691X.2015.1029147.

Petersen, K.U., Larsen, J.R., Deen, L., Flachs, E.M., Hærvig, K.K., Hull, S.D., Bonde, J.P.E., Tøttenborg, S.S., 2020. Per- and polyfluoroalkyl substances and male reproductive health: a systematic review of the epidemiological evidence. J. Toxicol. Environ. Health. Part B, Crit. Rev. 23, 276–291. https://doi.org/10.1080/10937404.2020.1798315.

Richterová, D., Govarts, E., Fábelová, L., Rausová, K., Rodriguez Martin, L., Gilles, L., Remy, S., Colles, A., Rambaud, L., Riou, M., Gabriel, C., Sarigiannis, D., Pedraza-Diaz, S., Ramos, J.J., Kosjek, T., Snoj Tratnik, J., Lignell, S., Gyllenhammar, I., Thomsen, C., Haug, L.S., Kolossa-Gehring, M., Vogel, N., Franken, C., Vanlarebeke, N., Bruckers, L., Stewart, L., Sepai, O., Schoeters, G., Uhl, M., Castaño, A., Esteban López, M., Göen, T., Palkovičová Murínová, Ľ., 2022. PFAS levels and determinants of variability in exposure in European teenagers - results from the HBM4EU aligned studies (2014-2021). Int. J. Hyg Environ. Health 247, 114057. https://doi.org/10.1016/j.ijheh.2022.114057.

Rickard, B.P., Rizvi, I., Fenton, S.E., 2022. Per- and poly-fluoroalkyl substances (PFAS) and female reproductive outcomes: PFAS elimination, endocrine-mediated effects, and disease. Toxicology 465, 153031. https://doi.org/10.1016/j.tox.2021.153031.

Salihovic, S., Lind, L., Larsson, A., Lind, P.M., 2020. Plasma perfluoroalkyls are associated with decreased levels of proteomic inflammatory markers in a cross-sectional study of an elderly population. Environ. Int. 145, 106099 https://doi.org/10.1016/j.envint.2020.106099.

Santonen, T., Alimonti, A., Bocca, B., Duca, R.C., Galea, K.S., Godderis, L., Göen, T., Gomes, B., Hanser, O., Iavicoli, I., Janasik, B., Jones, K., Kiilunen, M., Koch, H.M., Leese, E., Leso, V., Louro, H., Ndaw, S., Porras, S.P., Robert, A., Ruggieri, F., Scheepers, P.T.J., Silva, M.J., Viegas, S., Wasowicz, W., Castano, A., Sepai, O., 2019. Setting up a collaborative European human biological monitoring study on occupational exposure to hexavalent chromium. Environ. Res. 177, 108583 https://doi.org/10.1016/j.envres.2019.108583.

Santonen, T., Louro, H., Bocca, B., Bousoumah, R., Duca, R.C., Fucic, A., Galea, K.S., Godderis, L., Göen, T., Iavicoli, I., Janasik, B., Jones, K., Leese, E., Leso, V., Ndaw, S., Poels, K., Porras, S.P., Ruggieri, F., Silva, M.J., an van Nieuwenhuyse, Verdonck, J., Wasowicz, W., Tavares, A., Sepai, O., Scheepers, P.T.J., Viegas, S., 2023. The HBM4EU chromates study - outcomes and impacts on EU policies and occupational health practices. Int. J. Hyg Environ. Health 248, 114099. https://doi.org/10.1016/j.ijheh.2022.114099.

Santonen, T., Porras, S.P., Bocca, B., Bousoumah, R., Duca, R.C., Galea, K.S., Godderis, L., Göen, T., Hardy, E., Iavicoli, I., Janasik, B., Jones, K., Leese, E., Leso, V., Louro, H., Majery, N., Ndaw, S., Pinhal, H., Ruggieri, F., Silva, M.J., an van Nieuwenhuyse, Verdonck, J., Viegas, S., Wasowicz, W., Sepai, O., Scheepers, P.T.J., 2022. HBM4EU chromates study - overall results and recommendations for the biomonitoring of occupational exposure to hexavalent chromium. Environ. Res. 204, 111984 https://doi.org/10.1016/j.envres.2021.111984.

Savitz, D.A., 2007. Guest editorial: biomarkers of perfluorinated chemicals and birth weight. Environ. Health Perspect. 115, A528–A529. https://doi.org/10.1289/ehp.10923.

Schrenk, D., Bignami, M., Bodin, L., Chipman, J.K., Del Mazo, J., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L.R., Leblanc, J.-C., Nebbia, C.S., Nielsen, E., Ntzani, E., Petersen, A., Sand, S., Vleminckx, C., Wallace, H., Barregård, L.,

Ceccatelli, S., Cravedi, J.-P., Halldorsson, T.I., Haug, L.S., Johansson, N., Knutsen, H. K., Rose, M., Roudot, A.-C., van Loveren, H., Vollmer, G., Mackay, K., Riolo, F., Schwerdtle, T., 2020. Risk to human health related to the presence of perfluoroalkyl substances in food. EFSA J. 18, e06223 https://doi.org/10.2903/j.efsa.2020.6223.

Shrestha, S., Bloom, M.S., Yucel, R., Seegal, R.F., Wu, Q., Kannan, K., Rej, R., Fitzgerald, E.F., 2015. Perfluoroalkyl substances and thyroid function in older adults. Environ. Int. 75, 206–214. https://doi.org/10.1016/j.envint.2014.11.018.

Sinisalu, L., Yeung, L.W.Y., Wang, J., Pan, Y., Dai, J., Hyötyläinen, T., 2021. Prenatal exposure to poly-/per-fluoroalkyl substances is associated with alteration of lipid profiles in cord-blood. Metabolomics : Official journal of the Metabolomic Society 17, 103. https://doi.org/10.1007/s11306-021-01853-9.

Trier, X., Nielsen, N.J., Christensen, J.H., 2011. Structural isomers of polyfluorinated di- and tri-alkylated phosphate ester surfactants present in industrial blends and in microwave popcorn bags. Environ. Sci. Pollut. Res. Int. 18, 1422–1432. https://doi.org/10.1007/s11356-011-0488-2.

Tsai, M.-S., Lin, C.-C., Chen, M.-H., Hsieh, W.-S., Chen, P.-C., 2017. Perfluoroalkyl substances and thyroid hormones in cord blood. Environ. Pollut. 222, 543–548. https://doi.org/10.1016/j.envpol.2016.11.027.

Verner, M.-A., Loccisano, A.E., Morken, N.-H., Yoon, M., Wu, H., McDougall, R., Maisonet, M., Marcus, M., Kishi, R., Miyashita, C., Chen, M.-H., Hsieh, W.-S., Andersen, M.E., Clewell, H.J., Longnecker, M.P., 2015. Associations of perfluoroalkyl substances (PFAS) with lower birth weight: an evaluation of potential confounding by glomerular filtration rate using a physiologically based pharmacokinetic model (PBPK). Environ. Health Perspect. 123, 1317–1324. https://doi.org/10.1289/ehp.1408837.

Vicente, J.L., Gasol, R., Marnane, I., Ganzleben, C., Gilles, L., Buekers, J., Besselink, H., Colles, A., Gerofke, A., David, M., Barouki, R., Uhl, M., Sepai, O., Loots, I., Crabbé, A., Coertjens, D., Kolossa-Gehring, M., Schoeters, G., 2023. HBM4EU results support the chemicals' strategy for sustainability and the zero-pollution action plan. Int. J. Hyg Environ. Health 248, 114111. https://doi.org/10.1016/j.ijheh.2023.114111.

Vorkamp, K., Castaño, A., Antignac, J.-P., Boada, L.D., Cequier, E., Covaci, A., Esteban López, M., Haug, L.S., Kasper-Sonnenberg, M., Koch, H.M., Pérez Luzardo, O., Osīte, A., Rambaud, L., Pinorini, M.-T., Sabbioni, G., Thomsen, C., 2021. Biomarkers, matrices and analytical methods targeting human exposure to chemicals selected for a European human biomonitoring initiative. Environ. Int. 146, 106082 https://doi.org/10.1016/j.envint.2020.106082.

Wan, H.T., Zhao, Y.G., Wong, M.H., Lee, K.F., Yeung, W.S.B., Giesy, J.P., Wong, C.K.C., 2011. Testicular signaling is the potential target of perfluorooctanesulfonate-mediated subfertility in male mice. Biol. Reprod. 84, 1016–1023. https://doi.org/10.1095/biolreprod.110.089219.

Wang, Y., Adgent, M., Su, P.-H., Chen, H.-Y., Chen, P.-C., Hsiung, C.A., Wang, S.-L., 2016. Prenatal exposure to perfluorocarboxylic acids (PFCAs) and fetal and postnatal growth in the taiwan maternal and infant cohort study. Environ. Health Perspect. 124, 1794–1800. https://doi.org/10.1289/ehp.1509998.

Wit, M., Trujillo-Viera, J., Strohmeyer, A., Klingenspor, M., Hankir, M., Sumara, G., 2022. When fat meets the gut-focus on intestinal lipid handling in metabolic health and disease. EMBO Mol. Med. 14, e14742 https://doi.org/10.15252/emmm.202114742.

Xu, Y., Fletcher, T., Pineda, D., Lindh, C.H., Nilsson, C., Glynn, A., Vogs, C., Norström, K., Lilja, K., Jakobsson, K., Li, Y., 2020. Serum half-lives for short- and long-chain perfluoroalkyl acids after ceasing exposure from drinking water contaminated by firefighting foam. Environ. Health Perspect. 128, 77004 https://doi.org/10.1289/EHP6785.

Xu, Y., Nielsen, C., Li, Y., Hammarstrand, S., Andersson, E.M., Li, H., Olsson, D.S., Engström, K., Pineda, D., Lindh, C.H., Fletcher, T., Jakobsson, K., 2021. Serum perfluoroalkyl substances in residents following long-term drinking water contamination from firefighting foam in Ronneby, Sweden. Environ. Int. 147, 106333 https://doi.org/10.1016/j.envint.2020.106333.

Yang, Q., Wang, W., Liu, C., Wang, Y., Sun, K., 2016. Effect of PFOS on glucocorticoid-induced changes in human decidual stromal cells in the first trimester of pregnancy. Reprod. Toxicol. 63, 142–150. https://doi.org/10.1016/j.reprotox.2016.06.003.

Zare Jeddi, M., Soltanmohammadi, R., Barbieri, G., Fabricio, A.S.C., Pitter, G., Dalla Zuanna, T., Canova, C., 2021. To which extent are per-and poly-fluorinated substances associated to metabolic syndrome? Rev. Environ. Health. https://doi.org/10.1515/reveh-2020-0144.

Zhang, N., Wang, W.S., Li, W.J., Liu, C., Wang, Y., Sun, K., 2015. Reduction of progesterone, estradiol and hCG secretion by perfluorooctane sulfonate via induction of apoptosis in human placental syncytiotrophoblasts. Placenta 36, 575–580. https://doi.org/10.1016/j.placenta.2015.02.008.

Glossary

AEC: absolute eosinophil count
ADONA: ammonium 4,8-dioxa-3H-perfluorononanoate
AFFFs: aqueous film-forming foams
AOP: adverse outcome pathway
AR: androgen receptor
C8: perfluorooctanoic acid
CIC: combustion ion chromatography
CC16: club cell protein 16
EC: European Commission
ECP: eosinophil cationic protein
EDC: endocrine disrupting chemical
EFSA: European Food Safety Authority
EOF: extractable organofluorine
FT3: free triiodothyronine
FT4: free thyroxine
HBM: human biomonitoring
HBM4EU: European Human Biomonitoring Initiative
HFPO-DA (GenX): hexafluoropropylene oxide-dimer acid
HI: hazard index
HOMA-IR: homeostatic model assessment for insulin resistence
IG: immunoglobulin
IGF: insulin-like growth factor
IL: interleukin
P50: 50th percentile
P95: 95th percentile
PARC: partnership for the assessment of risks from chemicals
PFAA(s): perfluoroalkyl acid(s)
PFASs: per- and polyfluoroalkyl substances
PFBS: perfluorobutanoic acid
PFCA(s): perfluoroalkyl carboxylic acid(s)
PFDA: perfluorodecanoic acid
PFDoDA: perfluorododecanoic acid
PFHpA: perfluoroheptanoic acid
PFHxA: perfluorohexanoic acid
PFHxS: perfluorohexane sulfonate
PFNA: perfluoro-n-nonanoic acid or perfluoro-n-nonane carboxylate
PFOA: perfluoro-n-octanoic acid or perfluorooctane carboxylate
PFOS: perfluoro-n-octane sulfonate or perfluorooctane sulfonic acid
PFOSA: perfluorooctane sulfonamide
PFPeA: perfluoropentane acid
PFUnDA: perfluoroundecanoic acid
PPARs: peroxisome proliferator-activated receptors
PTFE: polytetrafluoroethylene
PXR: pregnane X receptor
QA/QC: quality assurance/quality control
RPF: relative potency factor
T3: triiodothyronine
T4: thyroxine
TOPA: total oxidizable precursor assay
TSH: thyroid stimulating hormone
TWI: tolerable weekly intake
VEGVR: vascular endothelial growth factor receptor

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Projecting the excess mortality due to heatwave and its characteristics under climate change, population and adaptation scenarios

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ABSTRACT

Background: Heatwaves have significant adverse effects on human health. The frequency, duration, and intensity of heatwaves are projected to increase dramatically, in the context of global warming. However, there are few comprehensive assessments of the health impact of heatwaves considering different definitions, and their characteristics under climate change scenarios.

Objective: We aimed to compare future excess mortality related to heatwaves among different definitions under climate change, population, and adaptation scenarios in China and further explore the mortality burden associated with heatwave characteristics.

Methods: Daily data during 2010–2019 were collected in Guangzhou, China. We adopted nine common heatwave definitions and applied quasi-Poisson models to estimate the effects of heatwaves and their characteristics' impact on mortality. We then projected the excess mortality associated with heatwaves and their characteristics concerning climate change, population, and adaptation scenarios.

Results: The relative risks of the nine common heatwave definitions ranged from 1.05 (95% CI: 1.01, 1.10) to 1.24 (95% CI: 1.13, 1.35). Heatwave-related excess mortality will consistently increase in the future decades considering multiple heatwave definitions, with more rapidly increasing rates under the Shared Socioeconomic Path5-8.5 and non-adaptability scenarios. Regarding heatwave characteristics, the intensity is the main factor involved in the threat of heatwaves. The increasing trend of characteristic-related mortality burden is similar to that of heatwaves, and the mortality burden caused by the duration of the heatwaves was the largest among all characteristics.

Conclusions: This study provides a comprehensive picture of the impact of heatwaves and their characteristics on public health under various climate change scenarios, population changes, and adaptive assumptions. The results may provide important public health implications for policymakers in planning climate change adaptation and mitigation policies, and implementing specific plans.

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1. Introduction

Global warming is an environmental issue of great concern as it increases the frequency, intensity, and duration of extreme weather and climate events (IPCC, 2014; Perkins-Kirkpatrick and Gibson, 2018; Trancoso et al., 2020). Heatwave, a type of extreme weather, can induce heat exhaustion, heat oedema, heat cramps, heat syncope, and heatstroke, thereby causing acute cerebrovascular accidents, chronic pulmonary conditions, cardiac conditions, kidney disorders, and psychiatric illness (Chen et al., 2018; McGregor, 2015). The devastating effects of major heatwaves over the past decades have been well documented (Guo et al., 2018; Ma et al., 2015; Patz et al., 2005; Robine et al., 2008; Yang et al., 2019). For instance, the European heatwave in the summer of 2003 caused more than 70,000 deaths (Robine et al., 2008), and nearly 55,000 people died as a result of the Russian heatwave in 2010 (Patz et al., 2005). Therefore, heatwave is still an important public health challenge.

To fully understand the overall risk of global warming, we need to understand how heatwaves can affect mortality in a changing climate scenario, particularly the extent to which the effect of heatwaves will be modified by heatwave characteristics. There have been a few studies projecting the mortality burden of future heatwaves. For example, a study using the definition of heatwave with daily maximum temperature reported that the city of Chicago could experience between 166 and 2217 excess deaths per year during 2081–2100 without considering adaptation scenarios and population change (Peng et al., 2011). Another study in the USA defined the heatwave using daily average temperature and found that heatwave-related excess deaths will increase from 36% to 365% in 2031–2080 compared to that during 1971–2020 (Guo et al., 2018), based on various population scenarios. However, the results of these studies are difficult to compare because of differences in the definitions of heatwaves, climate change scenarios, population change patterns, and adaptation assumptions. These uncertainties are particularly important in projecting future mortality burdens considering future hot weather or heatwaves (Sanderson et al., 2017). Therefore, a study considering all these uncertainties in quantifying future mortality burden in a changing climate is needed.

In addition, it is important to acknowledge future trends regarding the health effects of heatwave characteristics (such as intensity, duration, and timing), which could facilitate the development of targeted heatwave prevention and control policies. Previous investigations have demonstrated that certain heatwave characteristics will change significantly under the context of climate change (Barnett, 2007; IPCC, 2014; Perkins-Kirkpatrick and Gibson, 2018; Trancoso et al., 2020). For example, a study indicated that some tropical regions would experience up to 120 extra heatwave days if global warming of 5 °C is reached, and heatwave duration is projected to increase by 2–10 days/°C (Perkins-Kirkpatrick and Gibson, 2018). Recent attention has focused on the mortality risks of historical heatwave characteristics or their modifications (Anderson and Bell, 2011; Son et al., 2012; Sun et al., 2021), but the evidence regarding future trends of these mortality risks under climate change scenarios is unavailable.

In the present study, we aimed to project the excess mortality caused by heatwaves using diverse definitions under different climate change, population, and adaptation scenarios in Guangzhou, one of the largest cities in China with hot summers. We further differentiated the trends in excess mortality associated with heatwave characteristics and explored the influence of future population growth and adaptation scenarios. This study will contribute to a better understanding of heatwave-related health burdens in the complex context of global warming.

2. Materials and methods

2.1. The study site

Our study focused on Guangzhou, the capital city of Guangdong province, which covers an area of approximately 7434 km^2 and has almost 15.31 million permanent residents (Guangdong Statistics Bureau 2020) (Supplementary Fig. S1 in the appendix). In addition, Guangzhou is located in a subtropical coastal area with a marine subtropical monsoon climate and high temperatures during the hot season (May 1 to September 30), which makes residents in Guangzhou more likely to be exposed to heatwaves than those in temperate regions.

2.2. Data collection

2.2.1. Historical data on mortality and weather variables

The Guangzhou Centre for Disease Control and Prevention (http://www.gzcdc.org.cn/) provided daily mortality data from January 1, 2010 to December 31, 2019 (Supplementary Fig. S2). The 10th revision of the International Statistical Classification of Diseases (ICD-10) was used to classify the causes of death, and only all-cause mortality (A00-Z99) was selected. Corresponding daily meteorological data including daily maximum temperature, mean relative humidity, and atmospheric pressure were derived from the China Meteorological Data Centre (http://data.cma.cn/), which has only one basic monitoring station in Guangzhou, located at 113.33°E and 23.17°N. Moreover, the average daily concentrations of particulate matter $\leq$10 μg/m^3 in aerodynamic diameter (PM_{10}), fine particulate matter ($PM_{2.5}$), and ozone (O_3) were collected from the Environmental Protection Bureau of Guangzhou Municipality (http://www.gzepb.gov.cn), although $PM_{2.5}$ and O_3 data were only available from 2013.

2.2.2. Projected daily temperature series under climate change scenarios

The projected temperature series data for the period 1961–2100 were derived from the Coupled Model Intercomparison Project Phase 6 (CMIP6) using the statistical downscaling model NWAI-WG (De Li and Heping, 2012). In our study, 27 general circulation models (GCMs) and two emission scenarios provided by Scenario Model Intercomparison Project (ScenarioMIP) were used. These GCMs are representative of the range of future climate projections across the CMIP6 models (Supplementary Table S1 in the appendix) and the two emission scenarios resulting from the Tier-1 experiment under the ScenarioMIP, namely Shared Socioeconomic Path (SSP)2–4.5 and SSP5-8.5 (Brian et al., 2016; Eyring et al., 2016; van Vuuren et al., 2011). SSP2-4.5 and SSP5-8.5 are two scenarios assuming certain carbon emission control and less greenhouse gas emission, respectively, and are updates of the representative concentration pathway [RCP] 4.5, based on SSP2, and RCP 8.5 based on SSP5.

Specifically, we used the statistical downscaling model NWAI-WG to downscale the monthly gridded GCM data to daily data for the sites used in this study. The statistical downscaling model consisted of three major components: spatial downscaling, bias correction, and temporal downscaling. Special downscaling involved an inverse distance-weighted interpolation based on the centre of the nearest four grid points in the GCMs to avoid multiple counties within one grid sharing the same projected value. Bias correction of the GCM-projected monthly values and historically observed temperatures was performed using an equidistant quantile method. Finally, the daily time series of key climate variables, including the maximum and minimum temperatures for each site, were downscaled from the bias-corrected monthly GCM projections using a modified version of the stochastic weather generator (Richardson and Wright, 1984). Future climate data downscaled by this rapid and reliable statistical downscaling model have been widely used to assess the impact of climate change in many disciplines, including health sciences (Guo et al., 2016; Hundessa et al., 2018; Yang et al., 2021; Zhao et al., 2018). Finally, we calculated the average daily maximum temperatures of the 27 GCMs from 2010 to 2100 (Fig. 1).

2.2.3. Population scenarios

The annual population of each country during 2019–2100 were downloaded from the United Nations (UN) website (https://population.

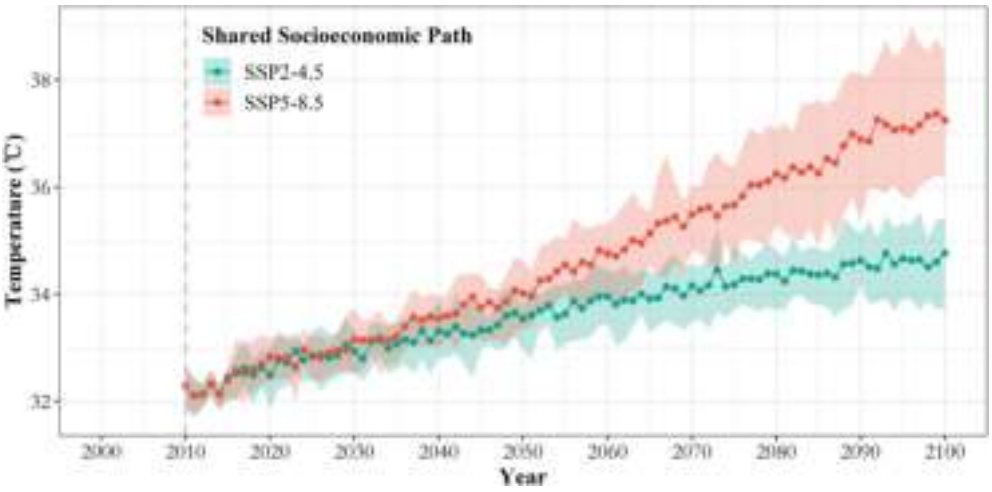

Fig. 1. The estimated and recalibrated annual average of daily maximum temperatures (in Guangzhou) during 2010–2100 (hot seasons) using 27 general circulation models (GCMs) under two climate change scenarios (SSP 2–4.5 and SSP5-8.5). The gray vertical line represents 2010 and the shaded areas indicate the inter quartile range (IQR) for the results of the ensemble of models (N = 27) for each year. All values correspond to the average value calculated from the 27 general circulation models. SSP: Shared socioeconomic paths.

un.org/wpp). The growth scenario included three fertility scenarios: low, medium, and high variants. Since only the annual future population data of all parts of China are available, we calculated the future annual population growth rates of all parts of China relative to the baseline population and then multiplied these data by the baseline population in Guangzhou to obtain the future population of this city (Supplementary Fig. S3). Baseline population data of Guangzhou during 2010–2019 were acquired from the Kwangtung Statistics Bureau (http://stats.gd.gov.cn/).

2.3. Data analysis

2.3.1. Heatwave definition

There is no uniform standard for the current definition of heatwave, but most studies incorporate the notion that intense heat lasts for several days. Generally, the definition of a heatwave depends on whether the daily maximum temperature or the daily average temperature is greater than an absolute or relative temperature threshold and whether the duration is greater than a certain threshold value (Guo et al., 2018; Wang et al., 2018; Yang et al., 2019). In addition, it is universally acknowledged that only warm seasons have the potential to have heatwave days (Guo et al., 2018; Peng et al., 2011; Yang et al., 2019); therefore, our statistical analyses and projections were restricted to the warm season (i.e., from May 1 to September 30) (Chen et al., 2022; Yang et al., 2019).

To compare the changes caused by different definitions, we chose nine commonly used definitions by combining three relative thresholds (92.5th, 95th, and 97.5th percentiles of daily maximal temperature, namely $T_{92.5}$, T_{95}, and $T_{97.5}$, respectively) with three durations ($\geq$2, $\geq$3, and $\geq$4 days, namely D_2, D_3, and D_4, respectively) (Supplementary Table S2). The most stringent definition ($HWD_4\_T_{97.5}$) was not included because no heatwave was identified in Guangzhou from 2010 to 2019 under this definition (Fig. 2). Daily maximum temperature was used as the heatwave definition because it can provide a better pattern for linking the effects of heat with daily mortality (Diaz et al., 2019; Guo et al., 2017).

2.3.2. The definition of heatwave characteristics

We characterized each heatwave by its intensity, duration, and timing in season. Heatwave intensity measured the degree of high temperature during the period of heatwave, defined as the difference between the maximum temperature on a heatwave day and the temperature threshold in the definition of heatwave. Heatwave duration was measured as the duration of the heatwave in days. Timing in season was measured as the day in the hot season when the heatwave started, defined as the difference in days between the onset of a heatwave in each hot season and the beginning of the hot season (May 1). To highlight the effect of timing in season, we used every 10 days as a unit. An example of the assignments for these three characteristics is provided in Supplementary Table S3.

2.3.3. Historical heatwave-mortality relationship

We estimated the current mortality risk from heatwaves using historical data. Specifically, we conducted the Poisson regression model allowing for overdispersion, as follows:

$$\mathrm{Log}(\mu_t) = \alpha + \mathrm{HW}_{t,l} + NS(\mathrm{Date}, 6) + \mathrm{Year}_t + NS(\mathrm{PM}_{10,t}, 2) + \mathrm{DOW}_t + \mathrm{Holiday}_t + \mathrm{Pre}_{t,\ l} + \mathrm{RH}_{t,\ l} \quad (1)$$

where μ_t is the expected number of deaths on day t; α is the intercept; Heatwave (HW_t) is considered as a 0–1 variable, which was assigned 1 for days with heatwaves and 0 for days without heatwaves, and a natural cubic spline with 3 degrees of freedom was used to capture the distributed lag effect of heatwaves over time; lag effect was set up to 10 days (Yang et al., 2019). However, we only used the cumulative lag effect of 3 days to calculate the relative risks (RR) of heatwaves because our pre-analysis confirmed that the significant lagged impact of heatwaves disappeared after three days (Supplementary Fig. S4). *NS*() denotes the natural cubic spline function. To account for seasonality, the day of the year ($Date_t$) with 6 degrees of freedom (df) was utilized, while an indicator variable for the year ($Year_t$) was employed to adjust for long-term trends in daily mortality. PM_{10} ($PM_{10,t}$) with 2 df was utilized to mitigate the impact of pollutants. (Chen et al., 2017); day of the week (Dow_t) and public holidays ($Holiday_t$) were included in the model as categorical variables. Cross-basis matrices of atmospheric pressure ($Pre_{t,\ l}$) and relative humidity ($RH_{t,l}$) were both produced by the distributed lag non-linear model with 6 df and 3 df natural cubic splines, respectively, for exposure and lag dimensions (up to 10 days). The choice of variables was in accordance with previous studies (Guo et al., 2018; Yang et al., 2019), and the selection of the model specifications was based on the Akaike information criterion for a quasi-Poisson model (Q-AIC).

2.3.4. Historical heatwave characteristics-mortality relationships

To study the characteristics of heatwaves, we used only the definition of $HWD_3\_T_{92.5}$, which was identified as the optimal heatwave definition in our recent study across 31 Chinese capital cities (Yang et al., 2019). To estimate the independent effects of each characteristic, we conducted a quasi-Poisson regression model that included all three characteristic variables (Barnett et al., 2012; Zeng et al., 2014). We also included the heatwave variable because the correlations among heatwave characteristics were significantly reduced after adjusting for this variable. The equation is as follows:

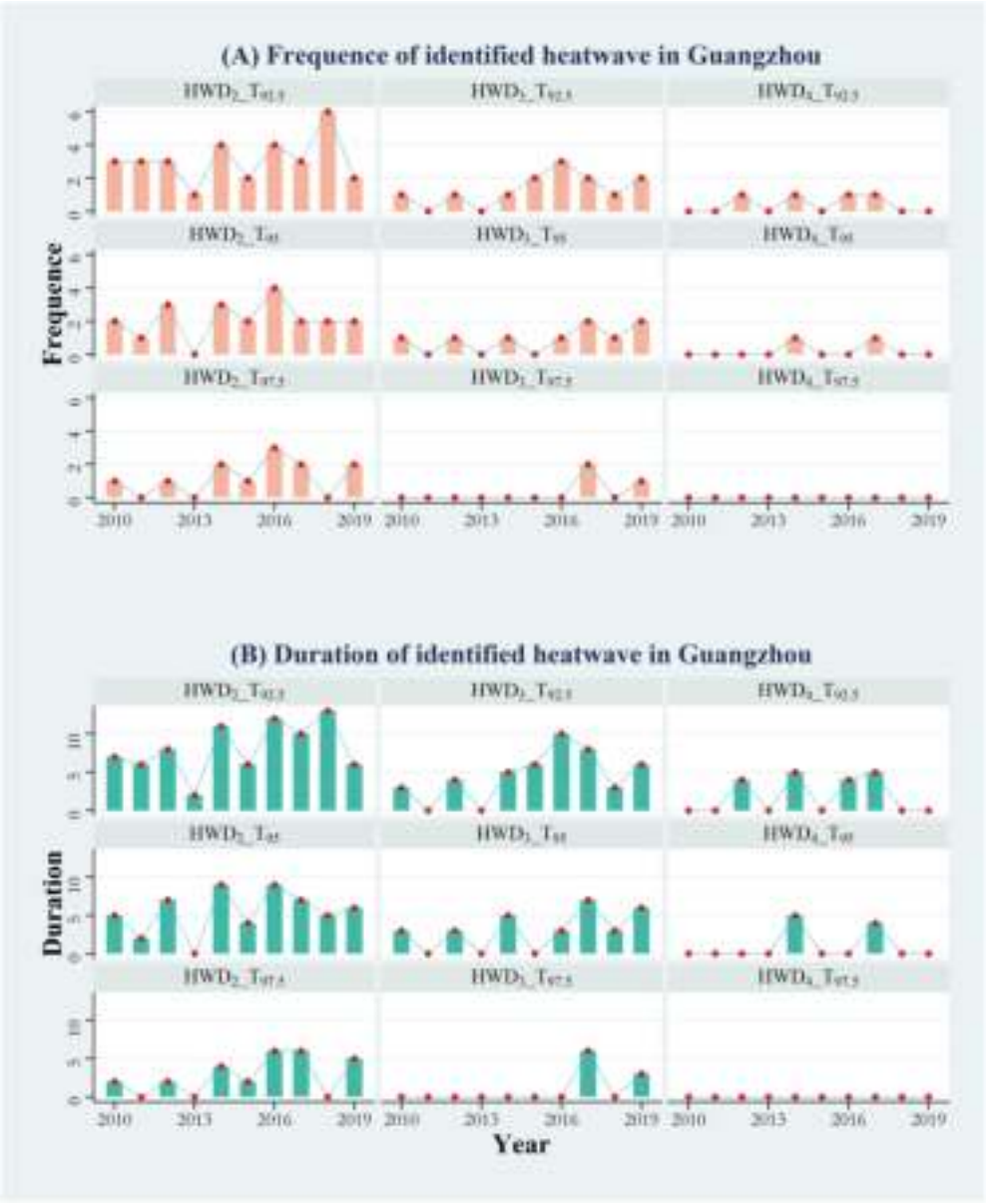

Fig. 2. The descriptive statistics of heatwaves. A: Annual sum of frequency of heatwaves in Guangzhou, China during 2010–2019 (hot season); B: Annual sum of duration of heatwaves in Guangzhou, China during 2010–2019 (hot season).

$$\mathrm{Log}(\mu_t) = \alpha + \mathrm{HI}_{t,l} + \mathrm{HD}_{t,l} + \mathrm{HT}_{t,l} + \mathrm{HW}_{t,l} + NS(\mathrm{Date}, 6) + \mathrm{Year}_t + NS(\mathrm{PM}_{10}{}_t, 2) + \mathrm{DOW}_t + \mathrm{Holiday}_t + \mathrm{Pre}_{t,\ l} + \mathrm{RH}_{t,\ l} \quad (2)$$

where $\mathrm{HI}_{t,l}$, $\mathrm{HD}_{t,l}$, and $\mathrm{HT}_{t,l}$ denote the cross-basis matrices of the intensity, duration, and timing of the heatwave, respectively. These three variables were all constructed using distributed lag models with a linear function for variables and a 3 df natural cubic spline for lag dimensions; the maximum lag effect was set to 10 days. However, we only considered the cumulative lag effect of 3 days to calculate the RR of characteristics because our pre-analysis confirmed that significant lag impacts of characteristics disappeared after 3 days (Supplementary Fig. S5). The choice of parameters was also determined using Q-AIC.

2.3.5. *Projection of future heatwave-attributable and characteristics-attributable mortality*

We set up two hypotheses: the population has/does not have heatwave adaptation (Diaz et al., 2019; Guo et al., 2018). Assuming that humans are capable of adapting/acclimatizing to gradually increasing temperatures, the relative threshold ($T_{threshold}$ will increase over time under the context of global warming) was used to identify heatwaves. Otherwise, the absolute threshold ($T_{threshold}$ was calculated from the 2010–2019 maximum temperature series and was constant over time) was used (See Supplementary Table S4 for an example of differences in the heatwave adaptation scenarios). Besides, we considered three patterns of population change: low, medium, and high variants.

Therefore, the number of excess deaths attributed to heatwaves or heatwave characteristics was calculated as follows:

$$\mathrm{ED_{hw}} = \mathrm{N} \times (\mathrm{RR}_{hw} - 1) \times \mathrm{DUR}_{hw}, \quad (3)$$

$$\mathrm{ED_{cha}} = \mathrm{N} \times \mathrm{ER_{cha}}, \quad (4)$$

$$\mathrm{N} = \mathrm{POP} \times \mathrm{MR} \quad (5)$$

where ED_{hw} and ED_{cha} is the expected number of excess deaths attributed to heatwave (ED_{hw}) and its characteristics (ED_{cha}), respectively, over a given period. N represents the annual average number of deaths on non-heatwave days, while POP and MR denote the annual population and the

mortality rate across all non-heatwave days from historical data (2010–2019). RR_{hw} is the relative risk of mortality attributed to a heatwave, and DUR_{hw} signifies the number of days identified as heatwave days each year. ER_{cha} is the percentage change of mortality (ER=RR-1) attributed to the three heatwave characteristics.

To quantify the overall impact of heatwaves on humans, we computed the decade having excess deaths attributed to heatwaves. This summary of the health impact of heatwaves incorporates changes in both the rate at which heatwaves occur and the number of heatwaves in the future (Peng et al., 2011). We calculated this summary of 2010–2019 for the "current" period and 2030–2039, 2050–2059, and 2090–2099 for different future periods under various climate change and population scenarios.

2.3.6. Uncertainty analysis

In addition to the uncertainties on heatwave adaptation by using two assumptions and the impact of population change, there are other uncertainties in future excess deaths, derived from the variance of the model coefficient and the variability of different GCMs, respectively. We quantified the above uncertainties by generating 1000 samples of coefficients estimated from historical data through Monte Carlo simulations, assuming that the estimated coefficients are normally distributed, and then generated results for each of the 27 GCMs (Guo et al., 2018). Then, the empirical confidence intervals (eCIs) corresponding to the 2.5th and 97.5th percentiles of the distribution of the results across the coefficients and 27 GCMs are reported to account for these uncertainties.

2.3.7. Sensitivity analysis

To verify the robustness of our main results, we extended the cumulative lag period of the impact of heatwaves and their characteristics to lags of 0–4, 0–7, and 0–10 days, respectively (Yang et al., 2019). Moreover, in the effect estimation of heatwave characteristics, we changed the definition of heatwaves to $HWD_2\_T_{95}$ and $HWD_3\_T_{95}$. To further examine the potential impact of air pollutants on the heatwave-mortality relationship, PM_{10} was replaced with fine particulate matter ($PM_{2.5}$) and ozone (O_3) one by one. But this part of the analysis was restricted to 2013–2019 as $PM_{2.5}$ and O_3 data were only available for this period. Controlling for PM_{10} rather than $PM_{2.5}$ and O_3 in the main model was due to better data availability for PM_{10}. We also considered a constant population scenario to better communicate with other projected studies without considering population changes.

All statistical analysis were conducted in the work environment of R software version 4.1.0 (R Foundation for Statistical Computing), with packages of "gnm" (Armstrong et al., 2014), "dlnm" (Gasparrini et al., 2010), and "pscl" (Achim and Weiss, 2008). Statistical significance was set at two-tailed p-value <0.05. The workflow of our study is shown in Fig. S6.

3. Result

3.1. Descriptive analysis

From 2010 to 2019, during the hot season in Guangzhou, 178,838 deaths were attributed to all causes, with an average of 117 deaths (range: 53–195) deaths per day. The overall annual average maximum temperature change in Guangzhou from 2010 to 2100 shows an increasing trend under SSP2-4.5 and SSP5-8.5 scenarios, and especially under SSP5-8.5 (Fig. 1).

Supplementary Table S5 shows the 10-year total duration of heatwaves under different definitions for four time periods. Supplementary Table S6 shows the trend of the 10-year average heatwave characteristics. Under the assumption of adaptation, the duration of the heatwave was generally three days, and the heatwave mostly occurred late in the hot season. There was no obvious difference between SSP2-4.5 and SSP5-8.5. Under the assumption of non-adaptation, the fluctuation in duration was severe, and the time of heatwave occurrence advanced sharply, which is more obvious under SSP5-8.5.

3.2. Effects of heatwave and its characteristics at baseline

Supplementary Fig. S7 displays the estimated cumulative relative risks of heatwaves on all-cause mortality at a lag of 0–3 days under different definitions. Point estimation of the effect of a heatwave generally increases when the heatwave definition becomes more stringent. Supplementary Fig. S8 displays the estimated cumulative relative risks of intensity, duration, and timing in season at a lag of 0–3 days, and the corresponding cumulative relative risk was 1.15 (95%CI: 1.04, 1.26), 1.07 (95%CI: 0.98, 1.15), and 0.99 (95%CI: 0.96, 1.01), respectively.

3.3. Heatwave-related burdens under climate change, population and adaptation scenarios

Figs. 3 and 4 show the mortality burden of eight heatwaves under two climate change scenarios and three population scenarios, without and with the assumption of adaptation, respectively. Under the assumption of non-adaptability, heatwave-related excess mortality consistently increased over decades among various heatwave definitions, with more rapid increase under SSP5-8.5. For example, under the definition of $HWD_3\_T_{92.5}$, heatwave-related excess deaths might be 2.13 (ten thousand) (95%eCI: 0.35, 4.40) in 2010–2019, while in 2090–99, heatwave-related excess deaths of the three population patterns low, medium, and high under SSP2-4.5 might be 13.32 (95%eCI: 1.03, 35.87), 19.49 (95%eCI: 1.50, 52.49) and 27.60 (95%eCI: 2.12, 74.34), respectively. Correspondingly, under SSP5-8.5, the deaths might be 43.40 (95%eCI: 5.19, 101.84), 63.57 (95%eCI: 7.52, 149.12), and 90.11 (95%eCI: 10.56, 211.33), respectively. Under the assumption of adaptability, there is no obvious difference in the trend of heatwave-related excess mortality between SSP2-4.5 and SSP5-8.5. Heatwave-related excess deaths under the definitions of $HWD_2\_T_{92.5}$, $HWD_2\_T_{95}$, and $HWD_2\_T_{97.5}$ might decrease from the 2010s–2090s under low and medium population patterns. We observed an increasing trend in heatwave-related excess deaths by decade for $HWD_3\_T_{92.5}$, $HWD_3\_T_{95}$, $HWD_3\_T_{97.5}$, $HWD_4\_T_{92.5}$, and $HWD_4\_T_{95}$. However, the growth rates were much lower than those under the assumption of non-adaptability. The estimates of deaths considering multiple definitions of heatwave, are presented in Supplementary Tables S7 and S8.

3.4. Heatwave characteristics-related burden under climate change, population and adaptation scenarios

Fig. 5 shows the burden of deaths related to the characteristics of heatwaves considering $HWD_3\_T_{92.5}$ in various scenarios. Compared with the intensity and timing of heatwave, the burden related to heatwave is mainly determined by its duration. In addition to the duration, under the assumption of non-adaptability, the intensity of the heatwave would cause more deaths than those caused by timing, particularly under the scenario of SSP5-8.5. Under the assumption of adaptability, the effect of heatwave timing was slightly greater than that of intensity. In addition, the trend in death burden caused by each heatwave characteristic was generally consistent with the trend in death burden caused by heatwaves. The specific number of deaths due to the characteristics of each scenario is shown in Supplementary Table S9.

In the sensitivity analyses, the relative risks of heatwaves did not change significantly when the lag of days was changed from 3 to 4, 7, and 10 days, and the majority of the confidence intervals became wider (Supplementary Figs. S9–S11). The relative risks of heatwaves were stable when PM_{10} was replaced with $PM_{2.5}$ and O_3 in the model, respectively (Supplementary Figs. S12–S13). Regarding the characteristics of heatwaves, there were no substantial differences observed in the relative risk of the characteristics when distinct lag days were employed (Supplementary Figs. S14–S16) or when different heatwave definitions

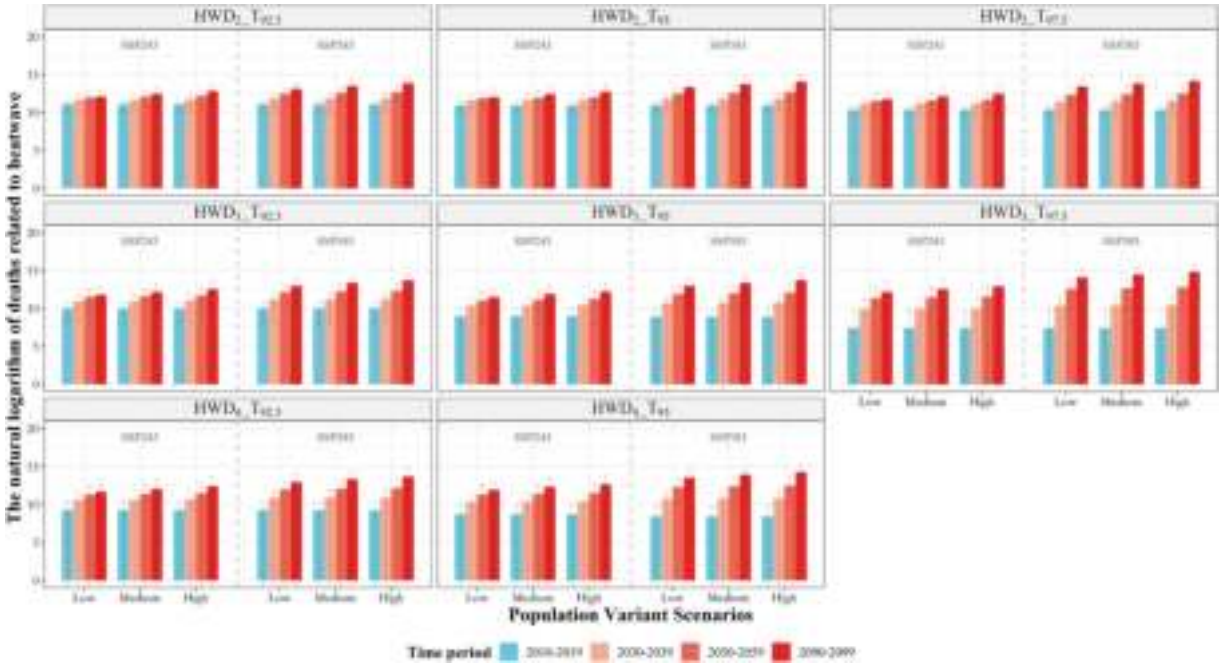

Fig. 3. The excess deaths of all causes related to eight different heatwaves in four periods, two shared socioeconomic paths, assumption of non-adaptability, and three population scenarios. The high–low line indicates 95% eCI; SSP: Shared socioeconomic paths. The Y axis is the natural logarithmic scale.

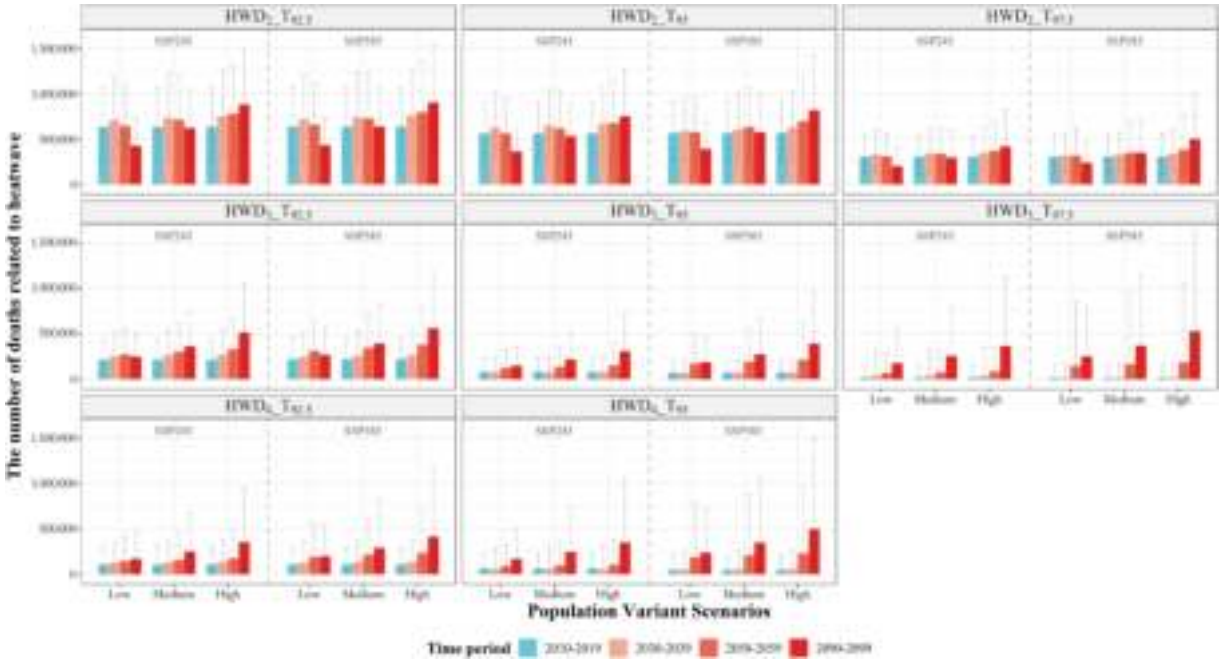

Fig. 4. The excess deaths of all causes related to eight different heatwaves in four periods, two shared socioeconomic paths, assumption of adaptability, and three population scenarios. The high–low line indicates 95% eCI; SSP: Shared socioeconomic paths.

were applied (Supplementary Figs. S17–S18). In addition, the number of deaths attributed to heatwaves and heatwave characteristics under the constant population scenario were very similar to those under the medium population scenario (Supplementary Figs. S19–S21).

4. Discussion

Based on 27 GCMs, two SSPs, three population growth patterns, and two adaptation assumptions, we predicted future changes in the heatwave- and heatwave characteristics-related death burden in Guangzhou. Our study found that the burden related to heatwaves was projected to increase significantly under each scenario, assuming that the population had not adapted to heatwaves. Under the assumption of adaptability, there is no sharp increase in heatwave-attributed mortality, especially under SPP2.4-5 and low population development scenarios. In addition, our study found that heatwave intensity may play a major role in the adverse effects caused by heatwave, and that prolonged heatwaves in the future could cause a huge death burden.

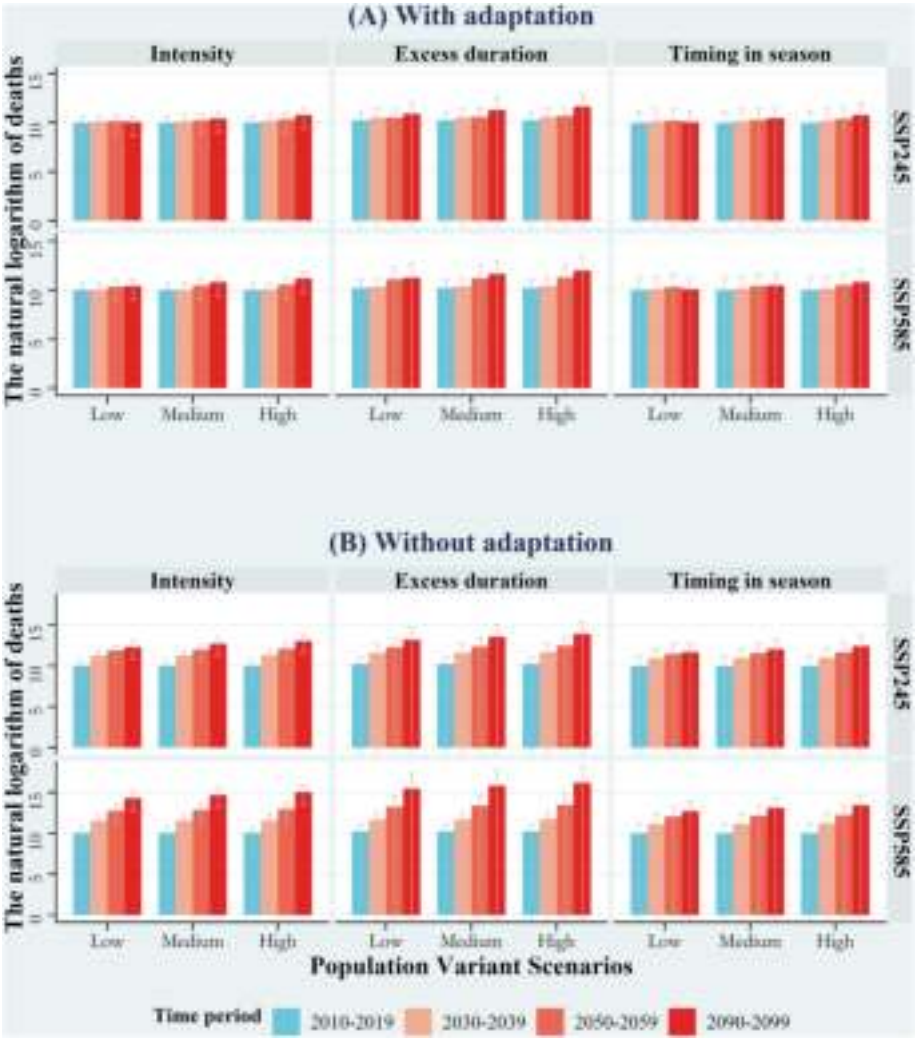

Fig. 5. The excess all-cause deaths related to the characteristics of $HWD_3\_T_{92.5}$ in four periods, two shared socioeconomic paths, assumption of adaptability or non-adaptability, and three population scenarios. A: assumption of adaptability. B: assumption of non-adaptability. The high–low line indicates 95% eCI; SSP: Shared socioeconomic paths. Timing in season in the figure refers to the excess deaths caused by the advance of the heatwave. The Y axis is the natural logarithmic scale.

4.1. Mortality risk related to heatwave characteristics

With the aggravation of global warming, temperatures will continue to rise in the future (Fig. 1), which will lead to a continuous increase in the frequency, intensity, and duration of heatwaves, as well as an earlier timing, especially in the high greenhouse-gases emissions scenario. Therefore, the exploration of risks related to these characteristics is constructive for the advanced planning of future resource allocation. Our study found that each 10-day advance of heatwave during the hot season was associated with a 1.25% (95% CI: −1.18%, 3.75%) increased risk of mortality, which may be because people are not well prepared for a heatwave immediately after a long period of cold season (Frost and Auliciems, 1993). In addition, the risk of mortality increased by 14.50% (95% CI: 3.66%, 26.47%) for each 1 °C increase from the heatwave threshold, and by 6.86% (95% CI: −0.34%, 14.59%) for each additional day of duration. One reason for this may be that longer and more intense heatwaves can place a heavy burden on the cardiovascular system (Barnett et al., 2012). Consistent with our results, an investigation (Anderson and Bell, 2011) based on 43 U.S. communities found that the mortality risk was increased by 2.49% for each 1 °F increase in heatwave intensity and decreased by 0.063% for each day later in the hot season. Another study (Son et al., 2012) conducted in seven major cities in Korea also found that mortality risk increased by 3.5% for each 1 °C increase during heatwave and decreased by 0.2% (95% CI: −1.6%, 1.2%) for a 1-day increase in the timing of heatwave in summer.

4.2. Future burden related to heatwave and its characteristics under assumption of non-adaptability

Regarding the mortality burden associated with heatwaves under climate change scenarios, our study shows that the trends of heatwave- and heatwave characteristics-related mortality are quite different under divergent adaptation assumptions. When we assume that individuals do not adapt to heat, heatwave-related mortality increases dramatically in any given scenario. Specifically, under $HWD_3\_T_{92.5}$, the number of deaths caused by heatwave in 2090–99 could range from 3.49 times to 48.77 times higher than that in 2010–19, which is consistent with that seen in previous studies (Diaz et al., 2019; Guo et al., 2018; Yang et al., 2021). For example, a study quantifying the excess deaths associated with heatwaves in the future, in 20 countries, reported that the change in excess deaths in 2031–2080 compared to 1971–2020, range from approximately 2000% in Colombia to 150% in Moldova (Guo et al., 2018). In addition, compared to the SSP2-4.5 scenario, the increase in heatwave-attributable deaths was extremely rapid under the SSP5-8.5 scenario, which is consistent with previous investigations (Diaz et al., 2019; Guo et al., 2018; Peng et al., 2011; Yang et al., 2021). Specifically, a multi-city study indicated that compared with the pathway of high CO_2 emissions, the number of heat-related deaths in the 2090s will be almost twice as high as that under the pathway of low CO_2. (Yang et al., 2021). As for the burden caused by heatwave characteristics, our study found that the burden caused by the duration of heatwaves will be much greater than other characteristics in the SSP5-8.5 and non-adaptive

scenarios, mainly because the continued increase in temperature will make the heatwaves last very long. These findings suggest that the development of climate change mitigation strategies and the implementation of effective climate policies are imperative to protect public health during the future heatwaves.

4.3. Future burden related to heatwave and its characteristics under assumption of adaptability

Contrarily, when assuming adaptability, our study revealed that in addition to the impact of global warming, the attributable deaths will also be largely influenced by the increasing rate of total population. For instance, under $HWD_3\_T_{92.5}$, the difference in attributable deaths between the SSP2-4.5 and SSP5-8.5 scenarios in 2090–99 would be 3.26 times, while the difference in attributable deaths between low and high population fertility would be 2.08 times in 2090–99. Additionally, we found that even under the low population scenario, the increase in future heatwave-related mortality burden could not be totally offset. These results agree with those of previous studies (Chen et al., 2022; Guo et al., 2018; Wang et al., 2018). Specifically, a study conducted in the US estimated that on account of adaptation, the overall heat-related mortality by 2050 would not change substantially compared to that in 2006, assuming a constant population (Wang et al., 2018). Our study also found that, similar to the duration, the timing and intensity of heatwaves can also contribute to the huge death burden of heatwaves. These findings underscore that mitigating the health losses due to heatwave would require not only the implementation of effective climate adaptation policies, but also the establishment of accurate early warning systems for heatwaves and the risk classification of heatwave characteristics.

4.4. Uncertainty assessment

It should be noted that although we considered scenarios based on the levels of greenhouse gas emissions, mortality baselines, population growth, and population adaptation, there still are other sources of uncertainty. First, we chose the SSP considering not only the representative concentration pathways (RCPs) but also various future social pathways as the emission pathway. Other mitigation scenarios, such as SSP1-2.6, can also be considered, although they may be too stringent. Second, although various heatwave definitions have been used to establish heatwave-related mortality baselines, we did not consider that the heatwave-mortality association might change over time (Guo et al., 2018). Third, in addition to changes in population growth, future trends in demographic structure (such as proportion of the elderly and those with low socioeconomic status) should also be considered when predicting the health burden from future climate change (Yang et al., 2021). Finally, for adaptation scenarios, it is reasonable to assume that the population has the capacity to adapt to heat gradually (Huber et al., 2022; Anderson and Bell, 2011; Guo et al., 2018; Wang et al., 2018), because of several adaptation interventions such as the implementation of prevention programs and improvements in health services, socioeconomic conditions, and household infrastructure. There are other strategies for the population's adaptation to heatwaves, such as if the populations are fully acclimated to rising temperatures including a complete adaptation for an increase in 1–4 °C, or using the temperature-mortality relationship from a hot (warmer) city to analogize the future temperature-mortality relationship in a warming city, which relies on a strong assumption that other confounding factors are the same in both cities, or including the interaction term of the average temperature in the hot season and the heatwaves (Wang et al., 2018), which merely indicates how people have responded to warmer summers in the past. This study considered only fully adapted and non-adapted scenarios, and more research is needed to consider more rigorous and nuanced adaptation scenarios in the field of climate change and health. Specifically, a recent study suggested that information on the pace of the population's adaptation from historical data could be integrated into predictions of future temperature-related mortality (Huber et al., 2022), although there are relatively few rigorous and detailed methods for heatwave adaptation. Future research is warranted to appropriately include these sources of uncertainty in the assessment of future heatwave-related mortality burden.

4.5. Limitations and outlook

This study has some limitations. First, there is no clear evidence that such a hypothetical adaptation will occur effectively and in a timely manner in the future (Smoyer, 1998). Further research is required to better quantify the population's adaptation when projecting the health risks of future heatwaves. Nevertheless, individuals have adapted and may continue to adapt to local climate change, to some extent, through adaptation interventions including those listed in Supplementary Table S10 (Anderson and Bell, 2011). Second, we did not consider the differences in the susceptibility of different groups of people to heatwaves. For example, previous studies have reported a higher vulnerability to heatwaves among the elderly (Ma et al., 2015; Yang et al., 2019), females (Barnett, 2007), and those with low education levels (Yang et al., 2013). Third, we assumed that heatwave-related relative risk (reflecting the resilience and vulnerability of the population) is constant in the future, which is virtually affected by behavioural changes, improvement of physical activity, healthier housing, and environmental changes through six levels of adaptation interventions, and also indirectly affects the process of physiological change (Costello et al., 2009). Fourth, because of data access issues, only all-cause death data were included in this study. Further assessment of the projections of future heatwave characteristics on cause-specific deaths is required. Finally, because the United Nations provides population data on a national basis, which is not particularly accurate for calculating the rate of population change in a fast-growing area, our study may have obtained a conservative estimate of future heat-related excess deaths. Further studies should consider diverse subpopulations, well-designed assumptions of adaptation, demographic shifts, and urbanisation patterns to provide a comprehensive picture of the impacts of global warming and climate change.

5. Conclusions

This is the first study to project the mortality burden of heatwave characteristics under climate change, population, and adaptation scenarios. Our findings can serve as a reference for formulating public health policies. First, it is important to formulate stricter air pollutant emission policies and implement a strategy of sustainable development, as the future heatwave-related mortality will be 2–8 times higher under SSP5-8.5 than under SSP2-4.5. Second, it is recommended that planning ahead for public resources, such as establishing urban cooling centres, is essential to ensure that populations are resilient to heatwave. As populations adapt to heatwaves, their health burden declines significantly. Third, based on the implementation of effective climate adaptation policies, the establishment of accurate heatwave warning systems can significantly reduce the adverse health effects of heatwaves, especially after the end of the cold season, when people are not yet well prepared for it.

CRediT authorship contribution statement

Jiangdong Liu: Formal analysis, Visualization, Writing-original draft. Hang Dong: Resources, Writing-review & editing. Mengmeng Li: Conceptualization, Resources, Writing-review & editing, Supervision. Ying Wu: Resources, Writing-review & editing. Chunlin Zhang: Resources, Writing-review & editing. Jinjian Chen: Writing-review & editing, Formal analysis. Zhou Yang: Writing-review & editing, Formal analysis. Guozhen Lin: Resources, Writing-review & editing. De Li Liu:

Resources, Writing-review & editing. Jun Yang: Conceptualization, Resources, Writing-review & editing, Funding acquisition, Project administration, Supervision.

Declaration of competing interest

The authors declare they have no actual or potential competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114157.

References

Achim, A.M., Weiss, A.P., 2008. No evidence for a differential deficit of reality monitoring in schizophrenia: a meta-analysis of the associative memory literature. Cognit. Neuropsychiatry 13, 369–384.
Anderson, G.B., Bell, M.L., 2011. Heat waves in the United States: mortality risk during heat waves and effect modification by heat wave characteristics in 43 U.S. communities. Environ. Health Perspect. 119, 210–218.
Armstrong, B.G., et al., 2014. Conditional Poisson models: a flexible alternative to conditional logistic case cross-over analysis. BMC Med. Res. Methodol. 14, 122.
Barnett, A.G., 2007. Temperature and cardiovascular deaths in the US elderly: changes over time. Epidemiology 18, 369–372.
Barnett, A.G., et al., 2012. Cold and heat waves in the United States. Environ. Res. 112, 218–224.
Brian, C.O., et al., 2016. The scenario model Intercomparison project (ScenarioMIP) for CMIP6. Geosci. Model Dev. (GMD) 9, 3461–3482.
Chen, F., et al., 2017. Does temperature modify the effect of PM(10) on mortality? A systematic review and meta-analysis. Environ. Pollut. 224, 326–335.
Chen, H., et al., 2022. Projections of heatwave-attributable mortality under climate change and future population scenarios in China. Lancet Reg Health West Pac 28, 100582.
Chen, R., et al., 2018. Association between ambient temperature and mortality risk and burden: time series study in 272 main Chinese cities. BMJ 363, k4306.
Costello, A., et al., 2009. Managing the health effects of climate change: lancet and university college london institute for global health commission. Lancet 373, 1693–1733.
De Li, L., Heping, Z., 2012. Statistical downscaling of daily climate variables for climate change impact assessment over New South Wales, Australia. Climatic Change 115, 629–666.
Diaz, J., et al., 2019. Mortality attributable to high temperatures over the 2021-2050 and 2051-2100 time horizons in Spain: adaptation and economic estimate. Environ. Res. 172, 475–485.
Eyring, V., et al., 2016. Overview of the coupled model Intercomparison project Phase 6 (CMIP6) experimental design and organization. Geosci. Model Dev. (GMD) 9, 1937–1958.
Frost, D.B., Auliciems, A., 1993. Myocardial infarct death, the population at risk, and temperature habituation. Int. J. Biometeorol. 37, 46–51.
Gasparrini, A., et al., 2010. Distributed lag non-linear models. Stat. Med. 29, 2224–2234.
Guo, Y., et al., 2016. Projecting future temperature-related mortality in three largest Australian cities. Environ. Pollut. 208, 66–73.
Guo, Y., et al., 2017. Heat wave and mortality: a multicountry, multicommunity study. Environ. Health Perspect. 125, 087006.
Guo, Y., et al., 2018. Quantifying excess deaths related to heatwaves under climate change scenarios: a multicountry time series modelling study. PLoS Med. 15, e1002629.
Huber, V., et al., 2022. Evidence of rapid adaptation integrated into projections of temperature-related excess mortality. Environ. Res. Lett. 17, 044075.
Hundessa, S., et al., 2018. Projecting environmental suitable areas for malaria transmission in China under climate change scenarios. Environ. Res. 162, 203–210.
IPCC, 2014. Climate Change 2013 – the Physical Science Basis: Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge.
Ma, W., et al., 2015. The short-term effect of heat waves on mortality and its modifiers in China: an analysis from 66 communities. Environ. Int. 75, 103–109.
McGregor, G.R., et al., 2015. Heatwaves and Health: Guidance on Warning-System Development. World Meteorological Organization and World Health Organization.
Patz, J.A., et al., 2005. Impact of regional climate change on human health. Nature 438, 310–317.
Peng, R.D., et al., 2011. Toward a quantitative estimate of future heat wave mortality under global climate change. Environ. Health Perspect. 119, 701–706.
Perkins-Kirkpatrick, S.E., Gibson, P.B., 2018. Author Correction: changes in regional heatwave characteristics as a function of increasing global temperature. Sci. Rep. 8, 4652.
Richardson, C., Wright, D., 1984. WGEN (Weather Generator): A Model for Generating Daily Weather Variables.
Robine, J.M., et al., 2008. Death toll exceeded 70,000 in Europe during the summer of 2003. C R Biol 331, 171–178.
Sanderson, M., et al., 2017. The use of climate information to estimate future mortality from high ambient temperature: a systematic literature review. PLoS One 12, e0180369.
Smoyer, K.E., 1998. A comparative analysis of heat waves and associated mortality in St. Louis, Missouri–1980 and 1995. Int. J. Biometeorol. 42, 44–50.
Son, J.Y., et al., 2012. The impact of heat waves on mortality in seven major cities in Korea. Environ. Health Perspect. 120, 566–571.
Sun, Z., et al., 2021. Heat wave characteristics, mortality and effect modification by temperature zones: a time-series study in 130 counties of China. Int. J. Epidemiol. 49, 1813–1822.
Trancoso, R., et al., 2020. Heatwaves intensification in Australia: a consistent trajectory across past, present and future. Sci. Total Environ. 742, 140521.
van Vuuren, D.P., et al., 2011. A proposal for a new scenario framework to support research and assessment in different climate research communities. Global Environ. Change 22, 21–35.
Wang, Y., et al., 2018. Accounting for adaptation and intensity in projecting heat wave-related mortality. Environ. Res. 161, 464–471.
Yang, J., et al., 2013. Impact of heat wave in 2005 on mortality in Guangzhou, China. Biomed. Environ. Sci. 26, 647–654.
Yang, J., et al., 2019. Heatwave and mortality in 31 major Chinese cities: definition, vulnerability and implications. Sci. Total Environ. 649, 695–702.
Yang, J., et al., 2021. Projecting heat-related excess mortality under climate change scenarios in China. Nat. Commun. 12, 1039.
Zeng, W., et al., 2014. The effect of heat waves on mortality and effect modifiers in four communities of Guangdong Province, China. Sci. Total Environ. 482–483, 214–221.
Zhao, Q., et al., 2018. Modeling the present and future incidence of pediatric hand, foot, and mouth Disease associated with ambient temperature in mainland China. Environ. Health Perspect. 126, 047010.

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Rapid extraction and analysis of oxidative stress and DNA damage biomarker 8-hydroxy-2′-deoxyguanosine (8-OHdG) in urine: Application to a study with pregnant women

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ABSTRACT

Oxidative stress is an important toxicity and genotoxicity mechanism of many chronic adverse health outcomes. This study developed a sensitive extraction method for urine matrix (based on lyophilization, without the need for pre-cleaning by solid phase extraction), coupled to LC-MS/MS analysis of the biomarker 8-hydroxy-2′-deoxyguanosine (8-OHdG). The methodology was validated in urine samples from a cohort of Spanish pregnant women collected during the first, second and third trimester of pregnancy, and urine samples collected within 24 h after delivery (n = 85). A detection and quantification limit of 0.01 and 0.05 μg/L, respectively, were established. The median 8-OHdG concentration was 2.18 μg/L (range 0.33–7.79); and the corresponding creatinine-adjusted concentrations ranged from 1.04 to 13.12 with median of 4.48 μg 8-OHdG/g creatinine. The concentrations of non-adjusted 8-OHdG significantly decreased ($p < 0.05$) in the 3rd trimester and post-delivery urine samples when compared to the 1st trimester levels. 8-OHdG concentrations were further studied in placenta samples matching the same urine samples (n = 26), with a median value of 1.3 ng 8-OHdG/g of tissue. Placental 8-OHdG concentrations were correlated with urinary levels of non-adjusted 8-OHdG in the 3rd trimester. Considering the small cohort size, results must be interpreted with caution, however statistical analyses revealed elevated urinary non-adjusted 8-OHdG levels in the 1st trimester of mothers that delivered boys compared to those who delivered girls ($p < 0.01$). Increased urinary non-adjusted 8-OHdG concentrations at the time of delivery were significantly associated with clinical records (any type of clinical record during pregnancy; $p < 0.05$). The novel extraction and analytical method for the assessment of 8-OHdG is applicable for sensitive analysis of multiple analytes or biomarkers in urine matrix. This method could also be applied for other matrices such as blood or tissues. Our findings show that 8-OHdG in urine of pregnant women could predict oxidative stress in placenta and can be related to characteristics such as maternal obesity, mode of delivery and newborn sex.

1. Introduction

Physiological processes as well as external physical and chemical factors continuously generate reactive oxygen species (ROS). Maintaining balance between ROS production and their removal is an important homeostatic process in all cells. Overproduction of ROS is known to induce pathologies via oxidative damage of important macromolecules and cellular structures (Valavanidis et al., 2009). Biomarkers of oxidative stress have been thoroughly investigated in medicine and toxicology but their importance for environmental health has only recently been postulated (Steffensen et al., 2020).

The hydroxyl radical, the most hazardous ROS, attacks cellular membranes, proteins or nucleic acids including both nuclear and mitochondrial DNA and RNA, where it forms abundant and stable adduct 8-hydroxy-2′-deoxyguanosine (8-oxodG; 8-OHdG) (Halliwell and Gutteridge, 1990; Marrocco et al., 2017). Oxidized DNA can be repaired by various mechanisms and the oxidized adducts are released into urine without further transformation (Fleming et al., 2015). Content of

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8-OHdG in urine and plasma is the most studied biomarker of DNA damage, and corresponding health outcomes such as cancer, neurodegenerative disorders and various other chronic diseases (Guo et al., 2017). Levels of 8-OHdG increase with smoking and aging, and with various occupational exposures to physical, chemical, or biological agents (Graille et al., 2020).

The urinary biomarkers of oxidative stress are broadly investigated in preventive or occupational medicine due to several benefits such as non-invasive sampling (Il'yasova et al., 2012; Ventura et al., 2021) or stable matrix less prone to secondary oxidation during sample handling. The biomarker concentrations in urine vary within and between days (Martinez-Moral and Kannan, 2019; Li et al., 2021), and the urinary creatinine is commonly used for normalization although the creatinine excretion rate is also known to be naturally variable (Garde et al., 2004). Levels of 8-OHdG have been investigated in various biomonitoring and epidemiological studies, including also highly vulnerable pregnant women (Zhang et al., 2021), where oxidative disbalance and increased ROS production were documented namely during the 1st trimester (Potdar et al., 2009).

The biomarkers of oxidative DNA damage can be analyzed by using the immunochemical enzyme-linked immunosorbent assays (ELISA) or by selective chromatography techniques with different detection rates (Graille et al., 2020). Because of analytical limitations of ELISA such as inherent lack of specificity (Song et al., 2009; Wu et al., 2004), liquid chromatography-mass spectrometry LC-MS/MS is becoming a preferred and golden standard technique for analyses of oxidative stress biomarkers in biological samples (Chen et al., 2020; Wang et al., 2016).

Although few studies suggested simple dilution or fast ultrafiltration of urine before LC-MS/MS analysis (Qian et al., 2021; Topic, 2014; Zhang et al., 2021), this usually resulted in lower sensitivity (LOQ 0.2–1 ng/mL) due to dilution and signal suppression. Moreover, faster contamination of ion source and shorter column life are also drawbacks of this approach. Correspondingly, pre-treatment of the sample is usually required to remove salts and organic interferences, and to enrich relatively low concentration of some biomarkers. The most commonly used pre-concentration method for urine is solid phase extraction (SPE) (Chen et al., 2020; Guo et al., 2017; Fan et al., 2012; Lu et al., 2016; Ren et al., 2016; Waits et al., 2020; Wang et al., 2016), where many types of sorbents and arrangements are available (Graille et al., 2020). However, multiple steps in SPE are not only laborious but may bring additional variability into the analytical process, and improvements in the extraction and analytical procedures are desirable.

The present study aimed to develop and validate a new and sensitive extraction and analytical protocol for urine samples based on lyophilization, extraction with isopropanol and LC-MS/MS without the need to employ SPE. The performance of the validated method was further demonstrated in a small study with pregnant women by determining the 8-OHdG concentration in several urine samples and the placenta collected at delivery. The correlation between 8-OHdG content in urine and placenta was also investigated, as well as the association between concentrations and data gathered from questionnaires and medical records of participants.

2. Materials and methods

2.1. Study design

This feasibility study was conducted under the umbrella of the Spanish "Childhood and Environment" (INMA) study. During pregnancy, repeated urine samples were collected, and additional samples (urine and placental tissue) were collected at delivery. For this purpose, 130 pregnant women were recruited at the University Hospital of Granada (Southern Spain), between 2013 and 2015. The basic information related to birth outcomes such as child sex, weeks of gestation, birth weight and child length were collected. All participants were aware of the objectives of the study and provided informed consent to access relevant data from hospital records, including socio-demographic and clinical information. The study followed the principles of the declaration of Helsinki and was approved by the Biomedical Research Ethics Committee of Granada.

2.2. Collection of urine and placental samples

Pregnant women (n = 130 in total) provided one urine sample at routine visits to the hospital in each trimester (week 12, 20 and 32 of pregnancy), plus one additional urine sample around the time of delivery (collected during their stay at the hospital). Thus, up to 4 maternal urine samples were collected – the 1st, 2nd, and 3rd trimester and one sample around 24h-post-delivery. All urine samples were collected in polypropylene tubes, aliquoted and immediately stored at −80 °C. The placenta was also collected at the time of delivery, weighted without fetal membranes/maternal decidua, and immediately stored at −80 °C. The frozen collected placentas were then aliquoted into small pieces including maternal and fetal sides as well as central and peripheral parts. In the present work, a subset of samples was used from those mothers who provided both urine samples and placenta. Thus, 26 placental samples and the matching 85 prenatal urine samples were randomly selected from the initial feasibility study, and were sent to Masaryk University, Brno (MU) for analysis. Samples were transported on dry ice and stored at −80 °C until extraction and analysis that was done during summer 2018.

2.3. Chemicals and reagents

Standard 8-hydroxy-2′-deoxyguanosine (8-OHdG, 98%) and 2′-deoxyguanosine monohydrate (2 dG, 99–100%) were obtained from Sigma-Aldrich (Merck). Isotopically labelled internal standard 15N5-8-hydroxy-2′-deoxyguanosine (>95%) was obtained from Cambridge Isotope Laboratories. MS grade acetonitrile, isopropanol and formic acid (FA, 99%) were purchased from BIOSOLVE BV (Netherlands).

2.4. Urine sample treatment and extraction procedure

For 8-OHdG assessment, the urine samples were thawed at room temperature and homogenized by vortex. The 10 μL of internal standard 15N5-8-OHdG (15N5-8-hydroxy-2′-deoxyguanosine; 1 μg/mL in 0.1% v/v formic acid) was added to 0.5 mL of each urine sample or to calibration solutions of 8-hydroxy-2′-deoxyguanosine (0, 0.05, 0.5, 5, 50 μg/L in 0.1% v/v formic acid) in 2 mL vials. Samples were well vortexed, then gradually frozen at −20 °C and −80 °C overnight and freeze-dried for 24h using freeze-drier (L10-55P, Gregor Instruments). After lyophilization, dry urine and calibration samples were re-suspended in 0.5 mL of isopropanol and extracted in cooled ultrasonic bath for 15 min. Other solvents such as acetonitrile and methanol were also tested for the extraction (data not shown) but the highest recovery was observed for isopropanol. Insoluble material was removed by centrifugation (12 000×*g*, 10 °C, 10 min), 350 μL of supernatants were transferred into a glass vial and evaporated under a stream of nitrogen to dryness (approx. 15 min). Dried extracts were re-dissolved in 250 μL of 0.1% v/v formic acid using cooled ultrasonic bath and vortex. Possible residual particles were removed by centrifugation using microspin filters (0.2 μm; cellulose acetate; Fisher Scientific; 10 000×*g*, 3 min, 10 °C). Filtrates in glass inserts in vials were stored at −20 °C under a nitrogen atmosphere until the analyses of 8-OHdG by LC-MS/MS.

For creatinine analyses, extraction of urine was done according to the previously published study (Dereziński et al., 2016). Analyses used LC-MS/MS method after acidification and dilution of urine in D3-creatitine internal standard solution.

2.5. Extraction of placenta for 8-OHdG analysis

Extraction of placenta was based on the previously published

methods (Bláhová et al., 2020). Briefly, DNA was extracted from the tissue by using a DNA isolation kit (DNeasy Blood & Tissue Kits, QIAGEN) according to the manufacturer's instructions in two replicates. Enzymatic digestion of isolated DNA was performed by 8-OHdG Assay Preparation Reagent Set (WAKO). To clean the extracts, ultrafiltration (Vivaspin 10 000 MWCO, PES) was used, and the final extract was acidified by formic acid and transferred into glass vials for analyses of 8-OHdG. For analyses of 2 dG, the samples were further diluted 200-fold. All samples were stored at −20 °C under a nitrogen atmosphere until analyses by LC-MS/MS.

2.6. Chromatography and mass spectrometry conditions

Analyses of 8-OHdG were performed with Waters Acquity LC chromatograph (Waters, Manchester, U.K.) consisting of a vacuum degasser, a binary pump, a thermostatted autosampler, and a column compartment. The column used was an Acquity UPLC BEH C18 (1.7 μm) (Waters) 100 × 2.1 mm equipped with a guard pre-column kept at 25 °C. Detection was performed on a Xevo TQ-S quadrupole mass spectrometer (Waters Manchester, U.K.) equipped with electrospray ionization. Analytes, after ESI ionization were detected in positive ion mode using tandem mass spectrometry with multiple reaction monitoring (MRM). Data were processed by MassLynxTM software (Manchester, U.K.).

The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile acidified by 0.1% formic acid (B). The binary pump gradient was linear (5% B at 0–1 min, then increases from 5% B at 1 min to 80% B at 5 min and 80% B was kept for 2 min) followed by 4 min column equilibration to the initial conditions (5% B). The flow rate was 0.2 mL/min, and 10 μL of individual sample from the thermostatted autosampler (10 °C) was injected for the analyses. The ionization parameters were as follows: capillary voltage, 2.5 kV; the source temperature and the desolvation temperature, 150 and 750 °C, respectively; the cone gas flow, 150 (L/h); the cone voltages, 30 V; the desolvation gas flow, 750 (L/h); and the collision gas flow, 0.15 mL/min. The following *m/z* transitions of 8-OHdG were monitored: *m/z* 284.1 > 168.1 (quantifier; collision energy 14V), *m/z* 284.1 > 140.1 (qualifier; collision energy 28V). An average ratio of quantifier (*m/z* 168.1) ions to qualifier (*m/z* 140.1) was stable in standard solution as well as during analyses of urine and placenta extracts and did not exceed 15% of relative standard deviation (RSD).

The *m/z* transition for internal standard 15N5-labled 8-OHdG were also monitored: *m/z* 289.1 > 173.1 (quantifier; collision energy 14V) and *m/z* 289.1 > 145.1 (qualifier; collision energy 28V). For analyses of a parent nucleoside 2′-deoxyguanosine monohydrate (2 dG), extracts of urine were 200-fold diluted, and 2 dG was detected by transition: *m/z* 268.0 > 152.0 (quantifier; 18V) and *m/z* 268.0 > 135.0 (qualifier; 37V). Concentrations of extracted 8-OHdG were corrected for the content of internal standard and expressed as microgram per litre of urine as well as microgram per gram of creatinine. For placenta, the content of 8-OHdG was normalized to the content of 2 dG and expressed in different units to allow for comparison with the literature – i.e. the molar ratio 8-OHdG per 10^5 2 dG, ng 8-OHdG per gram of placental tissue and ng 8-OHdG per mg of DNA (Bláhová et al., 2020).

2.7. Quality control and data analysis

Quality assurance and quality control samples, including blanks, spiked samples (5.0 ng/mL in 0.1% formic acid) and two urine samples (in house reference material with known lower and higher levels of 8-OHdG) were repeatedly extracted and included in the analysis of samples. Quality control samples (repeatedly extracted samples 5.0 ng/mL in 0.1% formic acid) were analyzed after every 20 urine extracts and found repeatability was acceptable (RSD ≤15%).

Statistical analyses and visualization of the data were performed using Statistica (StatSoft, Inc., Tulsa, OK, USA) and the R programming language, version 4.1.1 (R Core Team, 2021). To achieve a normal distribution and avoid negative values in graphical presentations, the data were log (x+1) transformed using natural logarithm. The paired *t*-test was used to compare urinary mean concentrations of 8-OHdG from different stages of pregnancy. Pearson's correlation coefficient was used to determine correlations between urine and placenta tissue concentrations of 8-OHdG. Due to a small sample size, the non-parametric Mann-Whitney *U* test was used when comparing 8-OHdG in groups with different sociodemographic characteristics or birth-related information.

3. Results

3.1. Validation of chemical analysis

The novel method used for extraction and analyses by LC-MS/MS in urine samples was validated according to the recommendations of Booth and Simon (2016).

Specificity, i.e. the ability of the method to measure the analyte in the presence of complex matrix, was evaluated by analysing the blanks. No peak was detected at the retention time of 8-OHdG. Quantifier/qualifier ratios (mean ± standard deviation) in different samples were as follows - standard solution 5.1 ± 0.6, urine samples 4.7 ± 0.7, placenta 4.8 ± 0.6. Overall, RSD of ratios did not exceed 15%. The precision of the method was evaluated by carrying out determinations of 8-OHdG replicates in three different urine extracts prepared from in house reference material (no certified reference material is currently commercially available (Topic, 2014)). The coefficient of variation was between 3.9–5.2% and 6.8–9.6% for intra-day (n = 5) and inter-day (n = 5) variability tests, respectively. Data are presented in Supplementary Table S1.

The instrumental limit of detection (LOD) and limit of quantification (LOQ) for 8-OHdG were determined for spiked concentrations in acidified water using a signal to noise ratio criteria (S/N). The limit of detection, 0.01 μg/L, was assessed as the concentration that yielded S/N ratio >3; and the limit of quantification, 0.05 μg/L, as the lowest amount of analyte in the sample that can be quantified, S/N > 10. A precision was further characterized at the LOQ level by injecting a standard of 8-OHdG 6-times (n = 6) into LC-MS/MS. The calculated coefficients of variation were 8.4% for determined concentration and 3.3% for the retention time repeatability. Linearity was assessed by repeated analysis of the calibration solutions (four points in two replicates) independently prepared, extracted and analyzed on 3 different days. The coefficients of variance (%) for 8-OHdG of the 3 independent calibration solutions did not exceed 24%. Calibration curve of stock solutions at four concentration levels from LOQ to 50 μg/L (0.05, 0.5, 5, 50 μg/L) were plotted between the responses of peak versus analyte concentrations corrected for responses of internal standard (10 μg/L of 15N5-8-OHdG in each standard concentration). This resulted in $r^2 > 0.999$.

The recoveries of the complete extraction and analytical procedure for 8-OHdG were 101 ± 5%, 98 ± 7% and 100 ± 5% for standard solutions of concentrations 0.5, 5 and 50 μg/L, respectively (five replicated experiments, n = 5). Standard solutions were always processed the same way as the urine samples (Supplementary Table S2).

Validation of the recovery was further performed by extracting three different urine samples from healthy volunteers with known concentrations of 8-OHdG (2.13 ± 0.11; 6.35 ± 0.31 and 10.07 ± 0.39 μg/L) after external spiking of two selected 8-OHdG concentrations (5 and 50 μg/L as low and high level of 8-OHdG, respectively, n = 2 for each concentration). The recovery for two spiked concentrations ranged from 84 to 106% (Supplementary Table S3). Lower spike of 8-OHdG (0.5 μg/L) could not be tested because of unavailability of sufficient amount of urine sample with comparable, i.e. low 8-OHdG concentration.

The stability of 8-OHdG in solution of 0.1% FA was determined by analysing reference standard in vials kept at −20 °C under nitrogen atmosphere. Tested solutions were stable for at least 1 month. The example of LC-MS/MS chromatogram obtained in multiple reaction

monitoring mode (MRM) for 8-OHdG and 15N5-8-OHdG in urine extract is shown in Supplementary Fig. S1.

3.2. Application of the validated extraction and analytical method

Validation of the new method was performed on urine samples from pregnant women collected throughout their pregnancy. Individual results are presented in Supplementary Table S4. The unadjusted median concentration of 8-OHdG in all urine samples (n = 85) was 2.18 μg/L (range 0.33–7.79). The median of concentration adjusted to creatinine was 4.48 μg/g creatinine (range 1.04–13.12) (Fig. 1). The median urinary concentration of creatinine was 457 mg/L (range 92–1270) (Supplementary Fig. S2). No significant differences in the concentrations of creatinine during the course of pregnancy were observed. The concentration of 8-OHdG significantly decreased ($p < 0.05$) in post-delivery urine samples (based on both unadjusted and creatinine adjusted values) when compared to urine samples obtain at the beginning of pregnancy (1st trimester). For unadjusted values, 8-OHdG (μg/L of urine) systematically decreased ($p < 0.05$) during pregnancy, comparing the 1st trimester with the 3rd trimester, and with urine at time of delivery (Fig. 1). Because of the small number of samples available, results for the urine collected during the 2nd trimester were included only in the overall statistics and were not analyzed in detail (Supplementary Table S4).

The concentration of 8-OHdG in placental tissue was also assessed, with a median concentration of 3.88 ng/mg of DNA (range: 1.43–7.34), which corresponded to 1.3 ng/g of tissue (0.63–3.62) or ratio of 1.5 8-OHdG per 10^5 of 2 dG (0.47–2.52) (Fig. 2).

As a next step, we studied correlations between 8-OHdG in urine at different pregnancy stages as well as correlations between urine and placenta concentrations. As expected, the correlations were observed for inter-correlated variables (concentrations in the same samples expressed in different units; results not shown). Interestingly, a significant correlation was observed between unadjusted urine concentration of 8-OHdG (μg/L urine) from the 3rd trimester with the concentration of 8-OHdG in placenta expressed as a ratio per 2 dG (Pearson's correlation coefficient = 0.4, $p < 0.05$) (Table 1 and Supplementary Fig. 3). The correlations for creatine-adjusted concentrations were not statistically significant.

Additional statistical analyses were performed to investigate associations between 8-OHdG and parameters from questionnaires and clinical records (placenta weight, head circumference, child sex, mother and child BMI, gestation age, mother age at delivery, tobacco habit, education level; for full data see Supplementary Table S4). Considering a small sample size, the results must be interpreted with caution. Nevertheless, the analysis showed some interesting associations (Fig. 3). Thus, mothers that delivered boys had significantly higher urinary 8-OHdG concentrations in the 1st trimester ($p < 0.01$). Slightly elevated urinary 8-OHdG concentrations at the time of delivery were associated with clinical signals (Table S4) during pregnancy (any type of clinical disease recorded during pregnancy; $p < 0.05$). For newborn sex, the differences remained robust ($p < 0.001$) also when controlling for potential confounding factors (mother's age, BMI, education, tobacco use during pregnancy, parity) (Fig. 3).

4. Discussion

The main objective of the present study was to develop and evaluate a simple, sensitive, and reproducible extraction method for determination of 8-OHdG, a widely used oxidative stress biomarker, in urine samples. Various analytical procedures have been previously described including simple dilution of urine samples followed by injection to LC-MS/MS (Al-Saleh et al., 2017; Qian et al., 2021). However, the injection of unprocessed urine has major limitations, namely for larger batches of samples because this approach severely contaminates the ion source in LC-MS/MS, causes signal suppression, leads to a shorter column life and results in insufficiently high detection limits (LOQ 0.2–1 ng/mL; Topic, 2014).

The main broadly used technique for analyte pre-concentration and removing of potential interferences is SPE, most commonly based on reversed phase or cation exchange cartridges (Fan et al., 2012; Potdar et al., 2009; Ravanat et al., 1998; Sabatini et al., 2004). For handling larger numbers of urine samples, automated on-line SPE instruments can be coupled to LC-MS/MS (Hu et al., 2010).

Our extraction method based on lyophilization showed low limits of detection and quantification (0.01 and 0.05 μg/L, respectively), well comparable or lower than limits previously reported for other extraction procedures (Topic, 2014). Actually, the first step, lyophilization, improves stability of urine samples for long-term storage and for easier processing or transport. Interestingly, the extraction methods based on similar principles - such as QuEChERS - are broadly used in

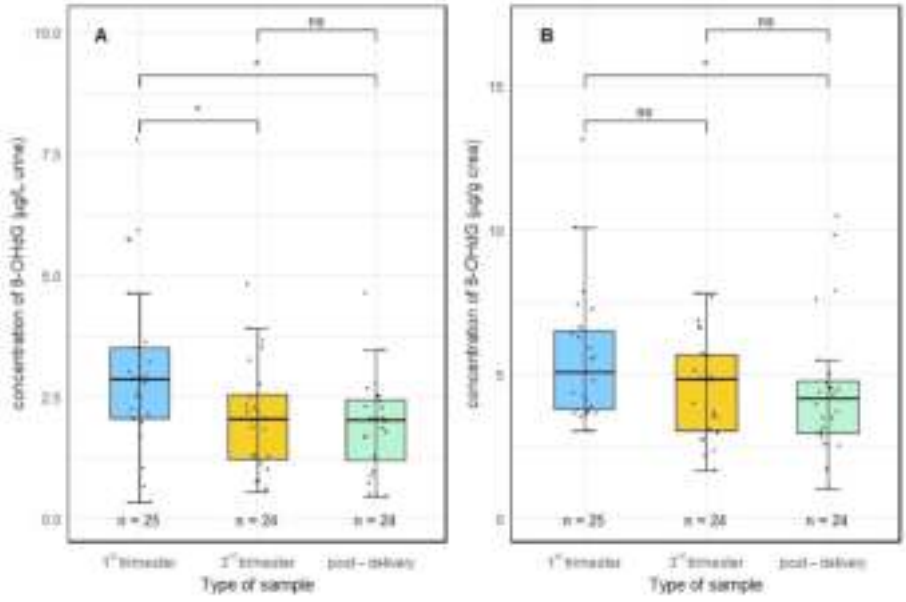

Fig. 1. Concentrations of 8-OHdG in urine samples (A - unadjusted urine concentrations, B – creatinine-adjusted concentrations) during the 1st and 3rd trimesters and at time of delivery (ns - statistically insignificant; *significant at $p \leq 0.05$ by paired *t*-test).

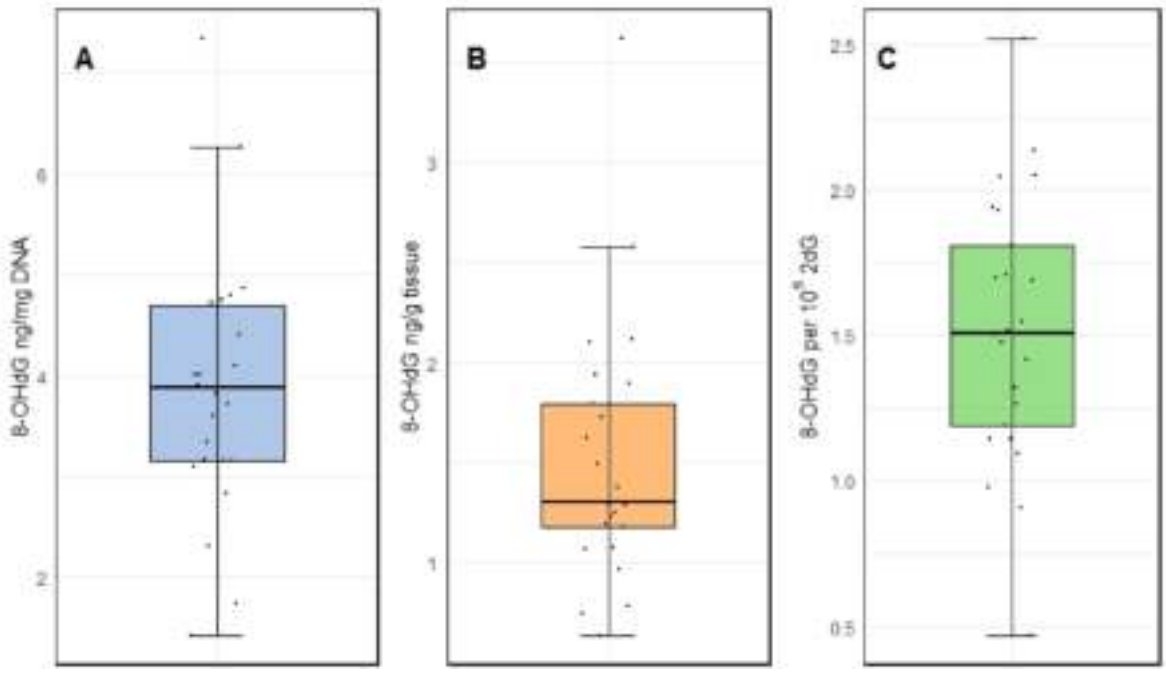

Fig. 2. Concentration of 8-OHdG in individual samples of placenta collected at time of delivery. Values expressed in different units – panel A: ng 8-OHdG/mg of DNA, panel B: ng 8-OHdG/g of tissue, panel C: molar ratio of 8-OHdG per 10^5 2 dG.

Table 1
Pearson correlation coefficients (r) and p-values among concentrations of 8-OHdG in urine and placenta.

Urine 8-OHdG	Placenta 8-OHdG					
	per 10^5 2 dG		ng/mg DNA		ng/g tissue	
	r	p-value	r	p-value	r	p-value
1st trimester (μg/L)	−0.11	0.593	−0.23	0.262	−0.18	0.393
3rd trimester (μg/L)	**0.4**	**0.047**	0.15	0.456	0.22	0.297
post-delivery (μg/L)	−0.17	0.403	−0.18	0.433	−0.17	0.415
1st trimester (μg/g creat.)	−0.1	0.625	−0.24	0.174	−0.03	0.879
3rd trimester (μg/g creat.)	0.38	0.059	0.27	0.176	0.32	0.119
post-delivery (μg/g creat.)	−0.02	0.91	−0.04	0.878	0.02	0.957

Significant correlation ($p \leq 0.05$) is highlighted in **bold**.

environmental analysis of e.g. pesticides in solid matrices such as soils or sediments (Anastassiades et al., 2003). However, to our knowledge, this is the first time that the suitability of the approach is shown for extraction and analysis of human biological liquid material such as lyophilized urine. Our additional studies indicate outstanding performance of the method also for other biomarkers and analytes such nucleotides and nucleosides and their methylated and hydroxylated variants (Janoš et al., 2023), benzotriazoles, benzothiazoles, and various other UV stabilizers, tyrosine derivatives etc. The present study thus suggests general applicability of the new method for many other analytes or biomarkers in urine or blood samples after lyophilization.

This developed method was used in a small cohort study of pregnant women to investigate 8-OHdG levels, their profiles during pregnancy and eventual associations with some determinants. The median concentration of 8-OHdG in all urine samples of participating pregnant women was 2.18 μg/L urine (or 4.48 μg/g of creatinine). These values are in line with some previous studies, where urinary levels of 8-OHdG were analyzed using the same detection technique LC-MS/MS. For example, interquartile range 1.96–3.67 pmol 8-oxodG/μmol creatinine (4.9–9.2 μg/g of creatinine) at 12 weeks of gestation was reported in a study from the United Kingdom (Potdar et al., 2009). Slightly higher concentrations of 8-OHdG with median 9.96 μg/L were observed in a study of pregnant women from Guangzhou, South China (Zhang et al., 2021). Approximately ten times higher median 8-OHdG concentrations were observed in some studies using ELISA methods, which has however been criticized for low specificity (Hu et al., 2004). For example, following values were reported: gravity adjusted median 130 μg/L in U. S. cohort (Ferguson et al., 2015), median 122 μg/L in the study from Northern Puerto Rico (Ferguson et al., 2014), geometric mean 122.6 ng/mL in a study of pregnant women from Boston, USA (Kim et al., 2019) and median in the birth cohort from China 315.2 μg 8-OHdG/g of creatinine (Pan et al., 2022). On the other hand, another study with pregnant women based on ELISA reported levels of 8-OHdG only slightly higher than those observed in the present paper - median 10.99 μg/g of creatinine (Al-Saleh et al., 2013) Similar median value (4.48 μg 8-OHdG/g of creatinine) based on ELISA was reported in a another study with non-smoker women (Lu et al., 2007). As it is apparent, using different techniques for quantification of urinary 8-OHdG makes comparison between cohorts difficult, and harmonization of measurement methodologies in human studies is needed.

Focusing on the differences in urine 8-OHdG in individual trimesters, the concentrations significantly decreased during the pregnancy and in post-delivery urine samples with the highest levels observed in the 1st trimester (Fig. 1). The observed increased production of ROS in urine of pregnant women is in line with other studies (Jauniaux et al., 2000; Myatt and Cui, 2004). It was also shown that the increased oxidative stress in the 1st trimester (measured as 8-OHdG in urine) can be associated with a risk of small gestational age (SGA) (Potdar et al., 2009). However, increasing amount of urination as well as variability of creatinine in different trimesters should be considered when assessing urinary biomarkers in cohorts of pregnant women (Lee et al., 2021).

Higher ROS production during pregnancy can be linked to higher oxygen demand due to increased metabolic rate. The median concentration of 8-OHdG in placenta tissue of participating pregnant women was 1.5 of 8-OHdG/10^5 2 dG, which is close to median reported in an older study investigating DNA adducts in placenta - control pregnant women median 0.86 of 8-OHdG per 10^5 2 dG (Daube et al., 1997).

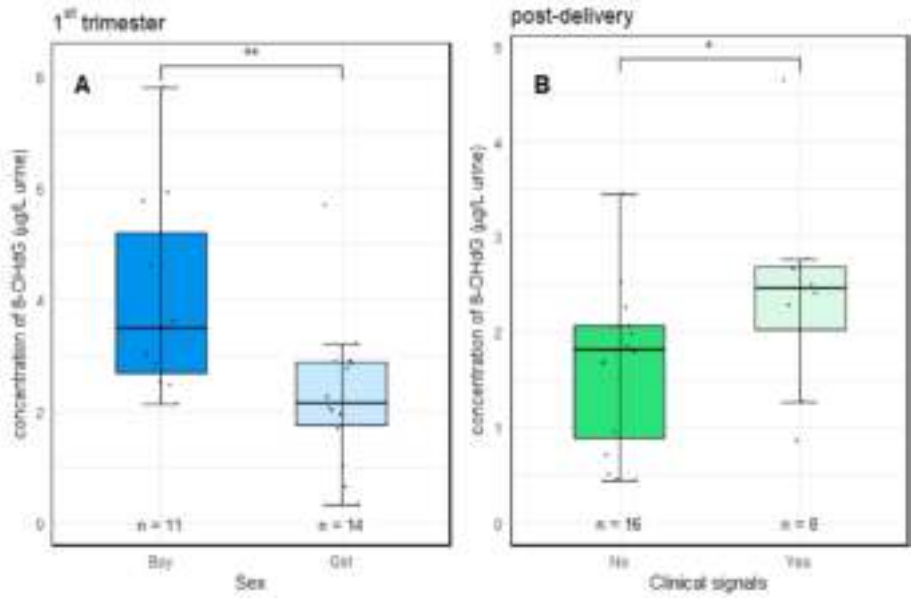

Fig. 3. Significant differences in concentrations of 8-OHdG (µg/L urine) between different sub-groups of samples. A – higher 8-OHdG concentrations in the 1st trimester urine at mothers that delivered boys; B – higher 8-OHdG concentrations (urine collected at time of delivery) of mothers with recorded clinical signals (any type of clinical records during pregnancy pooled) (* significant at $p \leq 0.05$; ** significant at $p \leq 0.01$ by Mann–Whitney *U* test).

The oxidative status of placenta was previously discussed as a marker of complicated pregnancy or adverse child health later in life (Al-Saleh et al., 2013; Dennery, 2010). In agreement, the present study showed higher 8-OHdG in urine of women with reported clinical signs (Fig. 3), and also correlations between 8-OHdG in urine at the end of pregnancy (the 3rd trimester) and 8-OHdG in placenta (Table 1). Overall, this indicate that urine 8-OHdG may be a good non-invasive early warning biomarker of oxidative damage in human tissues (such as placenta).

With regard to sampling of urine, 24h composite urine samples are considered to be the best option but this is not practical and samples are only rarely available in cohort studies (Graille et al., 2020). Correspondingly, it is recommended to adjust concentrations in commonly collected spot samples to creatinine, osmolality or specific gravity as a surrogate for 24-h collection. In the present study, the urinary concentrations of creatinine in pregnant women ranged between 0.1 and 1.3 g/L, which corresponds to values known to be associated with pregnancy (WHO, 1996). No significant changes in concentrations of creatinine were observed between urine collected in different time points (the 1st, 3rd trimester, at time of delivery). As expected, the correlations were observed for inter-correlated variables, i.e. concentrations in the same samples expressed in different units (unadjusted (µg 8-OHdG/L) or creatinine-adjusted µg 8-OHdG/g creatinine). Although some studies with healthy volunteers (e.g. Martinez-Moral and Kannan, 2019) showed that normalization to creatinine might decrease individual variability, we decided to report both unadjusted and adjusted values for 8-OHdG biomarker as it allows to compare the results with different existing studies.

Despite of a small number of participants, our study might indicate some novel associations related to oxidative stress biomarker. While most of the relationships between 8-OHdG and available questionnaire or clinical parameters were non-significant (including for example no effect of tobacco habits), there was an association with newborn sex. Levels of 8-OHdG in urine in 1st trimester were higher in mothers that gave birth to boys ($p < 0.01$). Additionally, higher urine 8-OHdG concentrations at the time of delivery were observed in mothers with clinical signals (any type of clinical disease recorded during pregnancy; $p < 0.05$; Fig. 3). These findings are in line with few previous studies that mentioned increase of oxidative stress in placenta in relationship to various characteristics such as maternal obesity, mode of delivery and medications, newborn sex or smoking habits (Burton et al., 2014; Daube et al., 1997; Hung et al., 2011).

There are some limitations of the present study. First, small sample size decreased statistical power, and the associations should be interpreted with caution. Second, reported correlations and/or differences based on parameters from questionnaires and clinical records were significant only in creatinine non-adjusted urinary concentrations. Thus, the influence of urine dilution could not be excluded. Future studies will help to validate the potential predictive power of urinary 8-OHdG concentrations as a non-invasive biomarker of oxidative stress-related diseases in pregnant women.

5. Conclusions

A sensitive extraction method for 8-OHdG in urine samples was developed. The method based on lyophilization does not require demanding solid phase extraction pre-cleaning, has a very low limit of detection of 0.01 µg/L, and seems generally applicable for many other analytes or biomarkers in urine or blood samples. The method was successfully applied for 8-OHdG analysis in urine of pregnant women, and the results indicated higher levels of oxidative stress during early pregnancy (1st trimester) with a decrease at the 3rd trimester and post-delivery urine samples. Despite of a small size, which is a major limitation of the present study, statistical analyses revealed elevated 1st trimester urinary 8-OHdG concentrations (non-adjusted) in mothers that delivered boys (compared to those who delivered girls) and increased 8-OHdG concentrations (non-adjusted) in the urine of mothers with clinical records (any type of clinical disease during pregnancy). Our findings further showed that 3rd trimester urinary 8-OHdG concentrations (non-adjusted) may correlate with oxidative stress in placental tissue.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114175.

References

Al-Saleh, I., Al-Rouqi, R., Elkhatib, R., Abduljabbar, M., Al-Rajudi, T., 2017. Risk assessment of environmental exposure to heavy metals in mothers and their respective infants. Int. J. Hyg Environ. Health 220, 1252–1278. https://doi.org/10.1016/j.ijheh.2017.07.010.

Al-Saleh, I., Alsabbahen, A., Shinwari, N., Billedo, G., Mashhour, A., Al-Sarraj, Y., Mohamed, G.E.D., Rabbah, A., 2013. Polycyclic aromatic hydrocarbons (PAHs) as determinants of various anthropometric measures of birth outcome. Sci. Total Environ. 444, 565–578. https://doi.org/10.1016/j.scitotenv.2012.12.021.

Anastassiades, M., Lehotay, S.J., Štajnbaher, D., Schenck, F.J., 2003. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for. J. AOAC Int. 86, 412–431.

Bláhová, L., Nováková, Z., Večeřa, Z., Vrlíková, L., Dočekal, B., Dumková, J., Křůmal, K., Mikuška, P., Buchtová, M., Hampl, A., Hilscherová, K., Bláha, L., 2020. The effects of nano-sized PbO on biomarkers of membrane disruption and DNA damage in a sub-chronic inhalation study on mice. Nanotoxicology 14, 214–231. https://doi.org/10.1080/17435390.2019.1685696.

Booth, B.P., Simon, W.C., 2016. Analytical method validation. New Drug Dev. Regul. Paradig. Clin. Pharmacol. Biopharm. 138–159. https://doi.org/10.1201/9780203026427-15.

Burton, G.J., Sebire, N.J., Myatt, L., Tannetta, D., Wang, Y.L., Sadovsky, Y., Staff, A.C., Redman, C.W., 2014. Optimising sample collection for placental research. Placenta 35, 9–22. https://doi.org/10.1016/j.placenta.2013.11.005.

Chen, Q., Hu, Y., Fang, Z., Ye, M., Li, J., Zhang, S., Yuan, Y., Guo, C., 2020. Elevated levels of oxidative nucleic acid modification markers in urine from gastric cancer patients: quantitative analysis by ultra performance liquid chromatography-tandem mass spectrometry. Front. Chem. 8, 1–7. https://doi.org/10.3389/fchem.2020.606495.

Daube, H., Scherer, G., Riedel, K., Ruppert, T., Tricker, a R., Rosenbaum, P., Adlkofer, F., 1997. DNA adducts in human placenta in relation to tobacco smoke exposure and plasma antioxidant status. J. Cancer Res. Clin. Oncol. 123, 141–151.

Dennery, P.A., 2010. Oxidative stress in development: nature or nurture? Free Radic. Biol. Med. 49, 1147–1151. https://doi.org/10.1016/j.freeradbiomed.2010.07.011.

Dereziński, P., Klupczyńska, A., Sawicki, W., Kokot, Z.J., 2016. Creatinine determination in urine by liquid chromatography-electrospray ionization-tandem mass spectrometry method. Acta Pol. Pharm. - Drug Res. 73, 303–313.

Fan, R., Wang, D., Mao, C., Ou, S., Lian, Z., Huang, S., Lin, Q., Ding, R., She, J., 2012. Preliminary study of children's exposure to PAHs and its association with 8-hydroxy-2'-deoxyguanosine in Guangzhou, China. Environ. Int. 42, 53–58. https://doi.org/10.1016/j.envint.2011.03.021.

Ferguson, K.K., McElrath, T.F., Chen, Y.H., Mukherjee, B., Meeker, J.D., 2015. Urinary phthalate metabolites and biomarkers of oxidative stress in pregnant women: a repeated measures analysis. Environ. Health Perspect. 123, 217–222. https://doi.org/10.1289/ehp.1307996.

Ferguson, K.K., Cantonwine, D.E., Rivera-Gonzalez, L.O., Loch-Caruso, R., Mukherjee, B., Anzalota Del Toro, L.V., Jimenez-Velez, B., Calafat, A.M., Ye, X., Alshawabkeh, A.N., Cordero, J.F., Meeker, J.D., 2014. Urinary phthalate metabolite associations with biomarkers of inflammation and oxidative stress across pregnancy in Puerto Rico. Environ. Sci. Technol. 48, 7018–7025.

Fleming, A.M., Alshykhly, O., Zhu, J., Muller, J.G., Burrows, C.J., 2015. Rates of chemical cleavage of DNA and RNA oligomers containing guanine oxidation products. Chem. Res. Toxicol. 28, 1292–1300. https://doi.org/10.1021/acs.chemrestox.5b00096.

Garde, A.H., Hansen, Å.M., Kristiansen, J., Knudsen, L.E., 2004. Comparison of uncertainties related to standardization of urine samples with volume and creatinine concentration. Ann. Occup. Hyg. 48, 171–179. https://doi.org/10.1093/annhyg/meh019.

Graille, M., Wild, P., Sauvain, J.J., Hemmendinger, M., Canu, I.G., Hopf, N.B., 2020. Urinary 8-OHDG as a biomarker for oxidative stress: a systematic literature review and meta-analysis. Int. J. Mol. Sci. 21, 1–24. https://doi.org/10.3390/ijms21113743.

Guo, C., Ding, P., Xie, C., Ye, C., Ye, M., Pan, C., Cao, X., Zhang, S., Zheng, S., 2017. Potential application of the oxidative nucleic acid damage biomarkers in detection of diseases. Oncotarget 8, 75767–75777. https://doi.org/10.18632/oncotarget.20801.

Halliwell, B., Gutteridge, J.M., 1990. Free radicals in biology and medicine. In: second ed. Trends in Biochemical Sciences. Elsevier. https://doi.org/10.1016/0968-0004(90)90254-9.

Hu, C.W., Chao, M.R., Sie, C.H., 2010. Urinary analysis of 8-oxo-7,8-dihydroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine by isotope-dilution LC-MS/MS with automated solid-phase extraction: study of 8-oxo-7,8-dihydroguanine stability. Free Radic. Biol. Med. 48, 89–97. https://doi.org/10.1016/j.freeradbiomed.2009.10.029.

Hu, C.W., Wu, M.T., Chao, M.R., Pan, C.H., Wang, C.J., Swenberg, J.A., Wu, K.Y., 2004. Comparison of analyses of urinary 8-hydroxy-2′-deoxyguanosine by isotope-dilution liquid chromatography with electrospray tandem mass spectrometry and by enzyme-linked immunosorbent assay. Rapid Commun. Mass Spectrom. 18, 505–510. https://doi.org/10.1002/rcm.1367.

Hung, T.H., Chen, S.F., Hsieh, T.T.A., Lo, L.M., Li, M.J., Yeh, Y.L., 2011. The associations between labor and delivery mode and maternal and placental oxidative stress. Reprod. Toxicol. 31, 144–150. https://doi.org/10.1016/j.reprotox.2010.11.009.

Il'yasova, D., Scarbrough, P., Spasojevic, I., 2012. Urinary biomarkers of oxidative status. Clin. Chim. Acta 413, 1446–1453. https://doi.org/10.1016/j.cca.2012.06.012.

Janoš, T., Ottenbros, I., Bláhová, L., Šenk, P., Šulc, L., Pálešová, N., Sheardová, J., Vlaanderen, J., Čupr, P., 2023. Effects of pesticide exposure on oxidative stress and DNA methylation urinary biomarkers in Czech adults and children from the CELSPAC-SPECIMEn cohort. Environ. Res. 222, 115368 https://doi.org/10.1016/j.envres.2023.115368.

Jauniaux, E., Watson, A.L., Hempstock, J., Bao, Y.P., Skepper, J.N., Burton, G.J., 2000. Onset of maternal arterial blood flow and placental oxidative stress: a possible factor in human early pregnancy failure. Am. J. Pathol. 157, 2111–2122. https://doi.org/10.1016/S0002-9440(10)64849-3.

Kim, S.S., Meeker, J.D., Keil, A.P., Aung, M.T., Bommarito, P.A., Cantonwine, D.E., McElrath, T.F., Ferguson, K.K., 2019. Exposure to 17 trace metals in pregnancy and associations with urinary oxidative stress biomarkers. Environ. Res. 179, 108854 https://doi.org/10.1016/j.envres.2019.108854.

Lee, G., Kim, S., Park, H., Lee, J., Lee, J.P., Kho, Y., Choi, G., Park, J., Worakhunpiset, S., Moon, H.B., Choi, K., 2021. Variability of urinary creatinine, specific gravity, and osmolality over the course of pregnancy: implications in exposure assessment among pregnant women. Environ. Res. 198 https://doi.org/10.1016/j.envres.2020.110473.

Li, Y.S., Kawasaki, Y., Watanabe, S., Ootsuyama, Y., Kasai, H., Kawai, K., 2021. Diurnal and day-to-day variation of urinary oxidative stress marker 8-hydroxy-2'-deoxyguanosine. J. Clin. Biochem. Nutr. 68, 18–22. https://doi.org/10.3164/JCBN.19-105.

Lu, C.Y., Ma, Y.C., Lin, J.M., Chuang, C.Y., Sung, F.C., 2007. Oxidative DNA damage estimated by urinary 8-hydroxydeoxyguanosine and indoor air pollution among non-smoking office employees. Environ. Res. 103, 331–337. https://doi.org/10.1016/j.envres.2006.08.009.

Lu, S., Ren, L., Fang, J., Ji, J., Liu, G., Zhang, J., Zhang, H., Luo, R., Lin, K., Fan, R., 2016. Trace elements are associated with urinary 8-hydroxy-2′-deoxyguanosine level: a case study of college students in Guangzhou, China. Environ. Sci. Pollut. Res. 23, 8484–8491. https://doi.org/10.1007/s11356-016-6104-8.

Marrocco, I., Altieri, F., Peluso, I., 2017. Measurement and clinical significance of biomarkers of oxidative stress in humans. Oxid. Med. Cell. Longev. https://doi.org/10.1155/2017/6501046, 2017.

Martinez-Moral, M.P., Kannan, K., 2019. How stable is oxidative stress level? An observational study of intra- and inter-individual variability in urinary oxidative stress biomarkers of DNA, proteins, and lipids in healthy individuals. Environ. Int. 123, 382–389. https://doi.org/10.1016/j.envint.2018.12.009.

Myatt, L., Cui, X., 2004. Oxidative stress in the placenta. Histochem. Cell Biol. 122, 369–382. https://doi.org/10.1007/s00418-004-0677-x.

Pan, C., Yu, J., Yao, Q., Lin, N., Lu, Z., Zhang, Y., Zhao, S., Wang, Z., Lei, X., Tian, Y., Gao, Y., 2022. Prenatal neonicotinoid insecticides Exposure, oxidative Stress, and birth outcomes. Environ. Int. 163, 107180.

Potdar, N., Singh, R., Mistry, V., Evans, M.D., Farmer, P.B., Konje, J.C., Cooke, M.S., 2009. First-trimester increase in oxidative stress and risk of small-for-gestational-age fetus. BJOG An Int. J. Obstet. Gynaecol. 116, 637–642. https://doi.org/10.1111/j.1471-0528.2008.02096.x.

Qian, X., Wan, Y., Wang, A., Xia, W., Yang, Z., He, Z., Xu, S., 2021. Urinary metabolites of multiple volatile organic compounds among general population in Wuhan, central China: inter-day reproducibility, seasonal difference, and their associations with oxidative stress biomarkers. Environ. Pollut. 289, 117913 https://doi.org/10.1016/j.envpol.2021.117913.

R Core Team, 2021. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL. https://www.R-project.org/.

Ravanat, J.L., Duretz, B., Guiller, A., Douki, T., Cadet, J., 1998. Isotope dilution high-performance liquid chromatography-electrospray tandem mass spectrometry assay for the measurement of 8-oxo-7,8-dihydro-2'-deoxyguanosine in biological samples. J. Chromatogr. B Biomed. Appl. 715, 349–356. https://doi.org/10.1016/S0378-4347(98)00259-X.

Ren, L., Fang, J., Liu, G., Zhang, J., Zhu, Z., Liu, H., Lin, K., Zhang, H., Lu, S., 2016. Simultaneous determination of urinary parabens, bisphenol A, triclosan, and 8-hydroxy-2′-deoxyguanosine by liquid chromatography coupled with electrospray ionization tandem mass spectrometry. Anal. Bioanal. Chem. 408, 2621–2629. https://doi.org/10.1007/s00216-016-9372-8.

Sabatini, L., Barbieri, A., Tosi, M., Roda, A., Violante, T.S., 2004. A method for routine quantitation of urinary 8-hydroxy-2′-deoxyguanosine based on solid-phase extraction and micro-high-performance liquid chromatography/electrospray ionization tandem mass spectrometry. RCM (Rapid Commun. Mass Spectrom.) 19/2, 147–152. https://doi.org/10.1002/rcm.1763.

Song, M.F., Li, Y.S., Ootsuyama, Y., Kasai, H., Kawai, K., Ohta, M., Eguchi, Y., Yamato, H., Matsumoto, Y., Yoshida, R., Ogawa, Y., 2009. Urea, the most abundant component in urine, cross-reacts with a commercial 8-OH-dG ELISA kit and contributes to overestimation of urinary 8-OH-dG. Free Radic. Biol. Med. 47, 41–46. https://doi.org/10.1016/j.freeradbiomed.2009.02.017.

Steffensen, I.L., Dirven, H., Couderq, S., David, A., D'cruz, S.C., Fernández, M.F., Mustieles, V., Rodríguez-Carillo, A., Hofer, T., 2020. Bisphenols and oxidative stress biomarkers— associations found in human studies, evaluation of methods used, and strengths and weaknesses of the biomarkers. Int. J. Environ. Res. Publ. Health 17. https://doi.org/10.3390/ijerph17103609.

Topic, A., 2014. Liquid chromatography-mass spectrometric methods fo quantitative analysis of oxidatively damaged DNA in biological fluids. Oxidatively modif. DNA lesions syst. Repar. Sources. Clin. Significance Methods Anal. 1–15.

Valavanidis, A., Vlachogianni, T., Fiotakis, C., 2009. 8-hydroxy-2' -deoxyguanosine (8-OHdG): a critical biomarker of oxidative stress and carcinogenesis. J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev. 27, 120–139. https://doi.org/10.1080/10590500902885684.

Ventura, C., Gomes, B.C., Oberemm, A., Louro, H., Huuskonen, P., Mustieles, V., Fernández, M.F., Ndaw, S., Mengelers, M., Luijten, M., Gundacker, C., Silva, M.J., 2021. Biomarkers of effect as determined in human biomonitoring studies on hexavalent chromium and cadmium in the period 2008–2020. Environ. Res. 197 https://doi.org/10.1016/j.envres.2021.110998.

Waits, A., Chen, H.C., Kuo, P.L., Wang, C.W., Huang, H. Bin, Chang, W.H., Shih, S.F., Huang, P.C., 2020. Urinary phthalate metabolites are associated with biomarkers of DNA damage and lipid peroxidation in pregnant women – Tainan Birth Cohort Study (TBCS). Environ. Res. 188, 109863 https://doi.org/10.1016/j.envres.2020.109863.

Wang, C.C., Chen, W.L., Lin, C.M., Lai, C.H., Loh, C.H., Chen, H.I., Liou, S.H., 2016. The relationship between plasma and urinary 8-hydroxy-2-deoxyguanosine biomarkers measured by liquid chromatography tandem mass spectrometry. Environ. Sci. Pollut. Res. 23, 17496–17502. https://doi.org/10.1007/s11356-016-6898-4.

WHO, 1996. Biological Monitoring of Chemical Exposure in the Workplace - Guidelines. World Health Organization, Geneva.

Wu, L.L., Chiou, C.C., Chang, P.Y., Wu, J.T., 2004. Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. Clin. Chim. Acta 339, 1–9. https://doi.org/10.1016/j.cccn.2003.09.010.

Zhang, Y.J., Wu, L.H., Wang, F., Liu, L.Y., Zeng, E.Y., Guo, Y., 2021. DNA oxidative damage in pregnant women upon exposure to conventional and alternative phthalates. Environ. Int. 156, 106743 https://doi.org/10.1016/j.envint.2021.106743.

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Review of the slippage factors from open defecation-free (ODF) status towards open defecation (OD) after the Community-Led Total Sanitation (CLTS) approach implementation

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ABSTRACT

Open-defecation (OD) is one of the most widespread sanitation practices in low-income countries. This practice often causes diarrheal diseases and 760,000 deaths per year. To eradicate OD, several approaches have been developed, including Community-Led-Total Sanitation (CLTS) which is a participatory and community approach. The specificity of CLTS is that it is managed by the community itself, as its name implies, and that no subsidies or financial contributions from outside the community are used in the construction of the facilities. Although, the CLTS is effective in the short-term for eradicating OD, the long-term results are not encouraging: Open-Defecation-Free (ODF) communities revert to OD or partially use latrines. The present research is based on literature review and authors investigation in Burkina Faso. It was conducted to provide a comprehensive understanding of the factors that affect the sustainability of ODF-status leading to slippage in communities. It was found that these factors can be grouped into five categories: behavioral and social, technological, organizational, and vulnerability factors. The last one, socio-political factors, is a contribution from the authors as it was not reported in the literature yet. The authors have proposed graphical synthesis of all the slippage factors and their associated categories in the ODF-communities. Finally, authors have suggested that to sustain ODF-status of communities: include all stages of the sanitation value chain (SVC) in the CLTS, the follow-up activities after achieving ODF-status must be planned well in advance, sanitation marketing should be developed and the sanctions against the practice of OD have to be reinforced. Governments and donors should pay particular attention to the following options: raising awareness and regular monitoring after ODF certification, encouraging research on sustainable and pro-poor sanitation technologies, and building the capacity of implementing actors including facilitators. While obtaining ODF status is materialized by a sign with the status on it, this paper drew the attention of CLTS implementers to the lack of materialization of slippage when it occurs, and the absence of studies on the evolution of the community sanitation scale after ODF-status.

1. Introduction

Globally, 3.6 billion people lack access to improved sanitation and 494 million people practice open-defecation (OD), of which 92% are living in rural areas of Central and Southern Asia and sub-Saharan Africa (JMP, 2021). United Nations has reaffirmed the importance of sanitation by including it in the Sustainable Development Goals (SDG6.2), which calls for an end to OD and for universal access to adequate and equitable sanitation (UN, 2015). To achieve the SDG6.2, several sanitation projects are being implemented. A sanitation project entails seeking an improvement of the living conditions of a population. These conditions are sanitary, environmental and economic. Funding is therefore mobilized and several stakeholders are involved. Unfortunately, funding has created a culture of dependence on subsidies by the beneficiary communities, leading them to stop making efforts to solve their sanitation problems and always reach out, even in situations where they have the

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means to do so by themselves (Kar and Pasteur, 2005). Additionally, financial supports have led to uneven adoption, sustainability problems and partial use of the sanitation facilities. OD through the fecal-oral contamination cycle continue to spread diseases. In response to this situation, behavior change (BC) approaches have been developed. They are fundamental to achieve sustainability in communities through the identification of new, and strengthening the existing positive practices (Afzal et al., 2022). It is considered central to the quest for a sustainable future and solves community problems, which require large scale shifts in human behavior with regard to long held habits (Yusliza et al., 2020). In the context of growing concern over the quality of human life and development of communities, BC interventions are considered essential to achieve sustainability in communities (Dickin and Gautam, 2019). Among BC approaches, there is the Community-Led Total Sanitation (CLTS) has emerged in 2000. CLTS has started in Bangladesh and has spread to rural areas in 66 countries in Asia, Africa, and America (Fig. 1). 36 countries have adopted CLTS as part of their national rural sanitation strategy and/or policy (Institute of Development Studies, 2019). Currently, CLTS is one of the most widely deployed behavioral hygiene and sanitation interventions (Zuin et al., 2019) (.

CLTS enables paradigm shift from teaching to communities facilitating their own analysis, from subsidizing the poor to communities doing it (Sah and Negussie, 2009). It enables a shift from top-down hierarchical standardization to bottom-up approach, and from investing on equipment to investing on human resources (UNICEF Mali, 2014). CLTS is meant to receive no funding unlike previous approaches. It focuses on the behavioral change needed to ensure lasting improvements. These include investing in community mobilization rather than infrastructure, and shifting the focus from building latrines for individual households to creating Open-Defecation-Free (ODF) villages. By raising awareness that "as long as even a minority continues to practice OD, everyone is at risk of disease", CLTS triggers the community's desire for collective change, pushes its members to action, mutual support, and locally solutions (Mukherjee and Mukherjee, 2017).

Attention to community participation has become a priority of the 2030 Agenda for Sustainable Development. SDG6.2 also supports participation of local community in improving water and sanitation management (UN Water, 2019). In this context, CLTS has a role to play in achieving this goal because it is participatory and has shown promise in addressing OD (Pickering et al., 2015). CLTS is currently considered by most international donors to be the most effective approach to scaling-up sanitation (Galvin, 2015). Being implemented for, with and by the community, CLTS should have had greater sustainability in terms of sanitation facilities use and hygienic behaviors than previous sanitation approaches. Unfortunately, experience reveals that this has not been the case in some communities. While the SDGs emphasize the need to reach everyone, there is growing evidences that CLTS outcomes may not be sustainable as expected (Robinson et al., 2016; USAID, 2018a). Poorer households more likely to revert to OD after a period of time (Odagiri et al., 2017; Robinson and Gnilo, 2016; USAID, 2017). Studies conducted by Cavill et al. (2015); Chambers and Myers (2016); Crocker et al. (2017a); Kouadio (2019); Abebe and Tucho (2020) revealed that after an initial excitement at the beginning of the CLTS implementation and the achievement of ODF-status, there was a slackening of communities often after two years of the change in behaviors advocated by CLTS are no longer followed. CLTS is now being questioned, particularly with regard to its methods, its results and the sustainability of the ODF-status.

Being a participatory approach, the objective of CLTS is to lead to sustainable behavioral changes. In spite of the community involvement in CLTS implementation, we are still witnessing a slackening. How then can we explain slippage (return of OD or the partial use of latrines) in some communities that had adopted CLTS and had been certified ODF 2–4 years ago?

Several authors have investigated on the sustainability or slippage of sanitation interventions in general and on CLTS in particular (Abdi, 2016; Abebe and Tucho, 2020; Ayalew et al., 2018; Barnard et al., 2013; Bhatt et al., 2019; Crocker et al., 2017; Galan et al., 2013; Garn et al., 2017; Kouadio, 2019; Osumanu et al., 2019; Patwa and Pandit, 2018). Crocker et al. (2017); Hanchett et al. (2011); Mukherjee et al. (2012) and Tyndale-Biscoe et al. (2013) have reported on sanitation outcomes and that the period of return to OD is 2–4 years after the end of CLTS.

This paper therefore identifies, categorizes, and analyzes the slippage factors towards OD following CLTS implementation. It is essentially based on literature review. It proposes a graph showing all the slippage factors and their associated categories. It highlights shortcomings and develops a set of actions that could be taken in order to improve the sustainability of ODF-status.

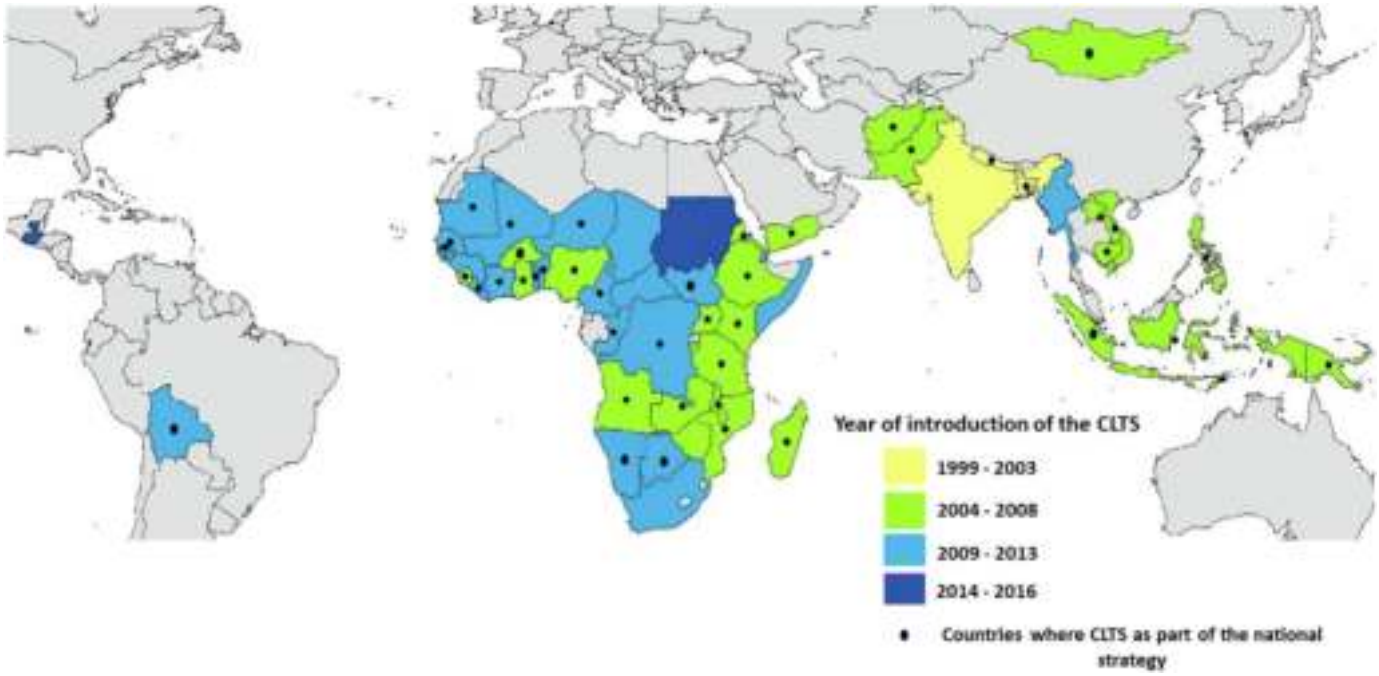

Fig. 1. Map of countries where CLTS has been introduced. Source: (Zuin et al., 2019).

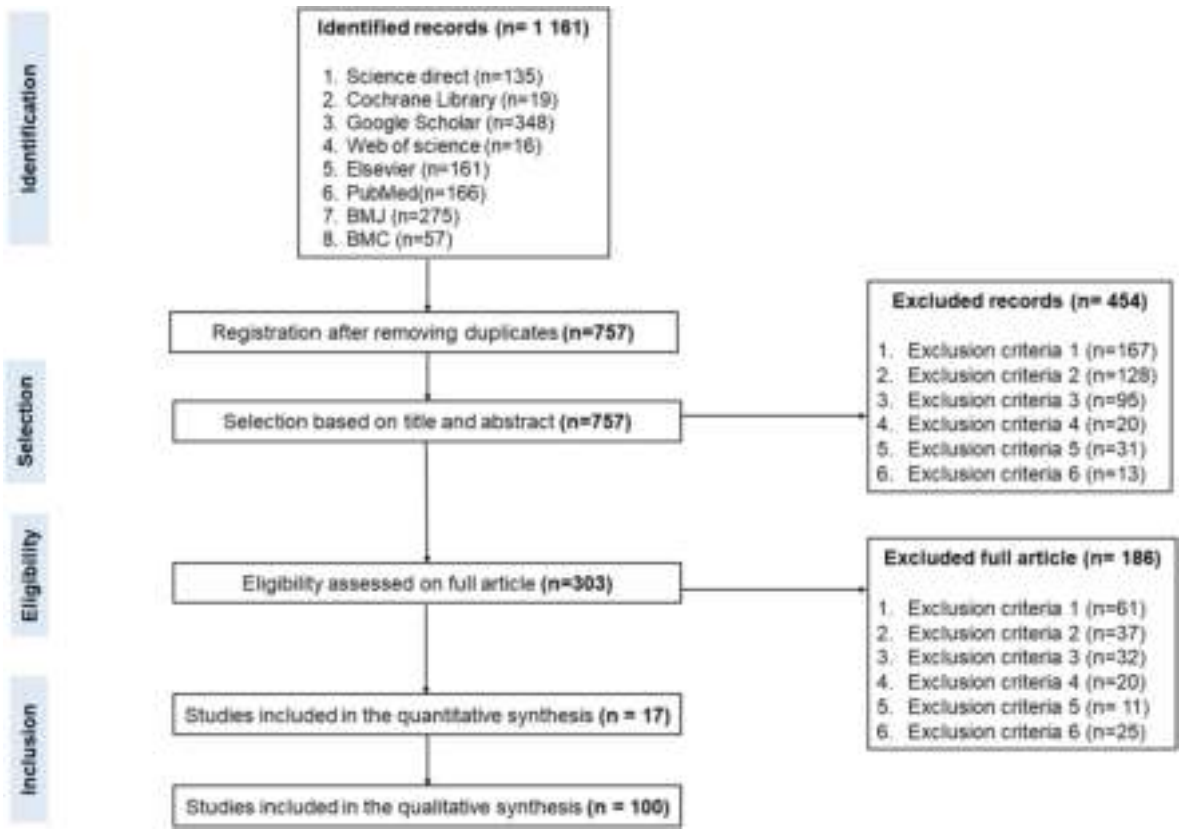

Fig. 2. Diagram of the 4 steps of the PRISMA method for our review.

2. Definition of the concept and different scenarios after CLTS implementation

2.1. Definition of sustainability and slippage in CLTS

For CLTS, sustainability refers to whole communities and their ODF-status (Cavill et al., 2015). Criteria for assessing ODF communities typically include the following criteria:

- Eradication of OD in the community.
- Private latrines, with a lid to cover the defecation hole and a roof for protection, that are hygienic, provide safe isolation of excreta, and maintain privacy.
- Use of the latrine by all members of the household and community.
- A hand-washing facility near the latrine with water, soap, towel and evidence of regular use.

Some countries have added other elements to be more stringent such as:

- Food hygiene (covering of food).
- Solid waste management and sewage disposal.
- Provision of institutional latrines in schools, markets and for passers-by.

"*Sustainability of ODF-status*" refers to maintenance of all these behaviors, conditions and facilities including the cleanliness and hygienic use of toilets by all in a community over time, and the safe management and disposal of faecal sludge (Cavill et al., 2015).

"*Slippage in CLTS*", defined as the return to previous unhygienic behaviors or the inability of some or all community members to continue to meet all ODF criteria (Hickling, 2019). However, the slippage criteria are country-specific and then tied to the definition of ODF-status and the criteria used in national certification protocols.

2.2. Different scenarios in the communities after the CLTS implementation

Like all previous approaches such as SARAR (Self-esteem; Associative; Resourcefulness; Action planning; Responsibility), PHAST (Participatory Hygiene and Sanitation Transformation), BCC (Behavioral Change Communication), CLTS also has its weaknesses, one of which is the short sustainability of the behavioral change operated and the latrines built through it. Follow-up studies have revealed variable long-term commitment to toilet use and maintenance in many areas (Bongartz et al., 2016). Two to three years after the introduction of the CLTS projects, three cases have emerged: NO-ODF TRIGGERED communities (communities that have not achieved ODF-status despite being triggered.), ODF communities (communities that have maintained ODF-status) and BACOD communities (communities that have reverted to OD after previously being certified ODF). This paper focuses on the latter BACOD communities.

Any innovation or change introduced in a community normally goes through a process of appropriation which, according to the theory of diffusion, takes place gradually (Everett, 1995). Individuals and groups usually undergo trial phases to match the newly introduced practices with their habits in order to find possible adaptations or to abandon the change. In this regard, Chambers and Myers (2016) reported partial use of latrines a few years after obtaining ODF-status, and rates of return to OD significantly vary between and within countries. This is evidence that the new practice has not become properly or deeply entrenched in the communities' habits. On the sustainability of CLTS, various findings have been made and it should be noted that the obstacles are likely to depend on the context (Coffey et al., 2017; Novotný et al., 2018).

3. Methods

This systematic review of the literature is based on the recommendations of the PRISMA method "*Preferred Reporting Item for Systematic Review and Meta-Analysis*" (Gedda, 2015; Mateo, 2020; Moher et al., 2009). To carry out this method, two software programs were used: Zotero, a free opensource bibliography manager (https://www.zotero.org/) and a Microsoft® Excel spreadsheet. The use of Zotero allows for simplified reference management. Excel is used to indicate decisions about the different steps of PRISMA. These decisions are then applied in Zotero. Finally, the comparison between Zotero and Excel allows us to verify the absence of errors at the different steps.

The first step was to query 8 databases (Science direct, Cochrane Library, Google Scholar, Web of Science, Elsevier, British Medical Journal (BMJ), BioMed Central (BMC), PubMed) and import the references into Zotero and then into Excel. As the CLTS was also implemented in a few sub-Saharan African countries, the majority of which are French-speaking, the databases were searched in English and French. The following search terms "open-defecation", "open defecation-free (ODF)", "slippage factors in CLTS", "categories of slippage factors in CLTS", "sustainability factors in CLTS", "community-led total sanitation (CLTS)", "rates of slippage in CLTS", "assainissement total piloté par la communauté (ATPC)", "FDAL", "facteurs de régression de l'ATPC" were used with Boolean operators such as "OR" or "AND" to obtain published articles and grey literature. These terms were entered in the same way in each of the 8 databases. CLTS being a relatively recent approach, the research has considered all publications without date limitation until August 2022. The search identified a total of 1161 references: 135 on Science direct, 19 on Cochrane Library, 348 on Google Scholar, 16 on Web of Science, 161 on Elsevier, 166 on PubMed, 275 on BMJ and 57 on BMC. These references were imported into Zotero.

The second step was to identify the duplicates, then to delete them. The risk of a systematic research with several databases is to identify the same reference several times. All the references exported to our Zotero library during the first step were copied into an Excel spreadsheet. In Zotero, the classification by title allows to visualize the duplicates. In Zotero, the classification by title allowed to visualize the duplicates. The total number of references identified with duplicates was 364. These duplicates were subsequently removed in Zotero and also in the Excel spreadsheet.

The third step was the identification of eligible articles based on the reading of the title and abstract. 6 exclusion criteria were selected and scored from 1 to 6. All articles whose titles and abstracts did not include the terms in English: "slippage factors of CLTS (1)", "sustainability factors of CLTS (2)" or "evaluation of CLTS implementation (3)" or in French "facteurs de régression de l'ATPC (4)", "facteurs de durabilité de l'ATPC (5)" or "évaluation de la mise en œuvre de l'ATPC (6)" has been excluded. The presence of an exclusion criteria was indicated in the Excel spreadsheet by a number from 1 to 6 in front of the reference. On this basis, 454 articles were excluded.

The fourth step was the evaluation of the eligibility of articles for inclusion in the journal on a full-read basis. This step provided OD slippage rates and also identified factors for OD slippage in countries where CLTS has been developed. The application of this research methodology allowed us to review 117 articles addressing the issue of OD slippage following the CLTS implementation. (see Fig. 2).

A categorization approach was used to group the slippage factors (qualitative data) with similarities in the same category. In addition to the qualitative data, quantitative data are also presented. The quantitative data focused only on the rates of slippage to OD in communities of 13 countries (Benin, Ethiopia, Eritrea, East Timor, Ghana, Indonesia, Kenya, Mali, Mauritania, Mozambique, Nepal, Sierra Leone and Uganda) that had previously adopted the CLTS approach and become ODF-certified in order to compare them with each other.

4. Results

4.1. *Open defecation-free slippage rate*

The slippage rate refers to the proportion of ODF-certified communities that, as a result of a cross-check were no longer compliant with their country's ODF criteria out of the total proportion of ODF-certified communities. The ODF slippage rate in Ethiopia was 15.9% (Abebe and Tucho, 2020). This rate was higher than a study conducted in Nepal 3.5% (Shrestha et al., 2018), Ghana 8.8% (Jiménez et al., 2017) and Indonesia 14.5% (Odagiri et al., 2017). Similarly, the slippage rate was higher than the average slippage rate of some African countries, which was 10–13% (Bongartz et al., 2016; Tyndale-Biscoe et al., 2013), in contrast, it was lower than East Timor 16.4% (Abdi, 2016), Benin 17.5%, Mali and Mauritania 24% (Jiménez et al., 2017), Kenya 22% (Tyndale-Biscoe et al., 2013), Eritrea 27% (UNICEF Eritrea, 2015), and Mozambique 31% (UNICEF, 2014). Indeed, Ghana, which had the highest gross domestic product (GDP) per capita (US$ 2260.9) among the African countries in our study, also recorded the lowest slippage rate to OD on the African continent. Mozambique, which had the lowest GDP (US$ 428.9), recorded the highest slippage rate among the 13 countries. Conversely, despite having the highest GDP among the 13 countries, Indonesia (US$ 3837.6) recorded a higher slippage rate than African countries such as Ghana and Uganda. Nepal which had the lowest slippage rate of all countries had a lower GDP than Indonesia (US$ 3837.6), Ghana (US$ 2260.9), Kenya (US$ 1872.1), Mauritania (US$ 1587.9) and East Timor (US$ 1353.8).

Fig. 3 shows the correlation between GDP per capita and ODF slippage rate in 13 countries located in Asia and Africa.

Except for Nepal and Ghana, the slippage rate to OD is above 10% in the other countries, irrespective of the continent. Through these data and the general trend in Fig. 3, there is no clear evidence that there is a correlation between the rate of slippage and the GDP per capita of country. GDP values were taken from https://data.worldbank.org/indicator/NY.GDP.PCAP.CD.

ODF slippage rates were calculated and estimated on the basis of the proportion of the total sample that did not have functioning latrines or the proportion of communities where visible signs of OD were observed during cross-checking. If ODF-status is equated only with the idea that a household has a functioning latrine and that there is no sign of OD near a household, then the slippage rates are underestimated. But if all of the ODF-status criteria (hand washing facilities with soap and water, a means of preventing flies from entering the pit, etc.) are applied, these slippage rates would be much higher than those presented.

4.2. *Open-defecation-free (ODF) to open-defecation (OD) status slippage factors*

The literature groups factors related to the slippage from ODF status to OD status into four categories: behavioral and social factors, natural and technological factors, vulnerability and poverty factors, and the organizational factors. These four categories are not fundamentally different, but are complementary.

4.2.1. *Behavioural and social factors*

The elements that make up this category of factors are:

- **Filled up pits and fear of pits filling up**

As a result of the exponential spread of CLTS campaigns, the number of full pits is high. When the pits fill up, there are four options: digging a new pit; emptying the pit; using it sparingly or returning to systematic OD. Digging a new pit in some cases can be challenging where there is limited space. The availability and price of pit-emptying services are causative factors in the decline of ODF-status in Bangladesh (Hanchett et al., 2011). In the rural areas of Laos, households that could not afford

Fig. 3. Correlation between GDP and ODF slippage rate in 13 countries after CLTS implementation.

the average cost of emptying pit of around US$50 reverted to OD (Opel and Cheuasongkham, 2015). In rural northern India, people want large, deep septic tanks to last a lifetime (Garn et al., 2017; Shah et al., 2013). Large tanks are less stable and have a risk of collapsing. Men may refrain from using the toilet by performing OD to reduce the frequency of pit emptying. Furthermore, a public latrine with a large number of people in a household will tend to fill up relatively much faster. This "rapid filling" requires frequent emptying consequently using a lot of financial resources. This factor is often a limitation in the sustainability of the ODF-status. An important question that is not raised when implementing the CLTS is the issue of emptying the filled pits. With the objective of putting an end to OD through the construction of latrines by the community itself, CLTS is limited only to the first phase of the sanitation value chain (SVC), "containment", overlooking the other phases (Emptying, Transport, Treatment and Reuse/Disposal). Not having anticipated this, some communities, three years after using their latrines, are facing a fait accompli and therefore return to OD. However, the excreta from the emptying of these pits may have economic value in some communities as fertilizer for agriculture. But, the acceptance of these products is not unanimous in all communities.

- **Pressure on usage and gender**

Public sanitation facilities may not be available when users need them, for example, when they are away from home or when facilities are closed at night (Caruso et al., 2017). As a result, queues can be expected, which can also discourage their use (Kulkarni et al., 2017). In large families, one latrine may not be sufficient for all members. Men may choose the OD to avoid queuing or to reduce line-ups in the morning, when children need to prepare for school. Similarly, SQUAT (Sanitation Quality, Use, Access and Trends) survey by Coffey et al. (2014) reported that in households with one latrine, men are less likely to use them than women. Men rationalize their ODs as valuing women's dignity by giving them unlimited access to the toilet. Also, men believe that toilets are not for them but for women, children, the very old, the sick and the disabled (Cole, 2013). Men may need to take longer to defecate than women or children and they opt to use OD because it allows more time for defecation and they will avoid the embarrassment of showing that they take longer (Chambers and Myers, 2016). A study report from Kenya showed that sharing latrine with neighbors has a significant contribution for ODF slippage (Singh and Balfour, 2015). Latrines are often shared and this situation penalizes the sustainability of ODF-status.

- **Dirt, smell, disgust, fear and cleaning**

Dirty and disgusting toilets do not encourage their use, making them unpleasant to clean and consequently leading to a return to OD. It is also argued that bad odors are often overlooked barrier to toilet adoption (Rheinländer et al., 2013). Studies by Yimam et al. (2014) in Ethiopia found that households with clean toilets were four times more likely to use them. People revert to OD, particularly when latrines are unattractive or not hygienically maintained (Dreibelbis et al., 2015). The sustainability of toilets clean depends on access to water, its distance, how it is transported, and who fetches it. Cavill et al. (2015) reported factors such as distance and the time and energy required to fetch water and clean toilets as contributing to slippage.

- **Socialization**

Socialization is a factor to slippage of ODF-status in some communities. These are communities where women are not free to move around. They prefer to go out as a group to practice OD at certain times of the day. These moments offer an opportunity for them to leave the house, to meet and exchange without men being present. A study in Odisha, India by Routray et al. (2015) found that socialization was an important factor contributing to low toilet use. According to the same authors, women reported that the OD gave them a rare opportunity to leave the house and spend time away from household chores and responsibilities.

- **Social and cultural standards**

A study in northern India has concluded that OD was rarely perceived as socially unacceptable (Coffey et al., 2015). Such communities are more likely to return to OD, and this return may not shock many people. In this regard, CLTS must overcome the force of such community habits, and in India, the deep-rooted beliefs about purity and pollution (Routray et al., 2015). Standards of body purity and pollution and notions of private space support the practice of OD at some distance from home, even when toilets are accessible. Many consider OD as a healthy activity that promotes purity and is good for health (Coffey et al., 2015). Further, during an event on November 24, 2018, during Sanitation Night in the village of Sokoula (Burkina), it was observed that some households were not using latrines. This fringe of the community who prefer OD than use the latrines citing the reason that: "*two holes should not look at each other*". Social norms and beliefs of purity are one of the factors in the slippage of ODF-status.

- **Taboos, beliefs and prohibitions**

The use of the same toilet, especially by fathers and their daughters-in-law in patriarchal societies can affect toilet use (Thys et al., 2015). In Idoma communities in Nigeria, it is taboo to defecate in a building or superstructure. Husbands have also refused to use the same toilet as with their wives and daughters (WaterAid, 2009). In Zambia, traditional taboos make it difficult for male heads of households to share toilets with their stepmothers, mature girls or younger children if they are at risk of being seen or if young children use the toilet immediately after their fathers (Thys et al., 2015). In Ethiopia, a study found that it was a taboo for men and women to share the same toilet. It is therefore reported that men continue OD to avoid this problem (Ashebir et al., 2013). In Nigeria, it is often believed that the hot air from the pit makes women more susceptible to disease. Due to this, women were less likely to use toilets than men (Abramovsky et al., 2015). In other communities, men do not wish to share a toilet with their daughters when they are menstruating for fear of being in contact with impure fluids. Therefore they resume OD at this time or forbid the women from using the toilet (Cavill et al., 2015). In Madagascar, in the community of Mandahazo, convincing people to abandon OD and start using latrines is difficult because latrines were believed to be linked to evil spirits that kill children, keeping excrement within four walls was taboo and attracted the devil (USAID, 2018b). Such beliefs and the sharing of latrines discourage people from using them (Coffey et al., 2014).

- **Perception around the faeces of children and people with mental disorders**

Safe disposal of children's excreta remains a challenge (Majorin et al., 2019; Miller-Petrie et al., 2016; Morita et al., 2016). Children's faeces are considered harmless and are not disposed of safely (Majorin et al., 2017), even though they may contain higher pathogen loads than adults (Lanata et al., 1998). Even those who have access to sanitation facilities often do not use them to dispose of children's faeces (Freeman et al., 2014; Miller-Petrie et al., 2016). Case studies in 42 sites in low, middle- and high-income countries found that all countries reported unsafe practices for disposing of children's faeces in households with improved sanitation facilities (Azage and Haile, 2015; Chebet et al., 2020; Mugel et al., 2022; Seidu et al., 2021). It is difficult to differentiate between the faeces of an adult or a child or even a person with a mental disorder within a community during a cross-check. It is difficult to persuade or control people with mental disorders to abandon the OD habit and their practices are most tolerated. This perception that some communities have of children's faeces being harmless contribute to slippage.

- **Level of education**

Anjum Altaf and Hughes (1994); Whittington et al. (1993) and Lauria et al. (1997) have shown a positive effect of the level of education on the willingness to invest in and use a sanitation service. According to these authors, a better-educated household is more aware of the positive externalities related to the use of a toilet. Milanesi (2007) points out that the lack of education negatively impacts the willingness of communities to invest in sanitation in Moshi, Tanzania. Laré et al. (2018) supports this view by adding that the low level of education of households generates less knowledge of the health risks incurred by the use of an unimproved latrine. There is a very strong relationship between women's education and their hygiene knowledge and behaviors. This means that more educated women are more successful in adopting and sustaining hygiene practices (Cairncross and Shordt, 2004).

"Shock and shame" methods were considered too extreme and although CLTS encouraged people to build toilets regardless of their education levels, the approach did not guarantee that they would be used (O'Reilly and Louis, 2014). The same authors acknowledged that most households in Himachal Pradesh (India) had built toilets but were not using them, so the program subsequently emphasized education and sanctions to encourage use. Sustainable behaviors result from giving high priority and adequate resources to hygiene promotion, sanitation, and education (Cairncross and Shordt, 2004). From the above, it follows that lack of sanctions and low levels of education are factors that lead to slippage to OD in the CLTS.

4.2.2. *Factors of vulnerability and poverty*

The elements that make up this category of factors are:

- **Financial difficulties of communities**

As a non-subsidy approach, CLTS increases the financial burdens on communities, although it is beneficial to them from a health perspective. The financial burden to maintain and keep toilets functional discourage some households. According to WHO (2019), cost is the most important factors influencing return to OD. In low-income settings, the cost of toilet construction and maintenance influences the initial and long-term adoption of CLTS (Hulland et al., 2015). This idea was reaffirmed by Venkataramanan et al. (2018) that the high cost of maintenance and reconstruction was one of the reasons for a return to OD. A study conducted in Odisha (India) by Barnard et al. (2013) found that financial hardship aspects accounted for over 75% of the causes of slippage to OD three years after CLTS implementation. Tyndale-Biscoe et al. (2013) found that external support and encouragement influenced household decisions: OD households reported lack of support as the third most important factor in their decision to abandon their latrine. Poverty may not only prevent but also discourage the poor from rebuilding new toilets in case of collapse or renovating damaged toilets (Mehta, 2011; Mehta and Movik, 2011). Financial constraints were therefore one of the most important factors causing the slippage. It would therefore be appropriate to question this approach which advocates for no financial support but encourages communities to achieve ODF-status through the promotion of shame.

- **Vulnerability, natural preference for OD, and convenience**

House and Cavill (2015) reported that there is low or non-inclusion of vulnerable people (women, elderly, disabled, etc.) in the triggering of CLTS. This situation is attributed to inadequate and unsuitable latrines for vulnerable people, who soon after certification return to their old OD behaviors. Besides the vulnerable people, there are people who naturally prefer OD. The survey on sanitation quality, use, access and trends in rural areas of northern India, found that 40% of respondents preferred OD because they found it more pleasant, comfortable, and convenient, even if their households had functional latrines. Access to sanitation facilities is a prerequisite for stopping OD, but it is not a sufficient condition (Barnard et al., 2013; Coffey et al., 2014).

To ensure sustainability, the construction of these facilities should be done by people with expertise in construction such as masons, and with quality materials considering natural constraints. We will discuss in the next section the material and technical factors leading to slippage at OD after CLTS implementation.

4.2.3. *Natural and technological factors*

The elements that make up this category of factors are:

- **Design, construction and use**

A material chosen according to natural constraints (slope, type of soil, flooding, etc.) is likely to be more durable than the one chosen with the sole objective of achieving ODF-status. It is undeniable that the quality, availability and cost of a material are linked. The quality of a material is not universal when the local realities and the scope of its use is considered. The structure and design of toilets affect usage in many ways: construction never completed, poor quality of material, small superstructure, lack of roof to serve as protection against rain, etc. are all

reported to undermine usage.

CLTS often results in low-durability latrines made from local materials, which contributes to return to OD (Cavill et al., 2015). In a study conducted by Crocker et al. (2017) on the sustainability of CLTS, many households (45% in Ethiopia and 6% in Ghana) had their latrines collapse in the year following certification. While households were clearly committed to continuing to use the latrines an annual latrine collapse rate of 45% discouraged households and ultimately drove them to OD.

Tyndale-Biscoe et al. (2013) found that poor quality construction and materials were a factor in the decision to abandon toilets. The study further reported that higher quality latrines were more likely to last and be maintained. A limitation of CLTS is that communities are often given the sole responsibility for choosing the technical design of the latrine, especially in areas where there are strong natural barriers. Often focusing on simple and quick solutions, people opt for latrine models that are easy to build without taking natural barriers into account. Communities are involved in strategies based on affordable solutions which ends up jeopardizing the sustainability of CLTS. A study in Nigeria found that the type of toilet affects usage rates. Septic tanks are most likely to be used while slabless pit latrines are the most likely to be avoided (Abramovsky et al., 2015). A study by Rotondo et al. (2009) conducted in Ghana, Mali, Niger and Nigeria found that 70.5% (95% CI 65.7–74.8) of the surveyed households had a dry pit latrine. A sensitivity analysis using polytomous regression to assess the sustainability of different sanitation technologies revealed that flush toilets were more likely to be sustainable than other types of improved latrines and obviously than dry pit latrines (Apanga et al., 2020).

Poor quality toilets without tight fitting lids are likely to produce foul odors, which discourages their use and leads to their abandonment. UNICEF Malawi's CLTS program after successfully implementing improved sanitation coverage by 2011, it received reports that households continued to face difficulties due to poorly designed latrines, often not watertight, which often collapse after a short period of use (Cole, 2013). In Ethiopia, Kenya, Uganda, and Sierra Leone, slippage was due to lack of advice on how to build or maintain good quality, sustainable latrines (Tyndale-Biscoe et al., 2013). CLTS approach needs to be improved technically, through the development of appropriate latrine standards.

Barnard et al. (2013) and Routray et al. (2015) have found that lack of toilet privacy was a factor that may also lead to slippage to OD. For example in eastern Zambia, some toilets have low walls, no roof, and no door locks (Thys et al., 2015). Other reasons for non-use of toilets are the difficulty of cleaning, lack of water for anal cleansing and, in India, lack of water for ritual bathing after defecation (Patil et al., 2014). According to Tyndale-Biscoe et al. (2013), failing toilets and the inability to maintain and repair them were often identified as justifications by those who returned to OD. Lastly, the sense of ownership must be considered. Those who construct their own latrines, as in the case of CLTS approach, have a sense of ownership and are much more likely to use and maintain them than those who have had their latrines built according to third-party models. For example, of all the toilets built for villagers during the first year of the Swachh Bharat Mission in India, 46% were in use (Chambers and Myers, 2016).

- **Non-assistance in the choice of materials**

CLTS does not give suggestions for latrine models or materials used to build the latrines. Based on the reported link between the sustainability of latrines and the type of construction materials, it would have been more logical to opt for sustainable latrines models from the onset, rather than trying to increase populations on the sanitation ladder over time. This "non-assistance" or "no-subsidy" principle, which is rarely questioned, is one of the causes of the slippage of the ODF-status in the communities where CLTS was triggered. According to Cole (2013), UNICEF Malawi decided to apply social marketing tools to improve its existing CLTS program. Afterwards, the program showed progress in increasing sanitation coverage. However, headquarters received various reports that households continued to experience difficulties due to poor latrine designs that often collapse after a short period of use. The degree of technical guidance provided to communities influences the sustainability of the latrines they build (Harter and Mosler, 2018; Sigler et al., 2015; Venkataramanan, 2016).

- **Natural barriers**

Cavill et al. (2015) have noticed that water bodies near households undermine the sustainable adoption and use of toilets because they provide convenient places to practice OD and clean oneself simultaneously. Lack of space to replace or dig new toilets in densely populated areas may also reduce sustainability. The authors further argue that the sustainability of handwashing and maintaining toilet cleanliness depends on access to water. However, lack of water in rural areas remains an enduring challenge. In some cultures, water is needed for cleaning after defecation, so its absence near households structure may encourage OD near surface water bodies (Routray et al., 2015). This problem limits the sustainability of CLTS, as do other natural barriers. The type of soil and the proximity of the water table is the most natural obstacle reported (RESEAU PROJECTION, 2015).

In hard rock environments, it is not possible to dig a toilet pit quickly. Frequent disasters such as floods which lead in collapsed pits and latrines have deterred ODF-communities from rebuilding their latrines, as reported in the Bangladesh study (Hanchett et al., 2011). Studies by Iyer and Pare Toe (2022) have shown the impact of climatic hazards (floods and droughts) on the sustainability of ODF-status in Burkina. Many households reverted to OD as a result of these disasters. Statements such as, "*When it rains our latrines collapse. The number of falling latrines cannot be counted. If you want to relieve yourself you take your bike and go into the bush"; "There is an old woman next door. Her wooden latrine has collapsed twice. She got tired of it and gave up and went back to OD"; "We use local materials to build our latrines which are not resistant to heavy rains. They can't even resist since it's dry wood and terra cotta I'm talking about"* were collected in the same study.

In such environment affected people return to the practice of OD or opt to use temporary toilets of poor quality. For example, when it rains, where latrines do not have a roof, there may be rise in groundwater table that prevents latrine from being used. During this period, the community returns to the OD. In environments with hydromorphic soil, the phenomenon of latrine collapsing is frequent and slows down work in some places (Protos, 2016). In Kayinja (Uganda), the proximity of the water table makes it very difficult for the community to build ordinary latrines. This is because of the unstable nature of the soil. As a result, the most vulnerable households cannot afford a latrine. They therefore use the latrines of their neighbors when these are available or practice OD or the cat method when these are not available (Wilbur and Jones, 2014).

4.2.4. *Organizational factors*

The elements that make up this category of factors are:

- **Failure of the leadership of the natural leader and lack of post-ODF follow-up**

Follow-up is a key element of the post-ODF process (Pasteur, 2017). Post-ODF follow-up is a very important condition or even a guarantee for the sustainability of ODF-status. Rigorous follow-up is inseparable from the sustainability of ODF-status (Thomas and Bevan, 2013). Therefore, the lack or insufficiency of post-ODF follow-up by the community and other leaders has jeopardized the sustainability of ODF-status in some communities. Venkataramanan et al. (2018) and Cavill et al. (2015) reported that lack of constant follow-up is one of reasons for poor latrine use and a return to OD. Often, governments and NGOs lack the longer-term resources for monitoring (Venkataramanan,

2012). As a result, follow-up activity is left to village committees, most of which do not function properly. These local committees are created on paper but less equipped. Follow-up and sensitizations require equipment such as means of transport (bike or motorcycle), loud-speakers and telephones that these committees do not have. In addition, very few of these committees receive training on how to raise awareness, and even fewer on how to follow-up and report to the municipality for centralized data. They struggle to play their role in maintaining this status over the long-term (Kouadio, 2019). These committees are made-up of community leaders. A failure of the monitoring committee is therefore synonymous with a failure of leadership. In CLTS, "natural leaders" are expected to emerge follow-up progress and encourage maintenance. Natural leaders are enthusiastic about eradicating OD in their region, although their initial commitment may erode over time as new priorities emerge (Galvin, 2015). As a result of monitoring challenge at leadership level, the goal of achieving total sanitation under CLTS to eliminate health risks in the long-term is far from becoming a reality in many communities.

- **External pressure and financing**

O'Reilly and Louis (2014) highlighted that the urgency of meeting the Millennium Development Goals, sanitation targets and the pressure on governments from the international community were important factors for investing in sanitation. Due to international pressure, subsidies under the CLTS were used in India to motivate people to build toilets. According to DGAEUE (2014), which is the General Directorate of Sanitation of Burkina Faso, CLTS is adapted and implemented in Burkina like India without taking into account its original specificity i.e. a non-subsidy approach. CLTS is partially funded by donors due to the high poverty rate in rural areas. With the subsidy, the financing of the infrastructures is partially the responsibility of the households, who contribute through the excavation and the construction of the superstructure (DGAEUE, 2014). This hybrid approach distorts the main principles of CLTS of total management of sanitation problems by the community and the exclusion of external financial assistance. In this hybrid approach, the "T" in referring to TOTAL in CLTS no longer makes sense. There is no longer total community ownership. The sustainability of ODF-status as a result of this hybrid approach could be fragile. The adaptation of CLTS by some governments by providing subsidies to communities and the pressure to achieve the SDGs could be the causes of the slippage of the ODF-status in certain communities because this is not different from previous subsidized sanitation approaches that have shown their limitations in terms of sustainability. Indeed, many governments & NGOs being obsessed by the idea of reaching the SDG at any cost, often distort the very essence of the CLTS (adaptation to their liking) by providing subsidies to communities which do not favor the real appropriation of latrines and the maintenance of good hygiene behaviors by communities in the long term, thus contributing to the slippage of ODF-status sometime after the end of project.

- **Lack of demand for sanitation and underlying control of implementing structures and funders**

Whittington et al. (1990) considers the analysis of the real demand of the populations as a guarantee of a better success of sanitation projects. According to Duflo (2010), development policies are most effective and sustainable when they are inspired by the needs of the populations concerned. But in the case of CLTS, there is no real demand from the communities to build latrines for them. This element, which is a guarantee of success and sustainability of sanitation projects, is absent in CLTS and could be one of the reasons for the return to OD after obtaining ODF-status.

According to Galvin (2015), one of the reasons for the failure of sanitation approaches to lead to sustainable behavior change by CTLS is that they are externally driven by the implementing structures or donors supporting CLTS approach. The entire CLTS approach is formulated and introduced by outsiders and international organizations that are often the drivers - World Bank, USAID, WSSCC, Bill and Melinda Gates Foundation, UNICEF, etc. (Deak, 2008; Global Sanitation Fund, 2016). In reality, there is an underlying element of control: communities may be implementing, but the path has been defined by these organizations (Galvin, 2015). These organizations, because of their influence and weight in the sanitation sector, may create an imbalance or selectivity bias, an application bias of CLTS approach different from that intended by the designer. The national governments particularly in Africa have little commitment to promote CLTS (Fig. 4). Sanitation sector is heavily dependent on external funding in Africa than in Asia (WHO, 2017). The lack of commitment of some governments to combine CLTS with a monitoring and continuous improvement system and failure to learn through sharing experience after certification are obstacles to the sustainability of CLTS (Zuin et al., 2019).

- **Low quality of CLTS implementation and inexperience of facilitators**

Since CLTS is implemented by the community, almost all factors tend to blame the communities. Although the community is at the center of this approach, it is not the only entity involved in the process. It is important to point out that the low quality of implementation of the trigger and the inexperience of some facilitators leads to fragile ODF-status. A weakly facilitated trigger generates a fragile ODF-status. No one in the world wants to ingest faeces. Once the idea is rooted in the mind of the community during the triggering, the resultant ODF-status that is likely to be sustainable. Comparing the results of 2 CLTS interventions in Ethiopia and Ghana, Crocker et al. (2017) found that in Ethiopia health workers were more effective facilitators than teachers, and that in Ghana, training of natural leaders has improved the outcomes and sustainability of CLTS. Crocker et al. (2016b) therefore concluded that in Ethiopia, teachers should be trained to support CLTS facilitation and that CLTS was not an appropriate intervention when baseline OD was low. From Ghana, Crocker et al. (2016a) concluded that natural leader training can lead to high reductions in OD and has permanent sustainability when targeted at socially cohesive villages. In both cases, CLTS should not be a stand-alone strategy to address sanitation, as it was not effective in all contexts, and resulted in latrines with low sustainability.

4.2.5. Socio-political factors

In addition to these four categories of factors found in the literature review that have led to slippage to OD after CLTS implementation, the authors considered that "socio-political factors" as well did cause the slippage of certain communities. The socio-political factors are not dependent on either the community or the CLTS implementing structure. This category arises from a research on the sustainability of CLTS conducted by the authors for UNICEF in Burkina in 2021 (Unpublished data).

The elements that make up this category of factors are:

- **Migration/community relocation**

Movement is inherent in low-income countries affected by political, terrorism, humanitarian crises, or within nomadic communities. In this type of countries, the migration or relocation of communities could be one of the factors of slippage towards the OD following CLTS implementation. Coming to new environments, often in different contexts some communities may lose ODF-status if the hygiene and sanitation practices advocated by CLTS are not deeply rooted in their habits. Throughout the life cycle of ODF communities, it is not envisaged that people from outside the CLTS communities might emigrate to the community after ODF-certification. For example, in countries facing the spread of terrorism or humanitarian crises, communities unaware of CLTS principles and fleeing terrorism may move to safer areas where

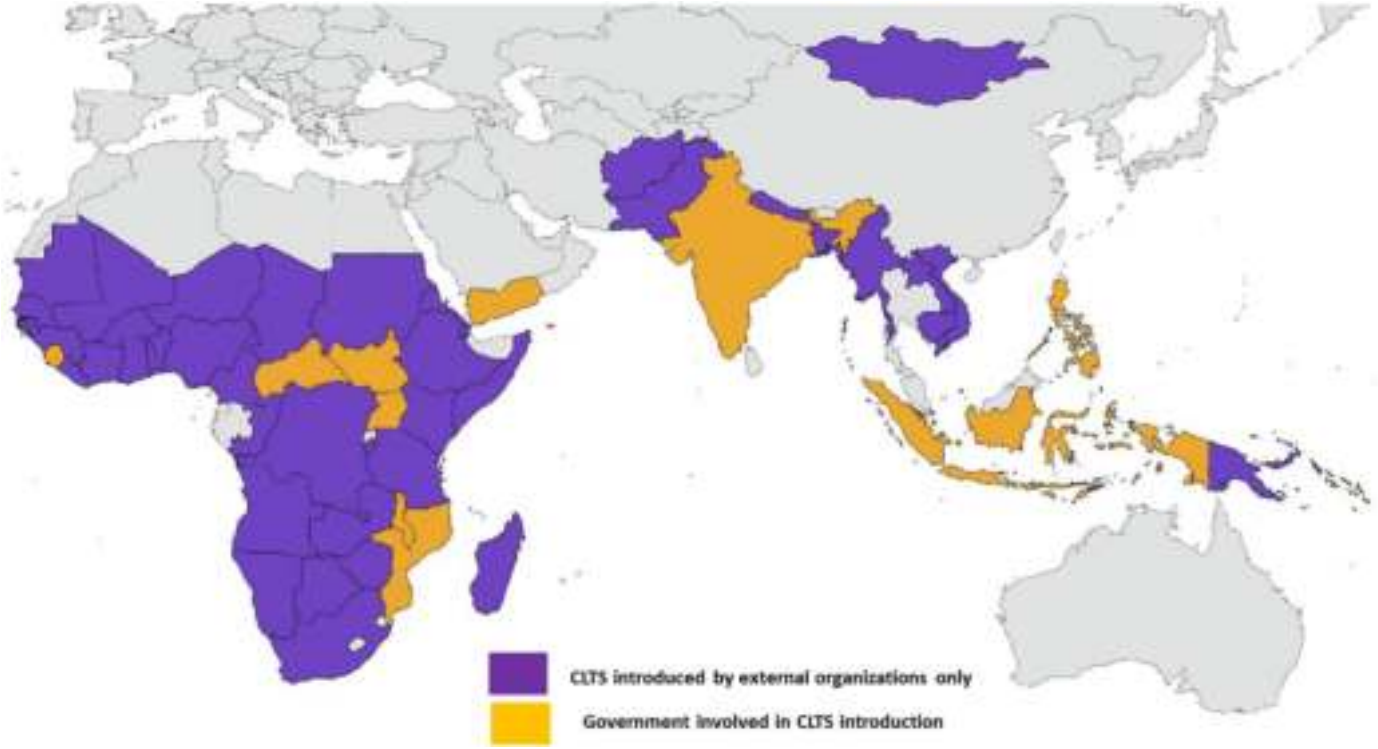

Fig. 4. Institutions that initially introduced CLTS in the 59 countries. External organizations (UNICEF, World Bank, etc.) played a role in all countries, either with (12/59, orange) or without (47/59, purple) initial government involvement. Sources: (Zuin et al., 2019). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

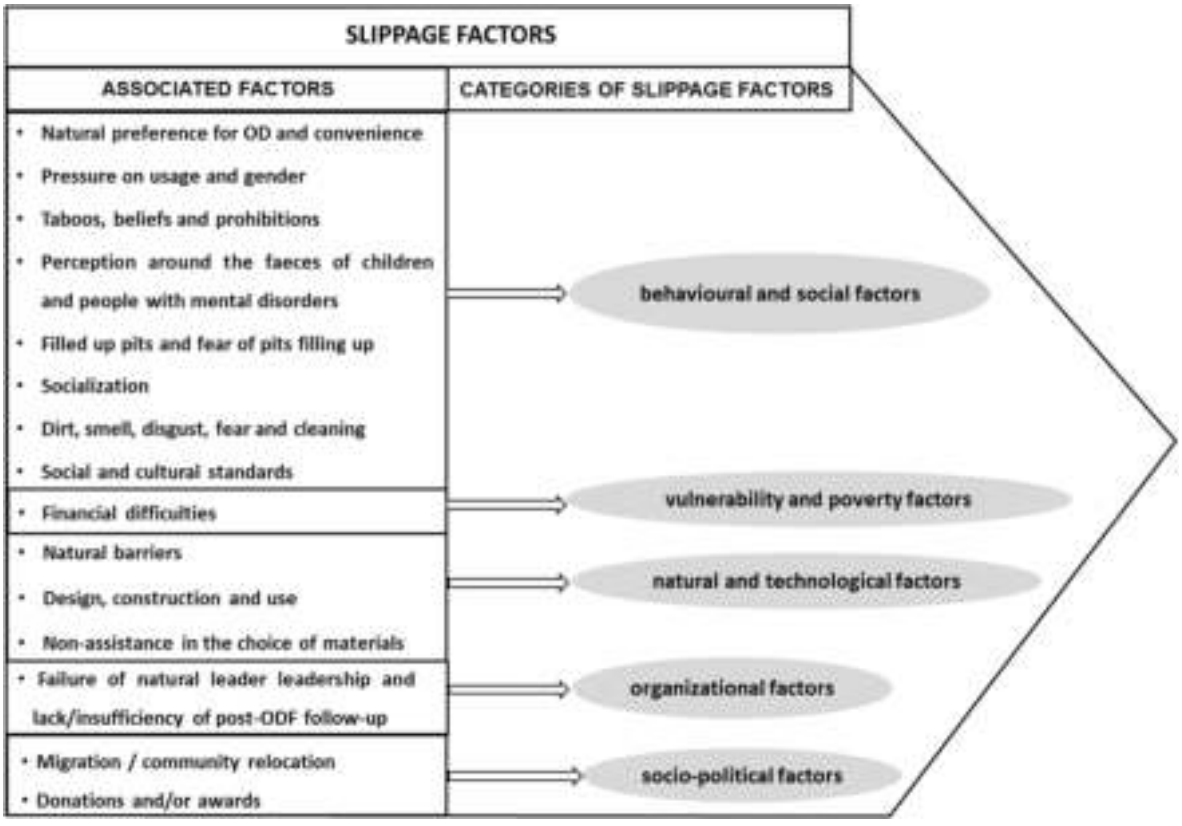

Fig. 5. Synoptic view of all slippage factors towards OD following CLTS implementation.

CLTS has been implemented. This mix of ODF-certified communities and communities with no knowledge of the health risks associated with the practice of OD, who cannot afford to build a house let alone a latrine due to lack of resources, could lead to slippage towards OD in the ODF village they have entered. This was the case in Burkina according to the study conducted by the authors for UNICEF Burkina in 2021 (Unpublished data).

- **Donations and/or awards**

Similar to community relocation, the literature hardly mentions gifts and rewards as factors that can influence slippage. Gifts and rewards instituted by the government can also distort and undermine the sustainability of ODF-status. This mainly applies where incentives have been used to achieve ODF. The communities may be motivated by the need to achieve targets solely for the acquisition of donations and not because they have understood the importance of having and using latrines.

Although CLTS has prohibited any subsidies or external financial contribution to the achievement of the works, it has not prohibited rewards as incentives. For example, certain infrastructures (schools, boreholes, etc.) are promised as rewards when ODF-status is achieved. These rewards sometimes represent a limit to the fundamental principles of CLTS. While rewards can be effective short-term incentives, they have drawbacks and can threaten the principles of CLTS. Indeed, communities may be diverted from their goal by working for the reward, when the target should be to achieve and maintain ODF-status. This was the case in Burkina according to the study conducted by the authors for UNICEF Burkina in 2021 (Unpublished data).

Fig. 5 is a graphical summary that provides a synoptic view of all factors grouped by category that cause ODF-status slippage after CLTS implementation.

5. Discussions

5.1. *Analysis of the slippage factors*

Sanitation interventions such as CLTS seek to change human patterns of OD, but rarely consider the socio-spatial dynamics and environmental factors that support the sustainability of toilet use (Jewitt, 2011). Projects ignore how family political relationships (e.g., women's lack of decision-making power) and variable access to resources (e.g., periodic water shortages) impact toilet use by all family members. Sanitation practitioners and researchers have recognized that toilet interventions must go beyond building toilets. Interventions should therefore include the social and economic factors that will lead to toilet adoption. Researchers have emphasized that toilet adoption depends on the provision of appropriate toilet designs (Devine, 2009), community involvement (Kar and Chambers, 2008), state involvement (Black and Fawcett, 2008), finding specific solutions at the local level (Waterkeyn and Cairncross, 2005), and understanding people's ideas and values regarding sanitation (Drangert and Nawab, 2011; Rheinländer et al., 2010). These elements are intrinsically linked for the sustainability of latrines and communities' hygiene and sanitation behaviors. Thus, neglect or even poor implementation of any of these elements is likely to lead to slippage. CLTS approach focuses primarily on the eradication of OD through the construction of latrines by the community.

CLTS approach is not interested in understanding people's ideas and values about sanitation but rather exposing communities to the fecal-oral cycle and the resulting health risks on them. It ignores the perceptions of the target community regarding OD and the motivations which drive them to practice OD. It does not integrate them in its approach but seeks above all and through a set of already predefined activities to show the communities the harmful effects of this practice on their health and the economic consequences that result from it. The critical starting point for ensuring sustainability of latrines and behaviors was to understand perceptions of impurity of different types of human waste and forms of excretion in communities. This is not the case in CLTS. The work of Robinson (2009) and Joshi et al. (2011), on the other hand, indicates that communities, even poor communities, already know good hygiene behaviors but lack the means and incentives to build facilities. In any case, when slippage occurs, that is when the implementing structures become interested in community perceptions of impurity, hygiene practices, excreta, and latrines. So, this should be done, assessed, and analyzed upstream well before the CLTS is triggered. However, it is important to bear in mind that, in many cases, often communities declared ODF were never really so. In reality, ODF is rarely an absolute. There are degrees of ODF.

5.2. *Analysis of the desire trigger "shame"*

Jenkins and Curtis (2005) found in rural Benin that the lack of desire for a toilet was the main reason people don't build them. This desire is elicited in CLTS during a participatory self-assessment process aimed at collective shame. But can we really talk about shame when a practice affects all members of a community and is culturally accepted? Clearly, the answer is no. The basis of shame in CLTS may therefore prove in some communities to be ineffective not only for building but also for maintaining long-term ODF-status.

5.3. *Analysis of the family situation and priorities*

Cost, lack of credit, maintenance, soil type, and family issues were identified as constraints to latrine adoption and ODF sustainability. The construction and maintenance of latrine require financial resources. CLTS is typically implemented in rural communities of low-income countries. These communities are financially limited and since CLTS does not provide financial support in most cases, they opt for local materials that are cheaper whether they are poor or good quality, durable or non-durable or not compatible with the natural conditions of the area. In many scenarios, the communities have to choose between solving family financial problem (death of a family member, lack of school fees, etc.) or maintaining latrines. Priority is mostly given to family problems to the detriment of latrines. This leads to a return to OD and thus a slippage in terms of the criteria for certification of ODF-status.

5.4. *Analysis of the slippage cross-checking process*

Sustainability statistics assume that communities were ODF at baseline and met all of the criteria for the cross-checking. Very few studies provide values for the slippage rate and there is a severe lack of a baseline situation before the CLTS was triggering. This slippage rate may be exaggerated if the original verification and certification was of lower quality than the cross-check. This may be the case when communities "fake it" during the cross-check. Very few programs include cross-checks after ODF-certification in their planning. Most often, implementing structures and their donors do not have a strategy for making continuous improvements after ODF-status or for reversing slippage. Surprisingly, even with the support of international organizations such as World Bank and UNICEF, the systems do not follow-up and collect data on the impact and sustainability of CLTS. To date, most analyses are based on evidence from selected cases based on authors views. It is difficult to determine whether slippage reports refer to household members returning to ODF or to the number of communities that were unable to maintain ODF-status or failed to meet other ODF criteria (such as handwashing or drinking water protection). Baseline data on latrine coverage is not collected systematically, leading to difficulties in estimating progress (Venkataramanan, 2016; Venkataramanan et al., 2015). The ODF program data, are generally not verified by third-party. This often leads to inaccuracies and inflation of results. More generally, implementing organizations may be naturally deterred from reporting disappointing results.

Any evaluation is credible if it is conducted externally by people who are not involved in the process. However, the mobilization of these outsiders and their expertise requires funding. So, where do we find the funding for an evaluation when the implementation is done by the community and does not require funding?

5.5. *Limitations in slippage materialization*

In majority of cases, obtaining the ODF-status is evidenced by a celebration party or displaying the ODF-status on a banner at strategic places in the village (at the entrance or exit). This way, everyone who comes to this community has an idea of the sanitation practices that take place there. In the opposite, there is no effigy of OD status to symbolize slippage of a community to OD following a counter-verification. A visualization of loss of ODF-status could lead the community to question itself and regain awareness of its sanitation situation, and subsequently develop mechanisms to counter the slippage and regain its status.

5.6. *Limitations to scaling up on the sanitation ladder during post-ODF follow-up or cross-checking*

The literature should also look at the level of the post-ODF follow-up or even during the cross-check to evaluate the increase of communities on the sanitation ladder (Fig. 6). The decrease in OD during the Ghana interventions was the same as an increase in household latrine ownership, but the quality and condition of latrines remained relatively constant over time. There was no substantial change in latrine quality in the year following the interventions (Crocker et al., 2017). Certainly, CLTS aims in the short-term to provide each household with at least one latrine and to end the OD, but in the long-term these households should be able to climb the sanitation ladder and reach the maximum according to their means. Studies should therefore be conducted to this end. Climbing the sanitation ladder for a household implies that sanitation behaviors have improved and are likely to be sustainable. Post-ODF monitoring should also consider the evolution on the sanitation ladder.

In addition to identifying slippage factors of ODF-status, why shouldn't we consider the reasons why some communities are parked at one level of the sanitation ladder even if it remains ODF?

As global water scarcity prevails, water-intensive flush latrines can no longer be considered the ideal target for access to safe sanitation. Indeed, in rural areas, communities are very often faced with water access problems. Global population growth and the effects of climate change will exacerbate this water access problem in these already vulnerable communities. The lack of water may also justify the choice of these communities to turn to less or no water-consuming types of latrines in CLTS. The flush latrines, even if they allow a safe evacuation of excreta, are costly and water intensive. It is therefore necessary to review the elements of the sanitation ladder by taking into account the scarcity of water resources and by developing safe, durable and less water consuming latrines.

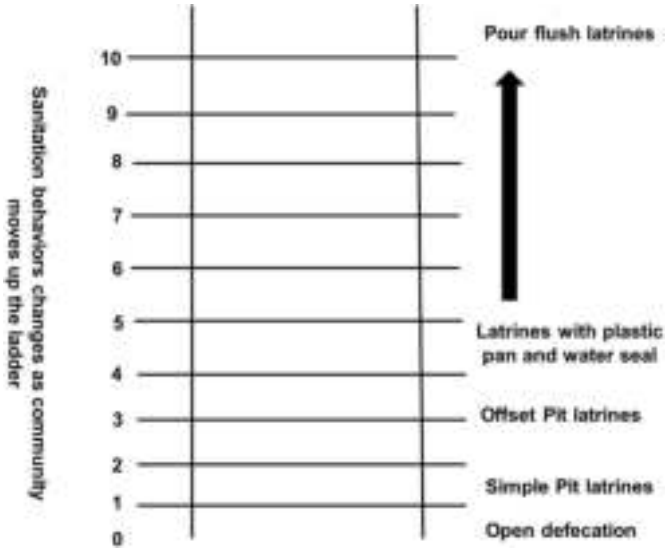

Fig. 6. CLTS sanitation ladder (Kar and Chambers, 2008).

5.7. *Suggested actions to sustain ODF status*

To reduce the rate of ODF-certified villages that slip back to OD, it is imperative to tailor solutions and strategies to each area. In order to limit instances of slippage towards OD, the following actions are suggested:

- **Include all stages of the sanitation value chain (SVC) in the CLTS**

With the objective of ending OD through the construction of latrines by the community itself, CLTS focuses only on the first stage of the SVC: "Containment". This is a limitation of CLTS, which was one of the reasons for the slippage to OD, as some households did not know what to do when the pits were full since they were not familiar with the other stages (Emptying, Transport, Treatment and Reuse/Disposal) of the SVC. For the CLTS to be sustainable, it would need to be either enhanced by including the other SVC phases or coupled with other services or approaches which include them. In addition to a post-ODF monitoring committee, CLTS should include the creation of a local association for emptying the sludge from the pits, and another to use the sludge as fertilizer for agriculture, or the training of the community in sludge treatment processes such as composting. These CLTS innovations will allow communities to not only limit the number of cases of slippage but also to provide financial benefits to the members of these associations.

- **Follow-up after achieving ODF status**

Post-certification follow-up is a crucial step for the sustainability of ODF-status. Follow-up visits are essential once ODF-status is achieved to maintain the new social standards advocated by CLTS. A frequency of two post-certification visits per month during the first year would produce good results. These follow-up visits should be done at the local level, by volunteer community members grouped in a monitoring and awareness-raising committee. This can be followed also by committed natural leaders whose voices are highly respected or by health workers. To facilitate the collection of data for monitoring and to have information in real time, new information and communication technologies can be popularized in the CLTS. Village committees in charge of post-ODF follow-up and awareness should be equipped with smartphones or tablets on which data collection applications such as Open-Data-Kit (ODK), CommCare or KoboCollect will be installed, allowing them to check whether or not there has been an improvement of the latrine compared to their last monitoring visits. This data, which will then be transferred to a larger scale (municipality, ministry), can provide insight into the evolution on sanitation ladder after the CLTS. The reliability of these results can be verified by taking photos, as well as GPS coordinates of the latrine. It will be possible to see if after a certain period of time, a household for example has made improvements to its latrine (by moving up the sanitation ladder), if it is the same latrine since the ODF certification, or if it has collapsed. Sensors connected to a cell phone application can also be installed in the pits to indicate the filling rate of the pit to its users so that they can better anticipate.

- **Promoting access and supply of materials: Development of Sanitation Marketing**

Better quality latrines are more likely to last and be floods. It is therefore timely to revise the CLTS approach, especially in the choice of

construction materials. CLTS quickly creates a demand for sanitation materials. While many households start with simple pit latrines, others opt for a higher standard and a more durable solution. In addition, those who start with pit latrines usually want to upgrade their toilets. Therefore, market access to good quality sanitation materials and services at an affordable price can be a prerequisite for sustainable latrine construction and maintenance of safe sanitation behavior. Construction materials should be provided through sanitation stores in or near ODF villages as part of the implementation of Sanitation Marketing. These stores will provide quality latrine building materials and increase income of the sanitation store owner. Another condition is that the households, the masons and the sanitation store owner have enough technical knowledge to monitor and guarantee the quality of the materials and the construction of the latrines. These services will generate revenue for the provider and will also be a means of combating slippage.

- **Sanctions against the practice of OD**

Sanctions (financial fines, taking or threatening to take photographs and publishing them; collecting faeces with the hand and putting them in the latrine, etc.) against those who continue to practice OD could play a role in combating the slippage. These sanctions can fall into two categories: those decided and enforced by the community and those legally imposed by the state. The implementation of sanctions should be facilitated with great caution as it can lead to the exclusion of the most vulnerable.

As CLTS has shown its limitations in several respects, including eliminating and sustaining community behavior change toward OD, it may be time to look at other approaches and concepts such as human-centered design to assess its results and evaluate its effectiveness on behavior change. Subsequently, comparing it to previous community-based approaches will help determine which of these approaches is best for a given context. Indeed, one context may be very favorable to one of the existing community-based approaches, while another is very suitable for human-centered design. It is an unexplored avenue to adapt 'human-centered design' to CLTS for better sustainability. An approach like "human-centered design" is a problem-solving technique that puts real people at the center of the development process, resulting in products and services that resonate with and are tailored to the needs of the target community. The goal is to keep users' desires, pain points and preferences in mind at every phase of the process. More intuitive and accessible products that can generate greater benefits are created because the target community has already approved the solution and feels more invested in using it. Human involvement usually occurs during the initial observation of the problem in context, brainstorming, conceptualization, design development and implementation of the solution. This approach enhances effectiveness and efficiency, improves human well-being, user satisfaction, accessibility, and sustainability, and counteracts potential negative effects of use on human health, safety, and performance. This could address many of the weaknesses of the CLTS approach.

6. Conclusion

The slippage towards OD following CLTS implementation is due to various factors namely: social and behavioral factors, technical and material factors, vulnerability and poverty factors, organizational factors and socio-political factors. We should avoid overestimating the CLTS as an approach in the elimination of OD by being aware of its weaknesses, including slippage to OD. CLTS approach was globally successful in encouraging households to build latrines. However, they have not succeeded in achieving long-term use of latrines and maintaining hygienic behaviors. It is also important to emphasize that adoption of a latrine alone cannot guarantee sustainability. It is therefore imperative to adapt solutions to each area, even to each village, and to ensure long-term mechanisms are in place to support change of behaviors on a sustainable basis. In view of the rapid return to OD of some ODF-certified communities and the poor quality of latrines achieved under CLTS, the question that needs to be asked is "Can CLTS be said to contribute to the actual achievement of SDGs?". Political and economic instability, natural disasters, and other external factors which are beyond the control of any project can threaten its sustainability. Finally, it should be pointed out that, when the end is short term and not sustainable it is important to look at the enabling and constraining factors necessary to achieve it. So, the most difficult thing is not to reach the top, but how to stay there in order to yield the much-desired results and maximise impact. Generating evidence on longer-term outcomes of sanitation interventions is a research priority.

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Declaration of competing interest

The authors declare no conflict of interest.

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References

Abdi, R., 2016. Open Defecation Free Sustainability Study in East Timor 2015-2016. Dili Water Aid.

Abebe, T.A., Tucho, G.T., 2020. Open defecation-free slippage and its associated factors in Ethiopia: a systematic review. Syst. Rev. 9, 1–15. https://doi.org/10.1186/s13643-020-01511-6.

Abramovsky, L., Augsburg, B., Oteiza, F., 2015. Sustainable total sanitation–Nigeria baseline report. Inst. Fisc. Stud. Collab. Ln-Depth Precis. Consult. Niger. https://www.econstor.eu/bitstream/10419/201775/1/R134.pdf.

Afzal, A., Javed, M., Jabeen, T., 2022. Integrated behaviour change intervention for sustainable community development: a KAP study of WASH in district Gujrat, Pakistan. J. Water, Sanit. Hyg. Dev. https://doi.org/10.2166/washdev.2022.243.

Anjum Altaf, M., Hughes, J.A., 1994. Measuring the demand for improved urban sanitation services: results of a contingent valuation study in Ouagadougou. Burkina Faso. Urban Stud. 31, 1763–1776.

Apanga, P.A., Garn, J.V., Sakas, Z., Freeman, M.C., 2020. Assessing the impact and equity of an integrated rural sanitation approach: a longitudinal evaluation in 11 sub-Saharan Africa and Asian countries. Int. J. Environ. Res. Publ. Health 17. https://doi.org/10.3390/ijerph17051808.

Ashebir, Y., Sharma, H.R., Alemu, K., Kebede, G., 2013. Latrine use among rural households in northern Ethiopia: a case study in Hawzien district, Tigray. Int. J. Environ. Stud. 70, 629–636. https://doi.org/10.1080/00207233.2013.835533.

Ayalew, A.M., Mekonnen, W.T., Abaya, S.W., Mekonnen, Z.A., 2018. Assessment of Diarrhea and its associated factors in under-five children among open defecation and open defecation-free rural settings of Dangla district, Northwest Ethiopia, 2018 J. Environ. Public Health, 4271915. https://doi.org/10.1155/2018/4271915.

Azage, M., Haile, D., 2015. Factors associated with safe child feces disposal practices in Ethiopia: evidence from demographic and health survey. Arch. Publ. Health 73, 40. https://doi.org/10.1186/s13690-015-0090-z.

Barnard, S., Routray, P., Majorin, F., Peletz, R., Boisson, S., Sinha, A., Clasen, T., 2013. Impact of Indian total sanitation campaign on latrine coverage and use: a cross-sectional study in Orissa three years following programme implementation. PLoS One 8, e71438. https://doi.org/10.1371/journal.pone.0071438.

Bhatt, N., Budhathoki, S.S., Lucero-Prisno, D.E.I., Shrestha, G., Bhattachan, M., Thapa, J., Sunny, A.K., Upadhyaya, P., Ghimire, A., Pokharel, P.K., 2019. What motivates open defecation? A qualitative study from a rural setting in Nepal. PLoS One 14, e0219246. https://doi.org/10.1371/journal.pone.0219246.

Black, M., Fawcett, B., 2008. The last taboo: opening the door on the global sanitation crisis. Int. J. Sustain. High Educ. 10 https://doi.org/10.4324/9781849776325.

Bongartz, P., Vernon, N., Fox, J., 2016. Sustainable Sanitation for All: Experiences, Challenges and Innovations. Practical Action. https://doi.org/10.3362/9781780449272.

Cairncross, S., Shordt, K., 2004. It does last! Some findings from a multi-country study of hygiene sustainability. Waterlines 22, 4–7. https://doi.org/10.3362/0262-8104.2004.003.

Caruso, B.A., Clasen, T.F., Hadley, C., Yount, K.M., Haardörfer, R., Rout, M., Dasmohapatra, M., Cooper, H.L., 2017. Understanding and defining sanitation insecurity: women's gendered experiences of urination, defecation and menstruation in rural Odisha, India. BMJ Glob. Health 2, e000414. https://doi.org/10.1136/bmjgh-2017-000414.

Cavill, S., Chambers, R., Vernon, N., 2015. Sustainability and CLTS: Taking Stock. IDS. https://opendocs.ids.ac.uk/opendocs/handle/20.500.12413/5859.

Chambers, R., Myers, J., 2016. Norms, Knowledge and Usage. Frontiers of CLTS : Innovations and Insights. IDS. https://opendocs.ids.ac.uk/opendocs/handle/20.500.12413/8960.

Chebet, J.J., Kilungo, A., Alaofè, H., Malebo, H., Katani, S., Nichter, M., 2020. Local perceptions, cultural beliefs, practices and changing perspectives of handling infant feces: a case study in a rural Geita district, North-Western Tanzania. Int. J. Environ. Res. Publ. Health 17. https://doi.org/10.3390/ijerph17093084.

Coffey, D., Gupta, A., Hathi, P., CoKhurana, N., Spears, D., Srivastav, N., 2014. Revealed preference for open defecation. Econ. Polit. Wkly. 49, 43–55.

Coffey, D., Gupta, A., Hathi, P., Spears, D., Srivastav, N., 2015. Culture and Health Transition-Understanding Sanitation Behavior in Rural North India. New Delhi, India, April: Unpublished Working Paper. Res. Inst. Compassionate Econ.

Coffey, D., Spears, D., Vyas, S., 2017. Switching to sanitation: understanding latrine adoption in a representative panel of rural Indian households. Soc. Sci. Med. 188, 41–50. https://doi.org/10.1016/j.socscimed.2017.07.001.

Cole, B., 2013. Développement d'un concept participatif pour l'assainissement. Aux Front. L'ATPC Innov. Impr. 1.

Crocker, J., Abodoo, E., Asamani, D., Domapielle, W., Gyapong, B., Bartram, J., 2016a. Impact evaluation of training natural leaders during a community-led total sanitation intervention: a cluster-randomized field trial in Ghana. Environ. Sci. Technol. 50, 8867–8875. https://doi.org/10.1021/acs.est.6b01557.

Crocker, J., Geremew, A., Atalie, F., Yetie, M., Bartram, J., 2016b. Teachers and sanitation promotion: an assessment of community-led total sanitation in Ethiopia. Environ. Sci. Technol. 50, 6517–6525. https://doi.org/10.1021/acs.est.6b01021.

Crocker, J., Saywell, D., Bartram, J., 2017. Sustainability of community-led total sanitation outcomes: evidence from Ethiopia and Ghana. Int. J. Hyg Environ. Health 220, 551–557. https://doi.org/10.1016/j.ijheh.2017.02.011.

Deak, A., 2008. Taking Community-Led Total Sanitation to Scale : Movement, Spread and Adaptation. Working Paper Series, vol. 298. IDS, Brighton.

Devine, J., 2009. Introducing SaniFOAM: A Framework to Analyze Sanitation Behaviors to Design Effective Sanitation Programs - Global Scaling up Sanitation Project. World Bank, Water and Sanitation Program (WSP), USA. http://documents.worldbank.org/curated/en/272351468334778050/Introducing-SaniFOAM-a-framework-to-analyze-sanitation-behaviors-to-design-effective-sanitation-programs.

DGAEUE, 2014. Guide d'orientation pour la mise en oeuvre de l'assainissement total pilote par les communautés-ATPC au BURKINA FASO, vol. 155p. https://www.pseau.org/outils/ouvrages/dgaeue_unicef_guide_d_orientation_pour_la_mise_en_oeuvre_de_l_assainissement_total_pilote_par_la_communaute_au_burkina_faso_2014.pdf.

Dickin, S., Gautam, O., 2019. Behaviour change sustainable sanitation Alliance. Available from. https://www.susana.org/en/workinggroups/behaviour-change#.

Drangert, J.-O., Nawab, B., 2011. A cultural–spatial analysis of excreting, recirculation of human excreta and health—the case of North West Frontier Province, Pakistan. Health Place 17, 57–66. https://doi.org/10.1016/j.healthplace.2010.08.012.

Dreibelbis, R., Jenkins, M., Chase, R.P., Torondel, B., Routray, P., Boisson, S., Clasen, T., Freeman, M.C., 2015. Development of a multidimensional scale to assess attitudinal determinants of sanitation uptake and use. Environ. Sci. Technol. 49, 13613–13621. https://doi.org/10.1021/acs.est.5b02985.

Duflo, E., 2010. Le développement humain (Lutter contre la pauvreté, volume 1). Le seuil. Lutter Contre Pauvr, Paris.

Everett, 1995. Diffusion of Innovation. Free Press, New York, 4th édition.

Freeman, M.C., Stocks, M.E., Cumming, O., Jeandron, A., Higgins, J.P.T., Wolf, J., Prüss-Ustün, A., Bonjour, S., Hunter, P.R., Fewtrell, L., Curtis, V., 2014. Hygiene and health: systematic review of handwashing practices worldwide and update of health effects. Trop. Med. Int. Health TM IH 19, 906–916. https://doi.org/10.1111/tmi.12339.

Galan, D.I., Kim, S.-S., Graham, J.P., 2013. Exploring changes in open defecation prevalence in sub-Saharan Africa based on national level indices. BMC Publ. Health 13, 527. https://doi.org/10.1186/1471-2458-13-527.

Galvin, M., 2015. Talking shit: is Community-Led Total Sanitation a radical and revolutionary approach to sanitation? WIREs Water 2, 9–20. https://doi.org/10.1002/wat2.1055.

Garn, J.V., Sclar, G.D., Freeman, M.C., Penakalapati, G., Alexander, K.T., Brooks, P., Rehfuess, E.A., Boisson, S., Medlicott, K.O., Clasen, T.F., 2017. The impact of sanitation interventions on latrine coverage and latrine use: a systematic review and meta-analysis. Int. J. Hyg Environ. Health 220, 329–340. https://doi.org/10.1016/j.ijheh.2016.10.001.

Gedda, M., 2015. Traduction française des lignes directrices PRISMA pour l'écriture et la lecture des revues systématiques et des méta-analyses. Kinésithérapie Rev. 15, 39–44. https://doi.org/10.1016/j.kine.2014.11.004.

Global Sanitation Fund, 2016. Global Sanitation Fund Progress Report 2016. Okechukwu Umelo. URL. https://okechukwuumelo.com/2016/04/14/global-sanitation-fund-progress-report/ (accessed 4.April.21).

Hanchett, S., Krieger, Laurie, Kahn, Mohidul Hoque, Kullmann, Craig, Ahmed, Rokeya, 2011. Long-term sustainability of improved sanitation in rural Bangladesh [WWW Document]. URL. https://openknowledge.worldbank.org/handle/10986/17347 (accessed 3.March.21).

Harter, M., Mosler, H.-J., 2018. Determining the effectiveness and mode of operation of Community-Led total Sanitation: the DEMO-CLTS study. https://www.globalwaters.org/sites/default/files/eawag-demo-clts-final-report.pdf.

Hickling, S., 2019. Tackling slippage", Frontiers of CLTS: innovations and insights 14 Brighton: IDS. https://opendocs.ids.ac.uk/opendocs/bitstream/handle/20.500.12413/14711/Issue%2014_Tackling%20slippage_FINAL.pdf?sequence=1&isAllowed=y.

House, S., Cavill, S., 2015. Rendre l'assainissement et l'hygiène plus sûrs–Réduire les vulnérabilités face à la violence'. Aux Front. L'ATPC Innov. Impr. https://opendocs.ids.ac.uk/opendocs/bitstream/handle/20.500.12413/6086/Issue%205%20-%20gender,%20violence%20and%20WASH%20French%20FINAL.pdf?sequence=9&isAllowed=y.

Hulland, K., Martin, N., Dreibelbis, R., Valliant, J.D., Winch, P., 2015. What factors affect sustained adoption of safe water, hygiene and sanitation technologies? A systematic review of literature. EPPI-Cent. Soc. Sci. Res. Unit UCL Inst. Educ. Univ. Coll. Lond. Lond. https://doi.org/10.13140/RG.2.1.5031.4329.

Institute of Development Studies, 2019. The CLTS approach Retrieved January 11, 2021, from. http://www.communityledtotalsanitation.org/page/clts-approach.

Iyer, R., Pare Toe, L., 2022. Impact of climate hazards on rural sanitation and hygiene practices in Burkina Faso. , SLH learning Brief 12, the sanitation learning hub, Brighton: IDS. https://doi.org/10.19088/SLH.2022.016.

Jenkins, M.W., Curtis, V., 2005. Achieving the "good life": why some people want latrines in rural Benin. Soc. Sci. Med. 61 https://doi.org/10.1016/j.socscimed.2005.04.036, 1982.

Jewitt, S., 2011. Geographies of shit: spatial and temporal variations in attitudes towards human waste. Prog. Hum. Geogr. 35, 608–626. https://doi.org/10.1177/0309132510394704.

Jiménez, A., Jawara, D., LeDeunff, H., Naylor, K.A., Scharp, C., 2017. Sustainability in practice: experiences from rural water and sanitation services in west Africa. Sustainability 9, 403. https://doi.org/10.3390/su9030403.

JMP, 2021. Progress on household drinking water, sanitation and hygiene 2000–2020: five years into the SDGs [WWW Document]. URL. https://www.who.int/publications-detail-redirect/9789240030848, 5.27.22.

Joshi, D., Fawcett, B., Mannan, F., 2011. Health, hygiene and appropriate sanitation: experiences and perceptions of the urban poor. Environment and Urbanization. Environ. Urbanization 23 (1), 91–111. https://doi.org/10.1177/0956247811398602, 23, 91–111.

Kar, K., Chambers, R., 2008. Handbook on community-led total sanitation. http://www.communityledtotalsanitation.org/sites/communityledtotalsanitation.org/files/cltshandbook.pdf.

Kar, K., Pasteur, K., 2005. Subsidy or Self-Respect? Community Led Total Sanitation. An Update on Recent Developments. Institute of Development Studies. https://opendocs.ids.ac.uk/opendocs/bitstream/handle/20.500.12413/4052/Wp257.pdf.

Kouadio, E.K., 2019. L'ATPC dans le Bounkani : la difficile appropriation par les acteurs locaux. Papiers de la Fondation Croix- Rouge. https://www.nzassa-revue.net/admin/img/paper/33.%20KOUASSI%20Kouadio%20Edouard.pdf.

Kulkarni, S., O'Reilly, K., Bhat, S., 2017. No relief: lived experiences of inadequate sanitation access of poor urban women in India. Gend. Dev. 25, 167–183. https://doi.org/10.1080/13552074.2017.1331531.

Lanata, C.F., Huttly, S.R., Yeager, B.A., 1998. Diarrhea: whose feces matter? Reflections from studies in a Peruvian shanty town. Pediatr. Infect. Dis. J. 17, 7–9.

Laré, A., Briand, A., Kéré, E.N., 2018. L'accès à l'assainissement dans les quartiers précaires de Ouagadougou Access to sanitation in precarious districts of Ouagadougou. Actual. Économique-Rev. Anal. Économique 94.

Lauria, D.T., Alfredo, H.C., Anthony, A.K., 1997. Final report on willingness to pay for improved water and sanitation in Dakar. In: Rep. Senegal Natl. Water Soc. Governement Senegal Word Bank Unpubl. Chap. Hill N. C.

Majorin, F., Torondel, B., Chan, G.K.S., Clasen, T., 2019. Interventions to improve disposal of child faeces for preventing diarrhoea and soil-transmitted helminth infection. Cochrane Database Syst. Rev. https://doi.org/10.1002/14651858.CD011055.pub2.

Majorin, F., Torondel, B., Routray, P., Rout, M., Clasen, T., 2017. Identifying potential sources of exposure along the child feces management pathway: a cross-sectional study among urban Slums in Odisha, India. Am. J. Trop. Med. Hyg. 97 https://doi.org/10.4269/ajtmh.16-0688.

Mateo, S., 2020. Procédure pour conduire avec succès une revue de littérature selon la méthode PRISMA. Kinésithérapie Rev. 20, 29–37. https://doi.org/10.1016/j.kine.2020.05.019.

Mehta, L., 2011. Introduction. Why shit matters: community led total sanitation and the sanitation challenge for the 21st century. Potential Community-Led Total Sanit. Rugby Pract. Action 1–22.

Mehta, L., Movik, S., 2011. The Dynamics and Sustainability of CLTS: Mapping Challenges and Pathways. Shit Matters: the Potential of Community-Led Total Sanitation. Practical Action Publishing. https://doi.org/10.3362/9781780440347.016.

Milanesi, J., 2007. La méthode d'évaluation contingente en question: critique, requalification et mesure de la demande en assainissement à Moshi (Tanzanie) (Pau).

Miller-Petrie, M.K., Voigt, L., McLennan, L., Cairncross, S., Jenkins, M.W., 2016. Infant and young child feces management and enabling products for their hygienic collection, transport, and disposal in Cambodia. Am. J. Trop. Med. Hyg. 94 https://doi.org/10.4269/ajtmh.15-0423.

Moher, D., Liberati, A., Tetzlaff, J., Altman, D.G., 2009. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. BMJ 339, b2535. https://doi.org/10.1136/bmj.b2535.

Morita, T., Godfrey, S., George, C.M., 2016. Systematic review of evidence on the effectiveness of safe child faeces disposal interventions. Trop. Med. Int. Health TM IH 21. https://doi.org/10.1111/tmi.12773.

Mugel, S.G., Clasen, T.F., Bauza, V., 2022. Global practices, geographic variation, and determinants of child feces disposal in 42 low- and middle-income countries: an

analysis of standardized cross-sectional national surveys from 2016 – 2020. Int. J. Hyg Environ. Health 245, 114024. https://doi.org/10.1016/j.ijheh.2022.114024.
Mukherjee, I., Mukherjee, N., 2017. Designing for sustainable outcomes: espousing behavioural change into co-production programmes. Polic. Soc. 37, 326–346. https://doi.org/10.1080/14494035.2018.1383032.
Mukherjee, N., Robiarto, A., Saputra, E., Wartono, D., 2012. Achieving and sustaining open defecation free communities: learning from East Java. In: Rep. WSP Wash. DC World Bank.
Novotný, J., Hasman, J., Lepič, M., 2018. Contextual factors and motivations affecting rural community sanitation in low- and middle-income countries: a systematic review. Int. J. Hyg Environ. Health 221, 121–133. https://doi.org/10.1016/j.ijheh.2017.10.018.
Odagiri, M., Muhammad, Z., Cronin, A.A., Gnilo, M.E., Mardikanto, A.K., Umam, K., Asamou, Y.T., 2017. Enabling factors for sustaining open defecation-free communities in rural Indonesia: a cross-sectional study. Int. J. Environ. Res. Publ. Health 14, 1572. https://doi.org/10.3390/ijerph14121572.
Opel, A., Cheuasongkham, P., 2015. Faecal Sludge Management Services in Rural Laos: Critical Gaps and Important Ways Forward, présentation, 19 mars 2015, Conférence internationale sur la gestion des boues fécales.
O'Reilly, K., Louis, E., 2014. The toilet tripod: understanding successful sanitation in rural India. Health Place 29. https://doi.org/10.1016/j.healthplace.2014.05.007.
Osumanu, I.K., Kosoe, E.A., Ategeeng, F., 2019. Determinants of open defecation in the wa municipality of Ghana: empirical findings highlighting Sociocultural and economic dynamics among households, 2019 J. Environ. Public Health 1–10. https://doi.org/10.1155/2019/3075840.
Pasteur, K., 2017. Keeping track: CLTS monitoring, certification and verification, CLTS knowledge hub learning paper. Brighton: IDS. https://www.pseau.org/outils/ouvrages/ids_sida_keeping_track_clts_monitoring_certification_and_verification_2017.pdf.
Patil, S.R., Arnold, B.F., Salvatore, A.L., Briceno, B., Ganguly, S., Jr, J.M.C., Gertler, P.J., 2014. The effect of India's total sanitation campaign on defecation behaviors and child health in rural Madhya Pradesh: a cluster randomized controlled trial. PLoS Med. 11, e1001709 https://doi.org/10.1371/journal.pmed.1001709.
Patwa, J., Pandit, N., 2018. Open defecation-free India by 2019: how villages are progressing? Indian J. Community Med. Off. Publ. Indian Assoc. Prev. Soc. Med. 43, 246–247. https://doi.org/10.4103/ijcm.IJCM_83_18.
Pickering, A.J., Djebbari, H., Lopez, C., Coulibaly, M., Alzua, M.L., 2015. Effect of a community-led sanitation intervention on child diarrhoea and child growth in rural Mali: a cluster-randomised controlled trial. Lancet Global Health 3, e701. https://doi.org/10.1016/S2214-109X(15)00144-8 e711.
Protos, 2016. Assainissement Total Piloté par la Communauté (ATPC) dans les programmes de Protos et de ses partenaires : Note de discussion sur les facteurs qui favorisent ou rendent difficile l'obtention et le maintien de l'état FDAL.
RESEAU PROJECTION, 2015. L'ATPC : une méthode efficace ? Compte-rendu de la rencontre Jeunes Professionnels. Disponible sur. https://www.reseauprojection.org/wiki/images/c/c2/CR_Rencontre_JP_AS_19022015_GCo+YL.pdf.
Rheinländer, T., Keraita, B., Konradsen, F., Samuelsen, H., Dalsgaard, A., 2013. Smell: an overlooked factor in sanitation promotion. Waterlines 106–112. https://doi.org/10.3362/1756-3488.2013.012.
Rheinländer, T., Samuelsen, H., Dalsgaard Konradsen, F., 2010. Hygiene and sanitation among ethnic minorities in Northern Vietnam: does government promotion match community priorities? Soc. Sci. Med. 71 https://doi.org/10.1016/j.socscimed.2010.06.014, 1982.
Robinson, A., 2009. Sustainability and Equity Aspects of Total Sanitation Programmes: A Study of Recent WaterAid-Supported Programmes in Nigeria. WaterAid, Londres. www.communityledtotalsanitation.org/sites/communityledtotalsanitation.org/files/Nigeria_CLTS_synthesis_report.pdf.
Robinson, A., Bond, M., Kidd, R., Mott, J., Tyndale-Biscoe, P., 2016. Final Evaluation: Pan African CLTS Program 2010-2015 (Plan Neth).
Robinson, A., Gnilo, M., 2016. Promoting choice: smart finance for rural sanitation development. Sustain. Sanit. Exp. Chall. Innov. https://doi.org/10.3362/9781780449272.014.
Rotondo, L.A., Ngondi, J., Rodgers, A.F., King, J.D., Kamissoko, Y., Amadou, A., Jip, N., Cromwell, E.A., Emerson, P.M., 2009. Evaluation of community intervention with pit latrines for trachoma control in Ghana, Mali, Niger and Nigeria. Int. Health 1, 154–162. https://doi.org/10.1016/j.inhe.2009.08.001.
Routray, P., Schmidt, W.-P., Boisson, S., Clasen, T., Jenkins, M., 2015. Socio-cultural and behavioural factors constraining latrine adoption in rural coastal Odisha: an exploratory qualitative study. BMC Publ. Health 15. https://doi.org/10.1186/s12889-015-2206-3.
Sah, S., Negussie, A., 2009. Community led total sanitation (CLTS): addressing the challenges of scale and sustainability in rural Africa. Desalination 248, 666–672. https://doi.org/10.1016/j.desal.2008.05.117.
Seidu, A.-A., Ahinkorah, B.O., Kissah-Korsah, K., Agbaglo, E., Dadzie, L.K., Ameyaw, E. K., Budu, E., Hagan, J.E.J., 2021. A multilevel analysis of individual and contextual factors associated with the practice of safe disposal of children's faeces in sub-Saharan Africa. PLoS One 16. https://doi.org/10.1371/journal.pone.0254774.
Shah, A., Thathachari, J., Agarwai, R., Karamchandani, A., 2013. White paper: a market led, evidence based approach to rural sanitation. https://beamexchange.org/uploads/filer_public/a7/cb/a7cb9261-ac24-4d01-a03b-6ae81b96110e/sanitation_whitepaper_-market_led_evidence_based_approach_to_rural_sanitation_compressed.pdf.
Shrestha, S., Ahmad, T., Shrestha, P.K., 2018. Sustainability of ODF in Nepal. Loughborough University. Conference contribution. https://hdl.handle.net/2134/35932.
Sigler, R., Mahmoudi, L., Graham, J.P., 2015. Analysis of behavioral change techniques in community-led total sanitation programs. Health Promot. Int. 30, 16–28. https://doi.org/10.1093/heapro/dau073.
Singh, S., Balfour, N., 2015. Sustainability of ODF Practices in Kenya. WASH Field Note Nairobi Kenya.
Thomas, A., Bevan, J., 2013. Developing and monitoring protocol for the elimination of open defecation in sub-Saharan Africa, 2013. www.communityledtotalsanitation.org/sites/communityledtotalsanitation.org/files/Thomas_and_Bevan_Elimination_of_open_defecation_SSA.pdf.
Thys, S., Mwape, K.E., Lefèvre, P., Dorny, P., Marcotty, T., Phiri, A.M., Phiri, I.K., Gabriël, S., 2015. Why latrines are not used: communities' perceptions and practices regarding latrines in a Taenia solium endemic rural area in Eastern Zambia. PLoS Neglected Trop. Dis. 9 https://doi.org/10.1371/journal.pntd.0003570.
Tyndale-Biscoe, Bond, M., Kidd, R., 2013. Plan International ODF Sustainability Study. Community-Led Total Sanit. Brighton Engl. https://www.communityledtotalsanitation.org/sites/communityledtotalsanitation.org/files/Plan_International_ODF_Sustainability_Study.pdf.
UN, 2015. Draft Outcome Document of the United Nations Summit for the Adoption of the Post-2015 Development Agenda. U. N. Digit. Libr. Syst. https://doi.org/10.1093/oxfordhb/9780199560103.003.0005.
UNICEF, 2014. Evaluation of the WASH Sector Strategy "Community Approaches to Total Sanitation"(CATS). Final Evaluation Report.
UNICEF Eritrea, 2015. National ODF Sustainability Assessment 2015.
UNICEF Mali, 2014. Guide pratique de l'Assainissement Total piloté par la Communauté au Mali. https://www.pseau.org/outils/ouvrages/unicef_guide_de_mise_en_oeuvre_de_l_assainissement_total_pilote_par_la_communaute_au_mali_2014.pdf.
UN Water, 2019. Target 6.B – Stakeholder Participation. Sdg6monitoring. URL. https://www.sdg6monitoring.org/indicators/target-6b/ (accessed 4.April.21).
USAID, 2018a. An examination of CLTS's contributions toward universal sanitation [WWW Document]. Exam. CLTSs Contrib. Univers. Sanit. Glob. URL. https://www.globalwaters.org/resources/assets/washpals/examination-cltss-contributions-toward-universal-sanitation (accessed 4.April.21).
USAID, 2018b. Déféquer avec le diable : cheminement d'un village malagasy vers une meilleure hygiène [WWW Document]. URL. https://www.usaid.gov/fr/Madagascar/news/defecating-devil-malagasy-village%E2%80%99s-journey-better-hygiene, 6.25.21.
USAID, 2017. Madagascar Rural Access to New Opportunities for Health and Prosperity (RANO-HP) Sustainability Evaluation.
Venkataramanan, V., 2016. CLTS Learning Series: Lessons from CLTS Implementation in Seven Countries. NC Chap. Hill.
Venkataramanan, V., 2012. Testing CLTS Approaches for Scalability: Systematic Literature Review. UNC Plan Int. USA Chap. Hill NC.
Venkataramanan, V., Bogle, J., Shannon, A., Rowe, R., 2015. Testing CLTS Approaches for Scalability. Lao PDR Learning Brief. UNC Water Institute, USA.
Venkataramanan, V., Crocker, J., Karon, A., Bartram, J., 2018. Community-led total sanitation: a mixed-methods systematic review of evidence and its quality. Environ. Health Perspect. https://doi.org/10.1289/EHP1965.
WaterAid, 2009. Towards Total Sanitation: Socio-Cultural Barriers and Triggers to Total Sanitation in West Africa, Water. Sanitation and Hygiene Institute.
Waterkeyn, J., Cairncross, S., 2005. Creating demand for sanitation and hygiene through Community Health Clubs: a cost-effective intervention in two districts in Zimbabwe. Soc. Sci. Med. 61 https://doi.org/10.1016/j.socscimed.2005.04.012, 1982.
Whittington, D., Briscoe, J., Mu, X., Barron, W., 1990. Estimating the willingness to pay for water services in developing countries: a case study of the use of contingent valuation surveys in southern Haiti. Econ. Dev. Cult. Change 38, 293–311. https://doi.org/10.1086/451794.
Whittington, D., Lauria, D.T., Choe, K., Hughes, J.A., Swarna, V., Wright, A.M., 1993. Household sanitation in Kumasi, Ghana: a description of current practices, attitudes, and perceptions. World Dev. 21, 733–748. https://doi.org/10.1016/0305-750X(93)90030-D.
WHO, 2019. Lignes directrices relatives à l'assainissement et à la santé [Guidelines on sanitation and health. Genève.
WHO, 2017. Integrating Neglected Tropical Diseases in Global Health and Development: Fourth WHO Report on Neglected Tropical Diseases. WHO, Geneva, Switzerland.
Wilbur, J., Jones, H., 2014. Handicap: Rendre l'ATPC véritablement accessible à tous. Aux Front. L'ATPC Innov. Impr. 3.
Yimam, Y.T., Gelaye, K.A., Chercos, D.H., 2014. Latrine utilization and associated factors among people living in rural areas of Denbia district, Northwest Ethiopia, 2013, a cross-sectional study. Pan Afr. Med. J. 18 https://doi.org/10.11604/pamj.2014.18.334.4206.
Yusliza, M.Y., Amirudin, A., Rahadi, R.A., Nik Sarah Athirah, N.A., Ramayah, T., Muhammad, Z., Dal Mas, F., Massaro, M., Saputra, J., Mokhlis, S., 2020. An investigation of pro-environmental behaviour and sustainable development in Malaysia. Sustainability 12. https://doi.org/10.3390/su12177083.
Zuin, V., Delaire, C., Peletz, R., Cock Esteb, A., Khush, R., Albert, J., 2019. Policy diffusion in the rural sanitation sector: lessons from community-led total sanitation (CLTS). World Dev. 124, 104643 https://doi.org/10.1016/j.worlddev.2019.104643.

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Tropical infections as occupational diseases among young volunteers in social projects

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ABSTRACT

Purpose: The trend of volunteering overseas has increased tremendously over the last decade. Volunteers often go to regions where they are exposed to the risk of tropical infections like malaria, dengue, typhoid fever and schistosomiasis. Health assessments have shown a high occurrence of tropical infections among young volunteers. Such tropical infections are notifiable in Germany, as they are covered by a separate branch of the social insurance system. However, there is still limited data on systematical improvement of medical prevention and health care for volunteers.

Methods: This retrospective study included 457 cases with a diagnosis for a tropical infection or typhoid fever from January 2016 to December 2019. Data sets were anonymised and then analysed with descriptive statistics first. Cases of volunteers sent abroad by "Weltwärts" were compared to cases of aid workers sent to non-industrial countries.

Results: A high occurrence of tropical infections as occupational diseases has been shown for volunteers compared to other (mostly older) aid workers being sent to tropical regions. The risk of acquiring a tropical infection was significantly higher in Africa compared to other tropical regions. Cases of malaria were reported significantly more often among the group of volunteers than among aid workers during the period under review. Medical check-ups after travel were rare among volunteers.

Conclusions: Data imply a disproportionate risk for malaria in Africa with a higher risk of acquiring malaria tropica in Sub-Saharan regions. Region-specific risks need to be addressed in training seminars in order to raise awareness among young volunteers before travel. Medical examinations after travel should be mandatory and specific to a particular region.

1. Introduction

In the last sixty years, the number of aid organizations sending aid workers abroad has multiplied. Aid workers were mostly experienced people with some kind of special training in their field of work. In the past two decades a new form of humanitarian work, called volunteering, was established and has since then increased tremendously. In 2008, the so called "Weltwärts" program was founded in Germany, sending young volunteers at the age of 18–28 to social projects worldwide for one year (Anonymous (2021b). This type of intercultural exchange which the young volunteers experience, promotes their personal development but also exposes them to new health risks they are not always aware of. As stated in other studies, "their idealism is often bigger than their risk perception" (Martin et al., 2012) and their risky behavior results in a higher number of frequent tropical infections like malaria (Anonymous, 2020b), dengue, typhoid fever or schistosomiasis (Kiehl, 2011). In order to prevent such diseases, volunteers being send to the tropical regions (tropics) must undergo a mandatory pre-travel medical examination and receive pre-travel health advice (Prüfer-Krämer et al., 2020). This preventive measure has been in effect since 2019.

By law, Weltwärts-volunteers with a German work contract being sent abroad are covered under the labour protection act just like any other workers in Germany (Anonymous, 2008). A suspected diagnosis for a tropical illness during the period of the voluntary work in the tropics must therefore be reported as occupational disease by the doctor and the employer if the procedural requirements according to BK-3104 (Anonymous, 2005) to get such a disease at work are fulfilled. If professional work in the tropics has exposed an employee to diseases that do

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not exist in Germany and it is likely that the disease was acquired at work and not otherwise, then contracting such a disease is considered a consequence of the work contract and fulfills therefore the procedural requirements as an occupational disease. This so-called "double causality" (diagnosis and exposure at work) may be required e.g. for Hepatitis B when a medical person is working with patients. However, if a diagnosis of Hepatitis B cannot be easily linked to risk exposure at work, e.g. in a bureau job, requirements are not fulfilled. The first case would fulfill the requirement of double causality, the latter normally not. In most countries occupational diseases are listed. In Germany, the diagnoses of tropical occupational diseases investigated in this study are coded as BK-3104.

It has been suspected that occupational diseases by infections occur more frequently among young volunteers than among the comparative group of mostly older aid worker (Martin et al., 2012). However, there is limited data and therefore any strategy to improve medical prevention and health care among young volunteers in the tropics is so far limited.

2. Material and methods

A total of 457 anonymised data sets with cases recommended by a third party expert to be recognized as occupational disease according to BK-3104 (occupational tropical disease, (Anonymous, 2005) have been analysed retrospectively. Reports from January 2016 to December 2019 were included in the analysis. More recent cases were excluded because most volunteers returned ahead of time because of the Covid-19 pandemic and their short stay abroad would have caused a significant bias. The cases consisted of notifications about young volunteers deployed by the German "Weltwärts" program as well as cases from mostly aid workers all addressed to the "Unfallversicherung für Bund und Bahn" (UVB; "accident insurance for government and railway") as statutory insurance agency. Disease frequencies were then analysed in geographic subgroups. Weltwärts-volunteers will be named "volunteers" in this paper. Recommendation for recognition depended on diagnostic reliability and suitable clinic. Diagnostic methods abroad were often limited and the quality of case reports differed significantly from extensive documentation to screenshots of handwritten laboratory results. Special attention was paid to the most occurring tropical diseases such as malaria, dengue, typhoid fever and schistosomiasis.

All data were compiled in an excel sheet and evaluated with SPSS (version 27). For the evaluation of differences between groups, data was checked for normal distribution. Such data was tested with Student t-test, others with non-parametric tests (Chi square test, Mann Withney *U* Test). $P < 0.05$ was defined as significant. The project was consulted by the ethics committee of RWTH Aachen University, Aachen/Germany (EK 078–21).

3. Results

This study included a total of 457 files of patients where an occupational disease was suspected. 290 (63.5%) of them were volunteers and 167 (36.5%) aid workers. 76% of the volunteers were females in contrast to 53% in the group of aid workers ($P < 0.001$). Mean age of the volunteers was 19 years and they stayed abroad for an average time of 11 months or 343 days (range 62–1198). The aid workers were significantly older (20–65 years, $P < 0.001$), with one third of all aid workers being between 28 and 34 years old at the time of departure from their working regions. They stayed significantly longer abroad ($p < 0.01$) for a mean of 2.5 years or 925 days (range 5–3756). The diseases leading to a notification followed the departure with a mean delay of 182 days (range 4–667) for volunteers and 710 days (range 5–3938) for aid workers ($p < 0.01$).

3.1. Travel destinations

A total of 14.052 volunteers were sent overseas to 65 different countries (Fig. 1) in the period under review (David Hingley, Engagement Global, personal communication, March 16, 2021). 45% were sent to Latin America, 36% to Africa, and 19% to Asia or Oceania (Table 1). Significant regional differences were found for diagnoses for tropical diseases classified as BK-3104 between volunteer cases from Africa and those from other parts worldwide ($p < 0.01$). 3.6% (185/5103) of all volunteer cases sent to Africa had a diagnosis of a tropical infection, in contrast to 1.7% (43/2641) sent to Asia and only 0.5% (32/6308) sent to Latin America for the period from 2015 to 2018 (Fig. 2).

All data for the comparison of the occurrence of tropical infections among aid worker came exclusively from the cases reported to the assurance company UVB. Even though the cases of aid workers came mainly from sending institutions like "Gesellschaft für internationale Zusammenarbeit" (GIZ) and "Arbeitsgemeinschaft für Entwicklungshilfe (AGEH)/Agiamondo", exact numbers of aid workers sent abroad for the period under review could not be provided. Therefore, only files of aid workers with a diagnosed tropical occupational disease were analysed in this study. Further, percentage rates of diagnoses conducted for the total number of aid workers could not be calculated. Out of all the analysed aid worker cases ($n = 167$), 67% of the cases were diagnosed with an occupational disease according to BK-3104 for aid workers who had been working or were at that time still working in Africa (112/167). In addition, 24% (41/167) had been to Asia and only 8% to Latin America. However, only in one case the aid worker had travelled within Europe.

3.2. Tropical illnesses

Among all reported diagnoses of occupational illnesses among volunteers ($n = 290$), the most common diagnosis was malaria with 54.5% (158/290, Table 2). Further, 19.7% (57/290) of volunteers got a diagnosis for dengue fever. In addition, typhoid fever was reported in 14.1% (41/290) and schistosomiasis in 5.9% (17/290). Besides, 8.6% of all reported diagnoses among volunteers were summarized as "other diseases" from which 10 out of the 25 cases were for amoebiasis and a few for giardiasis, chikungunya, or cholera.

For aid workers, only 37.1% (62/167) of the cases were reported with a malaria diagnosis and 19.8% had an infection with dengue (33/167, Table 2). Moreover, 23.4% (39/167) were diagnosed with schistosomiasis and only 4/167 had typhoid fever. The diagnosis rate for "other diagnoses" was 18.6% (31/167). Among these, 7 were for giardiasis, 6 for amoebiasis and 4 cases of chikungunya. For malaria, there was a significant difference between case numbers of volunteers and aid workers, with volunteers getting significantly more diagnoses ($p < 0.01$). Schistosomiasis on the other hand was diagnosed significantly more often among aid workers ($p < 0.01$) (Table 2).

3.2.1. Malaria cases in Africa

In this study, malaria was accepted as proven when a microscopy result or a rapid dipstick test was documented to be positive. In other cases, malaria was just clinically suspected and treated as such, leaving the question whether the diagnosis was correct or not. For all malaria cases among volunteers and aid workers ($n = 219$), all except one case were reported to have taken place in Africa and more than half of those in West Africa (127/219). Half of all malaria diagnoses in Africa were based on an infection with Plasmodium falciparum (114/219). In West Africa even 57% of the malaria cases were confirmed infections with Plasmodium falciparum (72/127; Fig. 4). Further, only three malaria cases were caused by Plasmodium vivax.

Volunteers, who lived and worked in Africa were significantly more at risk ($p < 0.01$) to get a diagnosis for one or even several tropical diseases. The risk of illness differed considerably from country to country. Out of 205 cases from volunteers who travelled to Togo between 2015 and 2018, only 18% (37) of cases were diagnosed as tropical diseases. For cases from volunteers in Benin, diagnosis rate was 14% (11/78) and 7% (52/776) for Ghana. For South Africa, the diagnosis rate for an occupational disease was made only in 2 cases out of 1352

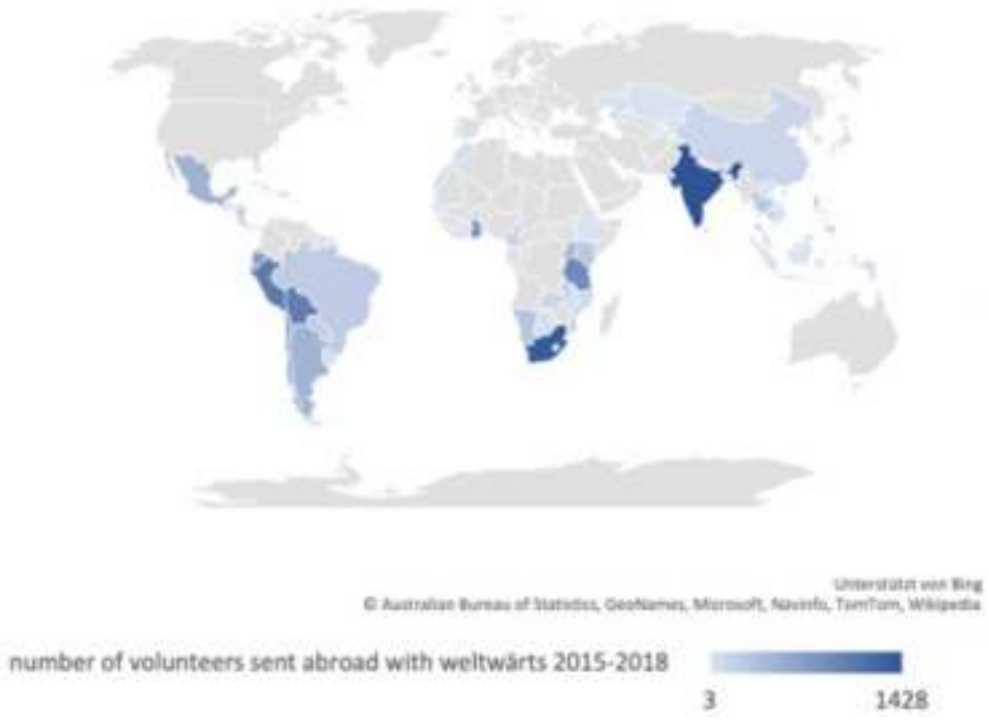

Fig. 1. Volunteers sent abroad 2015–2018 (n = 14.052).

Table 1
Travel destinations for the service abroad among diagnosed volunteers/aid workers (n = 457).

	Africa		Asia/ Oceania		Latin-America		Europe	
	n	%	n	%	n	%	n	%
volunteers	207	71	47	16	36	13	0	0
aid workers	112	67	41	24	13	8	1	1

volunteers who travelled there.

Compared to other malaria regions there was a significant higher risk for Africa but data did not prove a significant difference for different regions within Africa probably because of limited number of cases.

3.2.2. *Dengue cases in India*

Regarding the occurrence of dengue among volunteers and aid workers, data suggest that the incidence for dengue was significantly higher in India ($p < 0.01$) compared to the number of dengue cases among those who had travelled worldwide to other countries than India (64). A total of 26/90 dengue cases were diagnosed in India during the period under review or after the service there (Fig. 5) and 23 out of 26 were volunteers. There were only 6 cases from those who travelled to India with another diagnosis than dengue.

3.2.3. *Schistosomiasis and typhoid fever*

Diagnoses of schistosomiasis were only found among cases of volunteers and aid workers who had worked in Asia and Africa with significantly more cases in Uganda ($p < 0.01$). This corresponds to 30.4% (17/56) of all reported cases for schistosomiasis.

Cases of typhoid fever mainly occurred after volunteer service in Africa and significantly more in one country in America, Peru ($p < 0.01$), to which 33.3% (15/45) of the diagnoses reported can be attributed.

3.3. *Mandatory pre and post travel examinations*

Regarding the medical check-ups the volunteers and aid workers had received, this study has revealed that of all volunteers, only 38% (110/290) had undergone pre-travel medical checks, compared to 95% (159/167) among aid workers ($P < 0.001$, Table 3). In addition, only 23% (66/290) of the volunteers had received a follow-up examination after their return to Germany, whereas 86% (143/167) of the aid workers had one ($P < 0.001$). The suspected diagnosis was made in 96% (279/290) of the cases from volunteers in the host country and in 62% (104/167) of the cases from aid workers who were at that time abroad ($P < 0.01$; Table 3).

3.4. *Recognition as occupational disease*

The total recognition rate of diagnoses as occupational disease was almost equal among both groups being investigated (65% among volunteers and 64% among aid worker, Table 4). The proportion of diagnoses recognized as occupational disease differed depending on the disease. Out of malaria notifications, 84% among volunteers and 87% among aid worker were accepted. For dengue fever, it was about 79% of the volunteer cases and 70% of the aid worker's cases. None of the suspected cases for typhoid fever were solely recognized as occupational disease. In total, only 5 cases with the co-disease typhoid fever were recognized, all of them with a concomitant malaria as reason for the recognition. Only few cases of schistosomiasis met the criteria for recognition as occupational disease, 18% (3/17) among volunteers and 44% (17/39) among aid workers. About 28% (11/39) of the diagnoses of schistosomiasis among aid workers were made during a follow-up examination after return from abroad. Most of those (26/39) were made upon positive IgG anti-bodies. Furthermore, 31/39 schistosomiasis cases among aid worker occurred in Africa. For volunteers, only 3/17 were diagnosed during a follow-up and for those diagnosed abroad, in 62% of the cases, a rapid dipstick test for schistosomiasis was used, a method only used twice among aid workers (Table 4).

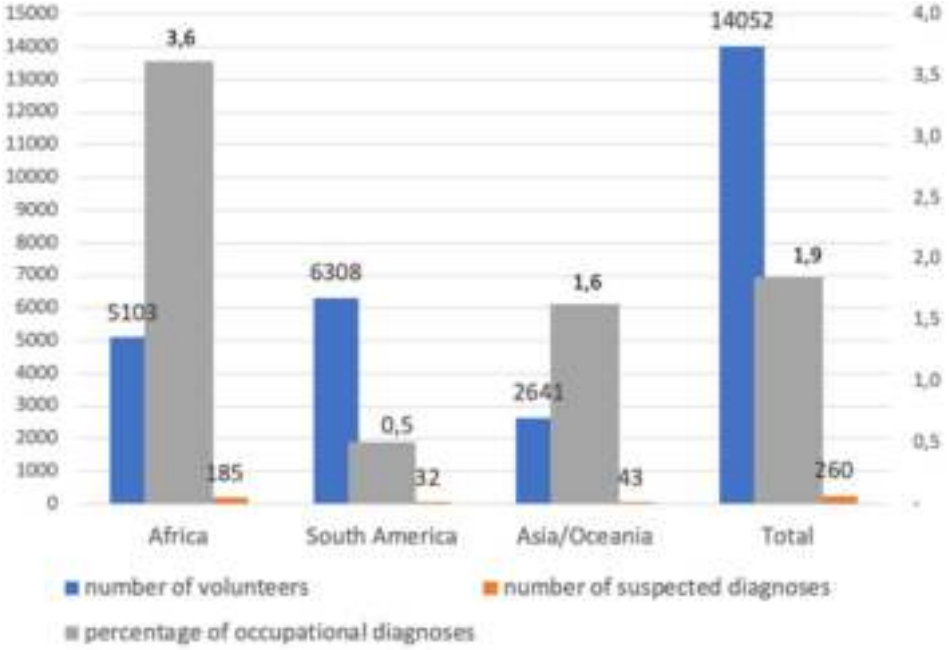

Fig. 2. Diagnosed percentages for all volunteers sent abroad 2015–2018 (n = 14.052) with the left y-axis showing the numbers of volunteers and the right y-axis the percentages of diagnoses.

Table 2
Appearance of tropical illnesses (n = 457).

diseases	volunteers		aid workers	
	n	%	n	%
malaria	158	54.5	62	37.1
dengue	57	19.7	33	19.8
Typhoid fever	41	14.1	4	2.4
schistosomiasis	17	5.9	39	23.4
other	25	8.6	31	18.6

Table 3
Mandatory examinations.

total collective (n = 457)	volunteers n = 290		aid worker n = 167	
	n	%	n	%
Diagnosis made abroad	279	96.2	104	62.3
Made pre-travel examination	110	37.9	159	95.2
Made post-travel examination	66	22.8	143	85.6
Recommendation as recognized occupational tropical disease	188	64.8	106	63.5

Table 4
Recommended recognition rate as an occupational disease (n = 457).

Disease	volunteers		aid workers	
	n	%	n	%
malaria	133	84.2	54	87.1
dengue	45	78.9	23	69.7
typhoid fever	4	9.8	1	25.0
schistosomiasis	3	17.6	17	43.6
Other	9	36.0	13	41.9

4. Discussion

4.1. Pre-travel preparations

"Occupational travel to tropical countries among Germans has more than doubled in the past five years" (Jansing et al., 2021). The number of young volunteers going abroad has also increased tremendously in the last decade (Anonymous, 2019), including the number of international travels in general. Health related issues occur for a good number of travellers. Therefore, this needs to be addressed, particularly regarding the higher exposure to tropical infections among volunteers and aid workers often living in rural regions for longer periods (months or even years). As other studies have already shown, risk behavior and risk perception among volunteers depends strongly on the intensity of pre travel training and medical advice given (Jansing et al., 2021), (Han et al., 2010). The importance of information on preventive behavior and education in self-management skills before travel was stated by a recent study (Sasayama et al., 2021). This particular study showed that even after having taken a health lecture, only 40% of people knew about the more dangerous risks like malaria and dengue. Obviously, just hearing about a health lecture is not sufficient for volunteers to understand the severity of existing health hazards they will be exposed to in the regions they visit. Specific training to develop self-management skills is therefore crucial for their better understanding of potential threats and how to react upon them. Studies have also addressed the issue of proper preparation (Sasayama et al., 2021). In another survey, only 70% of relief aid workers interviewed stated to have taken a pre-travel medical examination and only every fourth person had used a mosquito-net or repellents to protect themselves against malaria or dengue fever (Sharp et al., 2006). Regarding international travel, "malaria especially, continues to threaten international travellers due to inadequate perception of risk and sub-optimal pre-travel preparations" (Angelo et al., 2017). When our study was performed there was not yet a standardized training for the volunteers before departure. This was introduced step by step later and recommended now but is not yet mandantory for all organizations.

These previously described risky behaviors, seem indeed to represent one of the biggest risks, causing diseases such as malaria and dengue to

occur more frequently than necessary. Not only do volunteers behave risky despite pre-travel health advice (Martin et al., 2012), (Küpper et al., 2014), but studies have shown, especially regarding malaria prevention, that young people are clearly not well informed about this specific risk. Landman et al. (2013) reported that adherence to malaria prophylaxis was moderate due to the fear of long-term adverse events. One out of four even reported "not worrying about malaria" although living and working in high risk regions (Landman et al., 2013). This shows that there is a need of increasing information and training about prophylactic efficacy and likelihood of side effects.

Volunteers do not only have a higher risk profile than older aid workers, but they also travel to regions with underestimated health hazards resulting in possible health issues during and after their stay abroad. Therefore, not only pre-travel preparation is an ongoing issue but also the importance of medical follow ups. According to a study done in the United Kingdom in 2006–2007, 27% of volunteers returned from voluntary services overseas with ongoing unresolved medical problems (Bhatta et al., 2009) and more than one third of aid workers interviewed "reported worse health on return than before their mission" (Dahlgren et al., 2009). Another study found that 53% of the travellers presented themselves to hospital within one week of return and 96% within 6 months after coming back from overseas due to health problems" (O'Brien et al., 2001).

4.2. Sub-Saharan risk-regions and risk for malaria

For the occurrence of occupational diseases analysed in our study, a disproportionate risk of being diagnosed with a tropical disease, above all other diseases, malaria was detected in volunteers living in Africa and especially those in Sub-Saharan/West African regions (Fig. 3). Most of them were diagnosed with malaria and many of those with malaria tropica (Pl. falciparum). It is evident that the risk for malaria is higher there, as the region of West Africa is in fact stated by the World Health Organization (WHO) as a region with an all-year high malaria risk (Anonymous, 2020a). In 2020, 95% of all malaria cases were observed in Sub-Saharan-regions (Anonymus, 2021). International data has already shown, that "travel to Sub-Saharan Africa and Oceania was associated with the greatest relative risk of acquiring malaria" (Angelo et al., 2017). Nonetheless results from a survey for Spanish travellers showed again the lack of potential pre-travel preventive measures as more than one third of travelers to Sub-Saharan Africa received no malaria prophylaxis (Lopez-Velez and Bayas, 2007). Even more important is the preparation and importance of raising region-specific awareness for the existing health hazards for volunteers or travellers beforehand as these are the best measures to prevent an infection through a mosquito-bite.

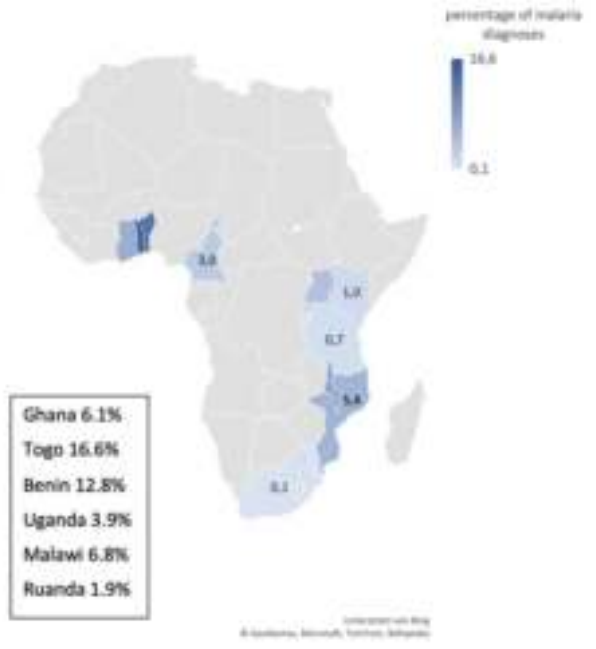

Fig. 3. Distribution [%] of malaria cases among volunteers in Africa 2015–2018 (n = 140).

In the current study, malaria was found to be diagnosed more often among volunteers than among humanitarian aid workers and other short or long-term aid workers for the time period investigated, even though the cases of aid workers analysed in this study showed a long travel history, travelling abroad over and over to risk countries due to their work contracts. According to the volunteer cases, their stay for the voluntary service abroad was often their first travel to a tropical region involving new health hazards. The time taken from departure up to diagnosis was significantly shorter for volunteers compared to the aid workers ($p < 0.01$), having most of the first diagnosis halfway through their voluntary year. Aid workers were diagnosed after a much longer stay abroad and data in this study showed that aid workers did not have more episodes of malaria whilst staying on average three times longer abroad, unlike volunteers who had up to five episodes of malaria during their shorter stay. These findings suggest continuously missing or not sufficient compliance for preventive measures, especially regarding mosquito bite prevention.

According to our findings, the risk of infection with malaria pathogen did not increase proportionally with the length of stay but there was a correlation between the stay in African regions and an infection with malaria pathogen, especially Plasmodium falciparum causing malaria tropica and harboring the risk of a severe or even life-threatening case of malaria (Fig. 4). A significant increased risk for the infection with Plasmodium falciparum in West Africa could not be proven. There could be up to one fourth more infections with Plasmodium falciparum since 23.6% of the malaria diagnoses were made upon a positive test result of a rapid diagnostic test (RDT). Unfortunately, the types of malaria pathogen tested positive could not be detected from the data of the RDT tests.

Other studies have likewise found, that especially travellers being exposed in Sub-Saharan regions were mostly diagnosed with malaria tropica (Angelo et al., 2017) and this supports our current hypothesis that there is a greater and often underestimated risk among young volunteers going abroad for the first time.

4.3. Suspicions for occupational diseases

More cases of suspected occupational diseases were reported among volunteers than among others, mostly aid workers, represented in this study. Malaria was shown to occur more frequently among the volunteers whereas the diagnosis for dengue fever occurred equally within both groups. The infection with dengue transmitted by a mosquito bite is only preventable with adequate use of repellents and mosquito-nets at night (Gupta and Rutledge, 1994) whereas an infection with malaria pathogen can additionally be prevented through adequate chemoprophylaxis (Nauck, 1956). Sadly, studies have shown how very little travellers know about the efficacy of the use of anti-malaria chemoprophylaxis or – even more effective – mosquito nets to sleep under them, and therefore the lack of taking or using them for prevention (Lopez-Velez and Bayas, 2007), (Landman et al., 2013). These findings suggest that volunteers do take higher risks in terms of prevention, causing more and repeated infections with malaria parasites and leading to preventable occupational diseases.

Similar behavior must be assumed regarding the lack in prevention measures taken to protect oneself against mosquito bites transferring dengue or also chikungunya. The lack of knowledge (Sasayama et al., 2021), risk perception or even willingness to protect oneself against dengue with bed nets and the daily use of repellents or

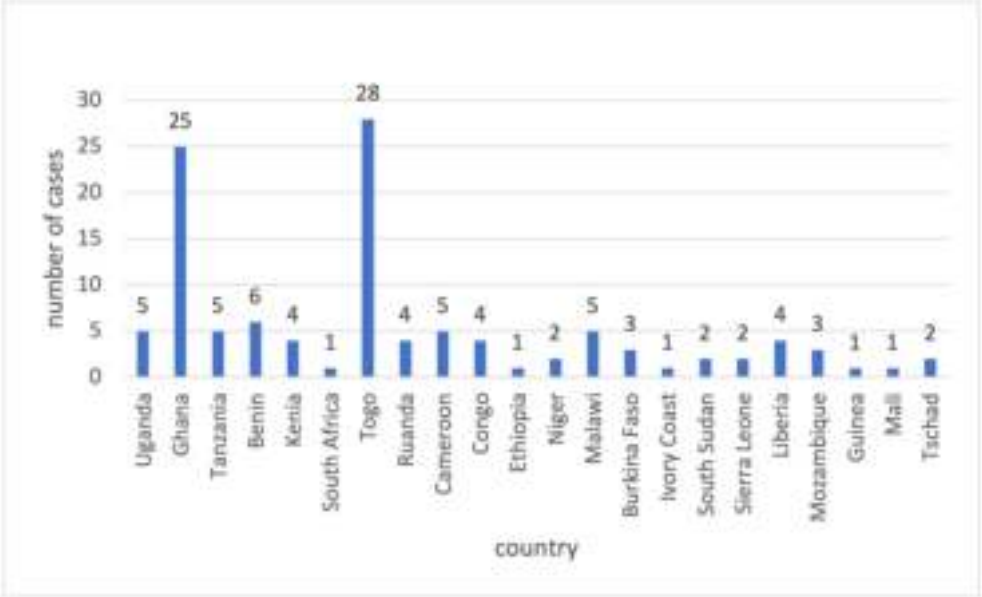

Fig. 4. Frequency of proven malaria tropica cases among volunteers in Africa (n = 114).

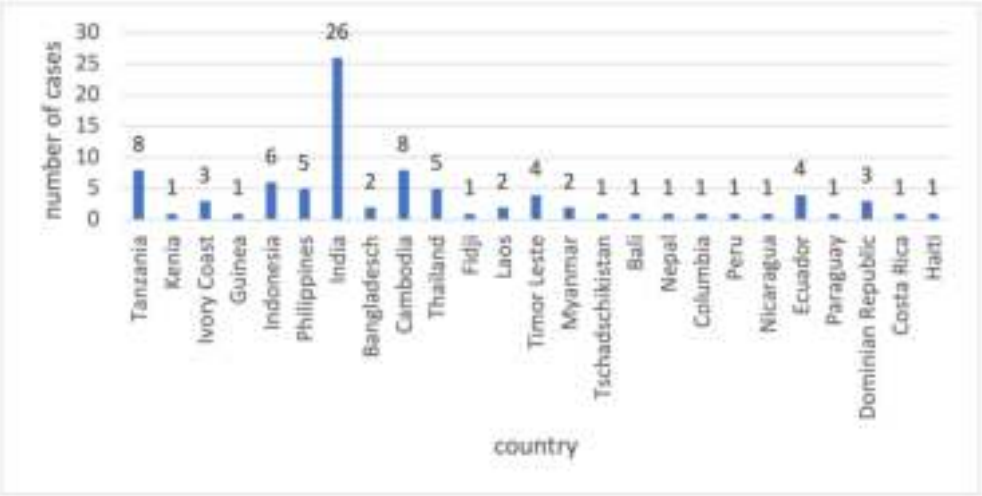

Fig. 5. Frequency of dengue cases among volunteers and aid workers (n = 90).

chemoprophylaxis especially among young volunteers leads again to potentially preventable case numbers of tropical occupational diseases (BK-3104). Considering the geographic localization of dengue cases investigated in this study, there were significantly more dengue cases and especially among volunteers who travelled to India for their service (Fig. 5). In current study, the different regions of India with dengue cases could not be differentiated as data were not detailed enough in the health reports analysed. Since India is a very popular country volunteers go to (Fig. 1), it is crucial to enlighten them on potentially risk of acquiring dengue fever whilst travelling there. The WHO classified dengue risk regions in Asia as representing 70% of the global burden of the disease (Anonymous, 2021c). A study analysing dengue in peace corps volunteers found the Caribbean to have highest number of dengue cases followed by East Asia and South/East Asia regions (Ferguson et al., 2016). In contrast, Latin America, where most volunteers are sent every year, did not report many cases of dengue fever. According to the WHO, a significant reduction of dengue cases was reported in the Americas in 2017 although dengue cases are increasing worldwide in the supervised risk regions with highest number of cases reported in Bangladesh, Malaysia, Philippines and Vietnam in Asia (Anonymous, 2021c). Attention must therefore also be paid to increasing risk for infection with dengue worldwide especially in tropical and subtropical regions as the number of dengue cases reported to the WHO increased over 8 fold in the last two decades putting half of the world's population at risk (Anonymous, 2021c). The difference between the general tendency and the decreasing number of cases in the Americas may be explained by extensive campaigns in South America, especially in Paraguay, to increase prophylaxis against vectors to fight chikungunya and zika infections. By this other vectorborne diseases like dengue were also reduced.

For the occurrence of typhoid (enteric) fever, no case has been registered as an occupational disease as a diagnosis based on positivity of the old and unreliable Widal test alone could not be accepted, especially if clinical signs were either not reported or not compatible with a diagnosis of typhoid fever. It should also be noted that our data about Peru cannot compared directly with those published by the local government since there infections with Salmonella typhiy and S. paratyphi are summed up together and due to local limitations (infrastructure,

medical training of staff, laboratory equipment, money …) all data must be interpreted carefully. Nonetheless attention should be paid to this infectious disease found in the tropics, highly correlating with a high fecal contamination of sanitary facilities in big cities, not only affecting locals but also travellers and workers (Abhilasha et al., 2008). As gastrointestinal symptoms are often self-limiting and diagnostic methods are insufficient, recognition of typhoid fever is often difficult because stool tests are often negative in the first week and serological tests may be positive at a late stage of disease only or never. The traditional Widal-Test shows a low sensitivity of 65.4%, specificity of 89.8%, and accuracy of 82.1% (Maheshwari et al., 2016), (Shahapur et al., 2021). Therefore, prevention measures regarding water transmitted diseases like typhoid fever must also be addressed during training seminars as volunteers do not always have access to safe drinking water and sanitary facilities (Martin et al., 2012). It may be expected that typhoid fever is more common when more people were sent to south-east Asia, especially to Nepal and India as these countries have the highest rate of typhoid fever worldwide (Karkey et al., 2016), (Abhilasha et al., 2008).

Infestations with schistosomiasis were significantly more frequently diagnosed among aid workers, mostly based on a positive serology after their return from overseas. This may be a consequence of the longer stay of aid workers. However, there are other factors which should be taken into consideration, e.g. individual behavior: Volunteers may have had some swimming or water diving activities which often takes place far from the shore while aid workers may have walked in the water at the shore to cool down. By this, their risk would be significantly higher than those of volunteers. Housing may also be an important factor: Do people get their water as rainwater or from a pond? Unfortunately, such factors are usually not documented or reported and therefore the underlying risk factors must remain as such. Only few cases of schistosomiasis were recommended by the third party expert to be accepted as occupational disease, due to the fact that in most cases, single serological tests do not constitute a diagnosis in the absence of parasitological proof and that the attribution of such a positive serology to a certain exposure is often impossible (Anonymous, 2017). A single serological test may be accepted as proven diagnosis, only if the person went to a risk area for the first time and the titers are high. However, an element of risk remains because in non-endemic countries some species of schistosomiasis occur, e.g. Cavu River in Corsica, and trichobilharzia occurs in Europe's swimming lakes if there are many ducks (Effelsberg, 1989). These factors may cause false-positive results. Some tests may also be negatively affected antibody cross reactions (Homsana et al., 2020). With such risk elements in mind a single serological test may be acceptable in some cases but a significant shift of titers is still "Gold Standard". Diagnostic methods like the Rapid dipstick point of care test (POC) are insufficient (Ochodo et al., 2015).

Interestingly, volunteers were mostly diagnosed in their host country with the above-mentioned rapid dipstick test. Not accepting a positive POC test as valid diagnosis doesn't exclude the possibility of a schistosomiasis as the test proves the contact to the pathogen but not an active infestation (Kapaun, 2004) (Casacuberta-Partal et al., 2020a). Two positive serology samples or microscopic detection of the pathogen are necessary to prove an active infection with schistosomiasis pathogen (Anonymous, 2017). These secure detection methods where fairly used among volunteers. A schistosomiasis screening for antibodies and preferably followed by the more sensitive up-converting phosphor lateral flow circulating anodic antigen (UPC-LF CAA) test should be encouraged after freshwater contact (Anonymous, 2017), (Casacuberta-Partal et al., 2020a). In high-endemic areas serology should be analysed 12 weeks after return (Anonymous, 2017), (Kapaun, 2004) to detect potentially active infestations with low burden of schistosomiasis pathogen after staying abroad, as travellers may be asymptomatic on first presentation (Casacuberta-Partal et al., 2020b) and infections need to be treated early in order to prevent long-term complications in the case of chronic schistosomiasis, as liver fibrosis (Zhong et al., 2022), bladder cancer (Efared et al., 2022) and other chronic inflammations (Musaigwa et al., 2022).

4.4. Personal protection measures

The effectiveness of repellents and the use of insecticide impregnated bed-nets should be encouraged in order to achieve better prevention. Pre-travel checkup and mandatory follow up examinations are crucial to be able to attribute a suspected diagnosis of an illness to the past journey and to accept it as an occupational disease which occurred during the actual journey.

Unfortunately, in this study it was impossible to assess whether volunteers took recommended prevention measures like malaria chemoprophylaxis or other, non-drug-related precautions like the use of repellents or mosquito-nets according to the risk associated to particular regions they were travelling to. However, Martin et al. reported that there are deficiencies in such strategies in young people, even more pronounced than in elderly persons (Martin et al., 2012). It was impossible to track what kind of specific training and quality of medical checkups were done before the departure. Further, investigations are necessary to assess the type and adequacy in terms of preventive measures taken before, during and after travel.

Another limitation of this study is the limitation on specific pathogens considered in this study. According to the regulations, other diseases than the ones listed as occupational diseases may be accepted as occupational disease by the insurance, if the place of work is the most likely circumstance where the infection occurred. The German regulations include two options here: Bk-3102 ("diseases which may be transmitted from animals to humans") or by the so-called "extension condition". This includes cases which are not listed as occupational disease but where the occupational exposure can be proven as the main or only risk. The former could be the case if an African tick bite fever was diagnosed. Rickettsioses were recently identified as rapidly emerging diseases and probably they are more common in returning travellers than malaria (Jensenius et al., 2003), (Jensenius et al., 2002), (Cherry et al., 2018), (Leder et al., 2013), (Bottieau et al., 2006). However, such diagnoses never occur so far in the applications for occupational disease. The latter option may be chosen when a person who is active in nature protection in national parks gets anthrax. Such cases rarely occur in Namibia and Zambia, probably also in other countries, e.g. Botswana (Rob Clifford, South Luangwa Valley, personal communication 2020). Because of the total number of cases especially concerning rickettsioses more awareness of the counselling physicians is desirable.

5. Conclusion

This study has shown that occupational diseases categorized as tropical diseases (BK-3104) occur significantly more often among young volunteers than among the (older) aid workers. A disproportional risk for malaria, especially malaria tropica, was detected among travellers to Africa. Further studies are necessary to investigate the behavior among young volunteers regarding the compliance with prevention and especially chemoprophylaxis for malaria as such measures are the key in reducing the occurrence for the most common tropical infections. Similar is the situation with the prevention of dengue, showing increasing numbers of cases worldwide and its potentially underestimated risk in India. Personal protective measures continue to be crucial. Using repellents like DEET, treating clothing with permethrin and sleeping under a mosquito net are the most effective personal prevention measures (Anonymous, 2021a). There is a need to address the region-specific risks more adequately in training seminars in order to raise awareness about existing health hazards associated with specific regions volunteers and aid workers will be visiting or staying, for the young volunteers to improve self-management skills before they travel abroad. Introducing a mandatory medical checkup after returning home will prove to be an important step to recognize illnesses early and to

prevent long-term consequences.

Author contributions

Preparation of the research project: YK, TK. Assembly of data for the research: YK. Conducting of statistical analysis: YK, TK. Interpretation of results: YK, TK. Manuscript preparation: YK, TK, BP. Literature review: YK.

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Declaration of competing interest

The authors have declared no conflicts of interest.

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References

Abhilasha, K., Amit, A., Buddha, B., Stephen, B., 2008. Kathmandu, Nepal: still an enteric fever capital of the world. J. Infec. Dev. Ctries. 2.

Angelo, K.M., Libman, M., Caumes, E., Hamer, D.H., Kain, K.C., Leder, K., Grobusch, M. P., Hagmann, S.H., Kozarsky, P., Lalloo, D.G., Lim, P.L., Patimeteeporn, C., Gautret, P., Odolini, S., Chappuis, F., Esposito, D.H., 2017. Malaria after International Travel: a GeoSentinel Analysis, pp. 2003–2016.

Anonymous, 2005. Merkblatt zur Bk Nr. 3104: Tropenkrankheiten, Fleckfieber. BArbBl 7, 48–62.

Anonymous, 2008. Verordnung zur arbeitsmedizinischen Vorsorge (ArbMedVV) Anhang arbeitsmedizinische Pflicht- und Angebotsvorsorge. Bundesministerium der Justiz und für Verbraucherschutz, BGB I, pp. 2771–2775.

Anonymous, 2017. S1-Leitlinie 042-005 „Diagnostik und Therapie der Schistosomiasis (Bilharziose). Deutsche Tropenmedizinische Gesellschaft und internationale Gesundheit, AWMF online. https://register.awmf.org/de/suche#keywords=schistosomiasis&sorting=relevance.

Anonymous, 2019. Weltwärts Zahlen und Fakten. Bundesministerium für wirtschaftliche Zusammenarbeit und Entwicklung.

Anonymous, 2020a. Weltkarte zum Malariarisiko (Stand 2020), Deutsche Gesellschaft für Tropenmedizin. Reisemedizin und globale Gesundheit e.V.

Anonymous, 2020b. World Malaria Report 2020. World Health Organization.

Anonymous, 2021a. Traveler’s Health Dengue. Center for disease control and prevention.

Anonymous, 2021b. Weltwärts - der entwicklungspolitische Freiwilligendienst, Bundesministerium für wirtschaftliche Zusammenarbeit und Entwicklung.

Anonymous, 2021c. WHO - Dengue and Severe Dengue. World Health Organization.

Anonymus, 2021. World Malaria Report 2021. WHO, tropeninstitut.de.

Bhatta, P., Simkhada, P., van Teijlingen, E., Maybin, S., 2009. A questionnaire study of voluntary service overseas (VSO) volunteers: health risk and problems encountered. JTM 16, 332–337.

Bottieau, E., Clerinx, J., Schrooten, W., Van den Enden, E., Wouters, R., Van Esbroeck, M., Vervoort, T., Demey, H., Colebunders, R., Van Gompel, A., Van den Ende, J., 2006. Etiology and outcome of fever after a stay in the tropics. Arch. Intern. Med. 166, 1642–1648.

Casacuberta-Partal, M., Janse, J.J., van Schuijlenburg, R., de Vries, J.J.C., Erkens, M.A. A., Suijk, K., van Aalst, M., Maas, J.J., Grobusch, M.P., van Genderen, P.J.J., de Dood, C., Corstjens, P., van Dam, G.J., van Lieshout, L., Roestenberg, M., 2020a. Antigen-based diagnosis of Schistosoma infection in travellers: a prospective study. J. Trav. Med. 27, 1–9.

Casacuberta-Partal, M., Janse, J.J., van Schuijlenburg, R., de Vries, J.J.C., Erkens, M.A. A., Suijk, K., van Aalst, M., Maas, J.J., Grobusch, M.P., van Genderen, P.J.J., de Dood, C., Corstjens, P.L.A.M., van Dam, G.J., van Lieshout, L., Roestenberg, M., 2020b. Antigen-based diagnosis of Schistosoma infection in travellers: a prospective study. J. Trav. Med. 27.

Cherry, C.C., Denison, A.M., Kato, C.Y., Thornton, K., Paddock, C.D., 2018. Diagnosis of spotted fever group rickettsioses in U.S. Travelers returning from Africa, 2007-2016. Am. J. Trop. Med. Hyg. 99, 136–142.

Dahlgren, A.L., DeRoo, L., Avril, J., Bise, G., Loutan, L., 2009. Health risks and risk-taking behaviors among international committee of the red cross (ICRC) expatriates returning from humanitarian missions. JTM 16, 382–390.

Efared, B., Bako, A.B.A., Idrissa, B., Alhousseini, D., Boureima, H.S., Sodé, H.C., Nouhou, H., 2022. Urinary bladder Schistosoma haematobium-related squamous cell carcinoma: a report of two fatal cases and literature review. Trop. Dis. Travel Med. Vaccines 8, 3.

Effelsberg, W., 1989. [Duck bilharziasis in the medical anthropologic perspective. Interview data as a principle for public health control measures]. Offentl Gesundheitswes 51, 123–127.

Ferguson, R.W., Henderson, S.J., Lee, E.A., Jung, P., 2016. Dengue in peace corps volunteers, 2000–14. J. Trav. Med. 23.

Gupta, R.K., Rutledge, L.C., 1994. Role of repellents in vector control and disease prevention. Am. J. Trop. Med. Hyg. 50, 82–86.

Han, P., Balaban, V., Marano, C., 2010. Travel characteristics and risk-taking attitudes in youths traveling to nonindustrialized countries. JTM 17, 316–321.

Homsana, A., Odermatt, P., Southisavath, P., Yajima, A., Sayasone, S., 2020. Cross-reaction of POC-CCA urine test for detection of Schistosoma mekongi in Lao PDR: a cross-sectional study. Infect. Dis. Poverty 9, 114.

Jansing, P., Morrison, A., Heggie, T.W., Küpper, T., 2021. Tropical infections as occupational diseases- Labor inspectorate physicians’ aspects of a complex problem. Health Promotion Physical Activity 15 (2), 21–28 (2021).

Jensenius, M., Fournier, P.E., Vene, S., Hoel, T., Hasle, G., Henriksen, A.Z., Hellum, K.B., Raoult, D., Myrvang, B., 2003. African tick bite fever in travelers to rural sub-Equatorial Africa. Clin. Infect. Dis. 36, 1411–1417.

Jensenius, M., Hoel, T., Raoult, D., Fournier, P.E., Kjelshus, H., Bruu, A.L., Myrvang, B., 2002. Seroepidemiology of Rickettsia africae infection in Norwegian travellers to rural Africa. Scand. J. Infect. Dis. 34, 93–96.

Kapaun, A., 2004. Labordiagnose der Schistosomiasis (Bilharziose). Laboratory diagnosis of schistosome infections. LaboratoriumsMedizin 28, 483–490.

Karkey, A., Jombart, T., Walker, A.W., Thompson, C.N., Torres, A., Dongol, S., Tran Vu Thieu, N., Pham Thanh, D., Tran Thi Ngoc, D., Voong Vinh, P., Singer, A.C., Parkhill, J., Thwaites, G., Basnyat, B., Ferguson, N., Baker, S., 2016. The ecological dynamics of fecal contamination and Salmonella typhi and Salmonella paratyphi A in municipal kathmandu drinking water. PLoS Neglected Trop. Dis. 10, e0004346.

Kiehl, W., 2011. Steckbriefe seltener und importierter Infektionskrankheiten, Parasitosen 3.25 Schistosomiasis. Berlin, Westkreuz-Druckerei Ahrens KG Berlin/Bonn. Robert-Koch-Institut, p. S. 145.

Küpper, T., Rieke, B., Neppach, K., Morrison, A., Martin, J., 2014. Health hazards and medical treatment of volunteers aged 18-30 years working in international social projects of non-governmental organizations (NGO). Trav. Med. Infect. Dis. 12, 385–395.

Landman, K.Z., Tan, K.R., Arguin, P.M., 2013. Adherence to Malaria Prophylaxis Among Peace Corps Volunteers in the Africa Region.

Leder, K., Torresi, J., Libman, M.D., Cramer, J.P., Castelli, F., Schlagenhauf, P., Wilder-Smith, A., Wilson, M.E., Keystone, J.S., Schwartz, E., Barnett, E.D., von Sonnenburg, F., Brownstein, J.S., Cheng, A.C., Sotir, M.J., Esposito, D.H., Freedman, D.O., 2013. GeoSentinel surveillance of illness in returned travelers, 2007-2011. Ann. Intern. Med. 158, 456–468.

Lopez-Velez, R., Bayas, J.M., 2007. Spanish travelers to high-risk areas in the tropics: airport survey of travel health knowledge, attitudes, and practices in vaccination and malaria prevention. JTM 14, 297–305.

Maheshwari, V., Kaore, N.M., Ramnani, V.K., Sarda, S., 2016. A comparative evaluation of different diagnostic modalities in the diagnosis of typhoid fever using a composite reference standard: a tertiary hospital based study in Central India. J. Clin. Diagn. Res. 10, DC01–DC04.

Martin, J., Rieke, B., Neppach, K., Hillebrandt, D., Kupper, T., 2012. Risks to young volunteers in international social projects. Ann. Occup. Hyg. 56, 242–252.

Musaigwa, F., Kamdem, S.D., Mpotje, T., Mosala, P., Abdel Aziz, N., Herbert, D.R., Brombacher, F., Nono, J.K., 2022. Schistosoma mansoni infection induces plasmablast and plasma cell death in the bone marrow and accelerates the decline of host vaccine responses. PLoS Pathog. 18, e1010327.

Nauck, E., 1956. Welche Richtlinien gelten heute für die Behandlung und die Prophylaxe der Malaria. DMW (Dtsch. Med. Wochenschr.) 81, 313.

O’Brien, D., Tobin S Fau - Brown, G.V., Brown Gv Fau - Torresi, J., Torresi, J., 2001. Fever in Returned Travelers: Review of Hospital Admissions for a 3-year Period. Clin Infect Dis 33 (5), 603–609.

Ochodo, E.A., Gopalakrishna, G., Spek, B., Reitsma, J.B., van Lieshout, L., Polman, K., Lamberton, P., Bossuyt, P.M., Leeflang, M.M., 2015. Circulating antigen tests and urine reagent strips for diagnosis of active schistosomiasis in endemic areas. Cochrane Database Syst. Rev. CD009579.

Prüfer-Krämer, L., Boecken, G., Steiner, F., Rieke, B., 2020. Mandatory medical examination pre and post travel for volunteers of the "weltwarts" programme in Germany. Flugmedizin Tropenmedizin Reisemedizin 27, 20–25.

Sasayama, K., Gilmour, S., Ota, E., 2021. Factors Affecting Disease Risk Perception and Self-Management Behaviours Among Japanese Long-Term Overseas Volunteers. Scientific research publishing.

Shahapur, P.R., Shahapur, R., Nimbal, A., Suvvari, T.K., Rg, D.S., Kandi, V., 2021. Traditional widal agglutination test versus rapid immunochromatographic test in the diagnosis of enteric fever: a prospective study from south India. Cureus 13, e18474.

Sharp, T.W., DeFraites, R.F., Thornton, S.A., Burans, J.P., Wallace, M.R., 2006. Illness in journalists and relief workers involved in international humanitarian assistance efforts in Somalia, 1992–93. JTM 2, 70–76.

Zhong, H., Gui, X., Hou, L., Lv, R., Jin, Y., 2022. From inflammation to fibrosis: novel insights into the roles of high mobility group protein box 1 in schistosome-induced liver damage. Pathogens 11, 289.

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Urinary concentration of phthalates and bisphenol A during minipuberty is associated with reproductive hormone concentrations in infant boys

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ABSTRACT

Background: The transient postnatal activation of the hypothalamic-pituitary-gonadal hormone axis is termed minipuberty and considered an important developmental period, which is highly sensitive to endocrine disruption. Here, we explore exposure-outcome associations during minipuberty between concentrations of potentially endocrine disrupting chemicals (EDCs) in urine of infant boys and their serum reproductive hormone concentrations.

Methods: In total, 36 boys participating in the COPENHAGEN Minipuberty Study had data available for both urine biomarkers of target endocrine disrupting chemicals and reproductive hormones in serum from samples collected on the same day. Serum concentrations of reproductive hormones were measured by immunoassays or by LC-MS/MS. Urinary concentrations of metabolites of 39 non-persisting chemicals, including phthalates and phenolic compounds, were measured by LC-MS/MS. Nineteen chemicals had concentrations above the limit of detection in ≥50% of children and were included in data analysis. Associations of urinary phthalate metabolite and phenol concentrations (in tertiles) with hormone outcomes (age- and sex-specific SD-scores) were analysed by linear regression. Primarily, we focused on the EU regulated phthalates; butylbenzyl phthalate (BBzP), di-isobutyl phthalate (DiBP), di-n-butyl phthalate (DnBP), and di-(2-ethylhexyl) phthalate (DEHP) as well as bisphenol A (BPA). Urinary metabolites of DiBP, DnBP and DEHP were summed and expressed as ∑DiBPm, ∑DnBPm and ∑DEHPm.

Results: Compared to boys in the lowest ∑DnBPm tertile, urinary concentration of ∑DnBPm was associated with concurrent higher luteinizing hormone (LH) and anti-Müllerian hormone (AMH) SD-scores as well as lower testosterone/LH ratio in boys in the middle ∑DnBPm tertile (estimates (CI 95%) 0.79 (0.04; 1.54), 0.91 (0.13; 1.68), and −0.88 (−1.58;-0.19), respectively). Further, higher insulin-like peptide 3 (INSL3) SD-scores and lower DHEAS SD-score in boys in the highest ∑DnBPm tertile (0.91 (0.12; 1.70) and −0.85 (−1.51;-0.18), respectively) were observed. In addition, boys in the middle and highest ∑DEHPm tertile had higher LH (1.07 (0.35; 1.79) and 0.71 (−0.01; 1.43), respectively) and in the highest ∑DEHPm tertile also higher AMH (0.85 (0.10; 1.61)) concentration SD-scores, respectively. Boys in the highest BPA tertile had significantly higher AMH and lower DHEAS concentration compared to boys in the lowest BPA tertile (1.28 (0.54; 2.02) and −0.73 (−1.45; −0.01)), respectively.

Discussion: Our findings indicate that exposure to chemicals with known or suspected endocrine disrupting potential, especially the EU-regulated DnBP, DEHP and BPA, may modify male reproductive hormone concentrations in infant boys suggesting that minipuberty is a critical window sensitive to endocrine disruption.

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1. Introduction

Abbreviations

AGD	Anogenital distance
CI	Confidence interval
EDCs	Endocrine disrupting chemicals
EFSA	European Food Safety Authorities
HPG-axis	Hypothalamic pituitary gonadal axis
LC/MS-MS	liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
SD-scores	Standard deviation scores
STAR protein	steroidogenic acute regulatory protein
TDI	tolerable daily intake

Minipuberty describes the early postnatal activation of the hypothalamic-pituitary-gonadal (HPG) axis characterised by a transient sex-specific increase in reproductive hormones in infants (Kuiri-Hänninen et al., 2014). Luteinizing hormone (LH) and follicle stimulating hormone (FSH) increase at one week of age, leading to testosterone and INSL3 production in boys peaking at 1–3 months of age (Busch et al., 2022; Kuiri-Hänninen et al., 2014), while the Sertoli cell markers anti-Müllerian hormone (AMH) and Inhibin B peak around 4–5 months of age (Busch et al., 2022). Thus, minipuberty stretches over the first six months of life during which gonadotropins and testicular hormones remain above childhood levels. Minipuberty is thought to play an important role in the sexual maturation of infants (Lucaccioni et al., 2021), involving genital development, body composition and cognitive function, although the period is still not completely understood (Becker and Hesse, 2020; Kuiri-Hänninen et al., 2014).

Endocrine disrupting chemicals (EDCs) interfere with hormone systems in living organisms, and they may adversely affect the synthesis, actions or metabolism of hormones (Yilmaz et al., 2019). Humans are especially sensitive to EDCs during fetal life and infancy (Yilmaz et al., 2019), both in terms of direct impact on endocrine systems but also due to the potential of long-term programming effects through changes in the developmental trajectories that could potentially affect health throughout life (Sánchez-Garrido et al., 2022). Thus, EDCs may affect gonadal- and neuro-development and subsequently growth, pubertal onset, fertility, and development of hormone-sensitive cancers (Yilmaz et al., 2019). Phthalates and bisphenols are examples of non-persistent chemicals with endocrine disrupting potential that are quickly metabolized and excreted within hours to days after exposure, mainly in urine. However, because humans are continuously exposed they have also been called "pseudo-persistent" or "continuously present" (Mackay et al., 2014).

Phthalates are widely used plasticisers found in plastic items, personal care products, paints and solvents (Berger et al., 2019; Runkel et al., 2022). The commonly used phthalates butylbenzyl phthalate (BBzP), di-iso-butyl phthalate (DiBP), Di-n-butyl phthalate (DnBP) and di-(2-ethyl-hexyl) phthalate (DEHP) have adverse anti-androgenic/estrogenic effects in animal studies (Howdeshell et al., 2017), including decreased testosterone production, reduced anogenital distance (AGD), and cryptorchidism in male rat offspring following prenatal exposures (Enangue Njembele and Tremblay, 2021; Foster et al., 2006; Sharpe, 2020). Human studies have also found associations of prenatal phthalate exposure with similar reproductive outcomes (Radke et al., 2018), including decreased AGD in some but not all studies (Bornehag et al., 2015; Jensen et al., 2016; Muerköster et al., 2020; Swan et al., 2015), and decreased testosterone/LH ratio during the hormone peak of minipuberty (Muerköster et al., 2020). Thus, adverse effects of prenatal exposure to endocrine disrupting phthalates are generally recognized. However, we hypothesise that early postnatal exposure may disrupt the developmental sensitive window of minipuberty. To our knowledge only one previous study has focused on this period, and observed that postnatal exposure to phthalates through breastmilk was associated with reduced testosterone and elevated LH and sex-hormone binding globulin (SHBG) serum concentrations in infant boys (Main et al., 2006).

Bisphenols are used in polycarbonate plastics and epoxy resins as well as numerous consumer products (Runkel et al., 2022). Especially bisphenol A (BPA) exposure has been associated with endocrine disruption and reprotoxic outcomes in animal as well as human studies (Kortenkamp et al., 2022; Ren et al., 2020). Human studies have linked prenatal BPA exposure to e.g. preterm birth, reduced male fertility and reduced sexual function later in life (Ma et al., 2019; Namat et al., 2021), and adverse neurodevelopmental effects have been suggested following prenatal BPA exposure (Minatoya and Kishi, 2021). Effects of postnatal BPA exposure have also been studied, and during minipuberty, BPA concentrations in breastmilk were negatively associated with body weight in infants (Çiftçi et al., 2021), while studies in young men found associations between BPA exposure and lower sperm count, impaired sperm motility, and elevated serum LH, estradiol and testosterone concentrations (Adoamnei et al., 2018; Lassen et al., 2014).

Due to a growing body of evidence of adverse effects, BBzP, DiBP, DnBP and DEHP, as well as BPA, have gradually been restricted in EU and are now on The European chemicals Agency's (ECHAs) list of substances of very high concern (SVHC)("ECHA Candidate List," 2019). However, in biomonitoring studies these chemicals are still detectable in human urine including from infants.

In the present study, we aimed to investigate whether postnatal exposures during minipuberty to the EU-regulated chemicals BBzP, DiBP, DnBP and DEHP, and BPA are associated with hormone concentrations in serum from healthy infant boys. Furthermore, in an exploratory approach, we investigated possible associations between male reproductive hormones and other phthalates, bisphenols, benzophenones and triclosan.

2. Methods

2.1. Materials and subjects

The current study is a sub-study based on data from The COPENHAGEN Minipuberty cohort (CPHminipub), a longitudinal birth and infancy cohort conducted in Copenhagen, Denmark (2016–2019), which mainly aimed to investigate the dynamics of the transient activation of the HPG axis in early postnatal life in boys and girls. In CPHminipub, children participated in six follow-up visits during their first year of life. Visits included a physical examination by trained physicians with focus on auxology and genital development and whenever possible, blood and urine samples were collected at each visit. Details regarding the full CPHminipub cohort have been described previously (Busch et al., 2021).

In the current sub-study, we included data only from boys and only from the boys for whom we had available data on serum hormone and urine chemical concentrations measured in samples collected at the same visit (n = 36) within the active window of minipuberty (see Fig. 1). The rationale for including exclusively boys was based on individual longitudinal hormone data previously published for the full CPHminipub cohort, which showed significantly larger intra-individual variation in serum concentration of some hormones among girls than boys as well as more samples with undetectable hormone concentrations among girls (Suppl. Fig 1 in Ljubicic et al., 2022). Furthermore, only 18 girls had available hormone and chemical data from same-visit samples.

2.2. Hormone measurements

Serum samples in infants were analysed for reproductive hormones

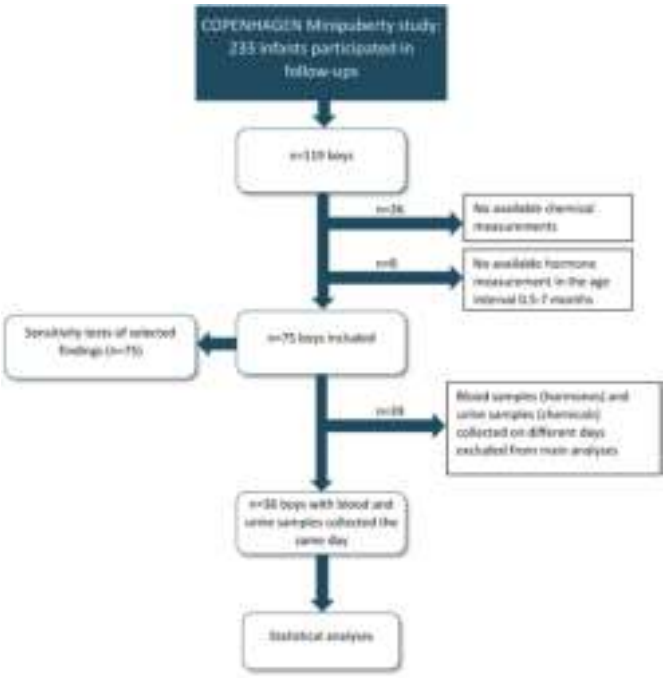

Fig. 1. Inclusion of participants
The 36 children with same day chemical and hormonal values were included in analyses.

as specified in detail previously (Busch et al., 2021) (see Table 1). In brief, LH and FSH were measured by time-resolved immunofluorometric assays (AutoDELFIA; PerkinElmer, Turku, Finland) with respective coefficients of variations (CV%) < 4% and <7%, sex hormone-binding globulin (SHBG) and anti-Müllerian hormone (AMH) were measured by chemiluminescence immunoassays (Access2, Beckman Coulter, Brea, CA, USA) with CV% <6% and< 4% respectively, and inhibin B was measured by enzyme-linked immunosorbent assays (ACTIVE® Inhibin B Gen II, Beckman Coulter) with CV% <7%. The following hormones were measured by isotope diluted on-line TurboFlow-liquid chromatography-tandem mass spectrometry (LC/MS-MS) in-house methods: dehydroepiandrosterone sulphate (DHEAS), CV% <7%; Δ4-androstenedione (androstenedione), CV% <4%; testosterone, CV% <3% and 17α-hydroxyprogesterone (17-OHP), CV% <3% (Søeborg et al., 2017) and INSL3, CV% <10% (Albrethsen et al., 2018). Longitudinal serum hormone concentrations for the full CPHminipub study have previously been published in (Busch et al., 2022) (boys) and (Ljubicic et al., 2022) (girls).

Table 1
Outcome variables included in our association analyses of chemical exposures during minipuberty.

Outcomes	Abbreviation	LOD	LOQ
Reproductive hormones			
Luteinizing hormone	LH	0.05 IU/L	
Follicle stimulating hormone	FSH	0.05 IU/L	
InhibinB	InhibinB	3 pg/mL	
Anti-Müllerian hormone	AMH	0.14 pmol/L	
Sex hormone binding globulin	SHBG	0.33 nmol/L	
Testosterone	–		0.012 nmol/L
Insulin-like peptide 3	INSL3		0.15 μg/L
17α-hydroxyprogesterone	17-OHP		0.1 nmol/L
Dehydroepiandrosterone sulphate	DHEAS		19 nmol/L
Δ4-androstenedione	Androstenedione		0.042 nmol/L
Hormone ratios			
Testosterone/luteinizing hormone	T/LH		
Testosterone/17α-hydroxyprogesterone	T/17-OHP		
Dehydroepiandrosterone sulphate/17α-hydroxyprogesterone	DHEAS/17-OHP		

LOD: Limit of detection. LOQ: Limit of quantification.

2.3. Chemical measurements

A sub-set of urine samples from CHPminipub were analysed for non-persistent chemicals with endocrine disrupting potential as part of two biomonitoring sub-studies to compare levels within and between parent-child trios and within the same child on different diets (Frederiksen et al., 2022a, 2022b). Selection of children for these previous sub-studies were based on whether their parents had provided a urine sample. In these samples, the total (free and conjugated) content of the metabolites of 15 phthalates and two phthalate substitutes (Frederiksen et al., 2022b), as well as the total (free and conjugated) content of seven benzophenones, eight bisphenols, and seven other polychlorinated- and phenolic substances (Frederiksen et al., 2022a), were measured by three different methods using isotope diluted on-line TurboFlow-LC-MS/MS as described in detail previously (Frederiksen et al., 2017, 2020). Measured urinary chemical concentrations were standardised for urinary dilution using the osmolality of individual samples (Frederiksen et al., 2022b).

2.4. Data analysis

In the present study, we included exclusively hormone data from blood samples collected in the age interval of 0.5–7 months to avoid the influence of residues of hormones of placental origin in the infant circulation shortly after birth as well as low or undetectable hormone concentrations for some hormones following the period of active minipuberty. As the infant urine samples originally were selected for two biomonitoring studies (Frederiksen et al., 2022a, 2022b) not all chemical measures available were performed on samples collected at timepoints suitable for the current study. Thus, to study associations between chemical concentrations measured in urine and hormone concentrations measured in serum, we matched hormone and chemical data from the most relevant samples as shown in Fig. 1 and described in detail below.

2.4.1. Matching of chemical and hormonal data and inclusion of participants

75/199 boys from CPHminipub had available hormone measurements within the age interval of 0.5–7 months of age as well as available chemical measurements in at least one urine sample. Of these 36 boys had hormones and chemicals measured in blood and urine samples collected at the same visit. For the remaining 39 boys for whom chemical and hormonal measurement were not available from the same visit, we matched the samples with the smallest available time gap (median (range) = 60 (21–194) days, urine collected before blood sample in 31/39).

Only data of the 36 boys with same-visit urine and blood samples were included in our primary association analyses of hormones and chemical exposure. If significant associations were observed and if we considered the associations biologically plausible, we repeated the analyses using data from all 75 boys with available hormone and chemical data. Characteristics of the 36 and 75 boys are presented in Table 2.

2.4.2. Outcomes

Hormones included as outcomes in our analyses were all

Table 2
Characteristics of included participants from CHPminipub.

	Boys with available hormone and chemical measurement within 0.5–7 months of age N = 75 Median (range)	Boys with available same-visit hormone and chemical measurement within 0.5–7 months of age N = 36 Median (range)
Birth weight (g)	3644 (2850–4566)	3685 (3040–4110)
Gestational age (week)	40 (37–42)	40.5 (37–41)
Age (days) at included urine collection	60.5 (6–247)	77.5 (31–182)
Age (days) at included blood collection	88 (20–204)	
Hormone SD-scores		
LH	−0.06 (−1.86–2.29)	−0.07 (−1.86–2.29)
FSH	−0.16 (−1.39–1.74)	−0.16 (−1.39–1.05)
Inhibin B	0.22 (−1.82–2.22)	0.13 (−1.82–1.98)
AMH	0.03 (−1.62–2.36)	0.16 (−1.57–2.36)
SHBG	−0.04 (−2.41–2.46)	−0.14 (−1.88–1.52)
Testosterone	0.17 (−1.68–3.07)	0.15 (−1.68–2.14)
INSL3	0.48 (−1.02–2.24)	0.53 (−1.00–2.09)
17α-hydroxyprogesterone	−0.002 (−2.71–1.94)	−0.03 (−2.71–1.74)
DHEAS	−0.01 (−2.04–2.18)	−0.23 (−1.70–1.90)
Δ4-androstenedione	−0.9 (−1.63–1.38)	−0.34 (−1.60–1.25)

reproductive hormones for which data was available: LH, FSH, inhibin B, AMH, testosterone, INSL3, SHBG, 17-OHP, androstenedione and DHEAS. In addition, we calculated ratios of the following hormones: Testosterone/LH (T/LH), Testosterone/17-OHP (T/17-OHP), and DHEAS/17-OHP (Table 1). T/17-OHP and DHEAS/17-OHP ratios are generally not used in a clinical setting but may be used as markers of the specific enzymatic activity at different steps in steroidogenesis, as well as the overall steroidogenic capacity. To adjust for age-dependent variations in our outcomes, we included hormone standard deviation scores (SD-scores), rather than the actual hormone concentrations in our analyses for all outcomes. Age specific hormone SD-scores were calculated in relation to a normal reference material consisting of longitudinal hormone data of all 119 healthy boys from the CHPminipub cohort as described by Busch et al. (2022). As indicated by the SD-scores reported in Table 2, the single hormone measurement included for the selected 36 boys as well as the 75 boys seem to be within the normal reference range without significant bias.

2.4.3. Exposure variables

Osmolality corrected (standardised for variation in urinary dilution) urinary concentrations of the measured chemicals were used as exposure biomarkers. Osmolality correction was chosen over urinary creatinine because it is less affected by body size and diet, which both change drastically during the first year of life. Another common method, specific gravity, may over- or underestimate concentrations in certain clinical conditions while osmolality is more versatile (Voinescu et al., 2002). Thus, the osmolality correction method is superior to the two other methods for this study. Chemicals with ≥50% of samples above the limit of detection (LOD) were included in the data analysis (n = 19/39 chemicals)(Frederiksen et al., 2022a, 2022b). We included 10 phthalates, two phthalate substitutes, four benzophenones, two bisphenols and triclosan. Names and abbreviations of the included chemicals can be found in Tables 3a and 3b, and in the following the abbreviations will be used. Osmolality corrected chemical concentrations (ng/mL) can be found in Table 4, and raw uncorrected chemical concentrations are presented in Table S4. The molar sum concentration of phthalate metabolites was multiplied by the molecular weight of the parent compound and summed as shown in Table 3a and described in detail in (Frederiksen et al., 2022b). Thus, measured metabolites of DnBP, DiBP, DEHP, DnHxP, DnHpP, DiNP, DiDP, DINCH and DEHTP

Table 3a
Included phthalates, their respective metabolites and limits of detection (LOD).

Phthalate diester	Abbreviation	Urine metabolite	Abbreviation	LOD (ng/mL)
Di-methyl phthalate	DMP	Mono-methyl phthalate	MMP	0.27
Di-ethyl phthalate	DEP	Mono-ethyl phthalate	MEP	0.43
Butylbenzyl phthalate	BBzP	Mono-benzyl phthalate	MBzP	0.03
Di-iso-butyl phthalate	DiBP	Mono-iso-butyl phthalate	MiBP	0.16
		Mono-(2-hydroxy-iso-butyl) phthalate	2OH-MiBP	0.25
Di-n-butyl phthalate	DnBP	Mono-n-butyl phthalate	MnBP	0.17
		Mono-(3-hydroxybutyl) phthalate	3OH–MnBP	0.25
Di-(2-ethyl-hexyl) phthalate	DEHP	Mono-(2-ethyl-hexyl) phthalate	MEHP	0.07
		Mono-(2-ethyl-5-hydroxyhexyl) phthalate	5OH-MEHP	0.05
		Mono- (2-ethyl-5-oxohexyl) phthalate	5oxo-MEHP	0.06
		Mono-(2-ethyl-5-carboxypentyl) phthalate	5cx-MEPP	0.07
		Mono-(2-carboxymethyl-hexyl) phthalate	2cx-MMHP	0.12
Di-n-hexyl phthalate	DnHxP	Mono-n-hexyl phthalate	MnHxP	0.04
		Mono-(5-hydroxyhexyl) phthalate	5OH-MHxP	0.02
		Mono-(5-carboxypentyl) phthalate	5cx-MPeP	0.02
Di-n-heptyl phthalate	DnHpP	Mono-n-heptyl phthalate	MnHpP	0.03
		Mono-(6-hydroxyheptyl) phthalate	6OH-MHpP	0.01
		Mono-(6-carboxyhexyl) phthalate	6cx-MHxP	0.03
Di-iso-nonyl phthalate	DiNP	Mono-iso-nonyl phthalate	MiNP	0.14
		Mono-hydroxy-iso-nonyl phthalate	OH-MiNP	0.03
		Mono-oxo-iso-nonyl phthalate	oxo-MiNP	0.02
		Mono-carboxy-iso-octyl phthalate	cx-MiOP	0.04
Di-iso-decylphthalate	DiDP	Mono-iso-decyl phthalate	MiDP	0.16
		Mono-(hydroxy-iso-decyl) phthalate	OH-MiDP	0.02
		Mono-(oxo-iso-decyl) phthalate	oxo-MiDP	0.02
		Mono-(carboxy-iso-nonyl) phthalate	cx-MiNP	0.02
Phthalate substitutes				
Di-iso-nonyl-cyclohexane-1,2-dicarboxylate	DINCH	Cyclohexane-1,2-dicarboxylate-mono-(hydroxyl-iso-nonyl) ester	OH-MiNCH	0.03
		Cyclohexane-1,2-dicarboxylate-mono-(carboxy-iso-octyl) ester	cx-MiOCH	0.02
Di-2-ethylhexyl terephthalate	DEHTP	Mono-(2-ethyl-5-hydroxy-hexyl) terephthalate	5OH-MEHTP	0.04

(continued on next page)

Table 3a (*continued*)

Phthalate diester	Abbreviation	Urine metabolite	Abbreviation	LOD (ng/mL)
		Mono-(2-ethyl-5-oxo-hexyl) terephthalate	5oxo-MEHTP	0.03
		Mono-(2-ethyl-5-caboxyl-pentyl) terephthalate	5cx-MEPTP	0.02
		Mono-(2-caboxyl-methyl-hexyl) terephthalate	2cx-MMHTP	0.02

Sums of selected phthalate metabolite and substitutes[a]

SDiBPm	molar sum of MiBP and 2OH-MiBP expressed as DiBP
SDnBPm	molar sum of MnBP and 3OH–MnBP expressed as DnBP
SDEHPm	molar sum of MEHP, 5OH-MEHP, 5oxo-MEHP, 5cx-MEPP and 2cx-MMHP expressed as DEHP
SDnHxPm	molar sum of MnHxP, 5OH-MHxP and 5cx-MPeP expressed as DnHxP
SDnHpPm	molar sum of MnHpP, 6OH-MHpP and 6cx-MHxP expressed as DnHpP
SDiNPm	molar sum of MiNP, OH-MiNP, oxo-MiNP and cx-MiOP expressed as DiNP
SDiDPm	molar sum of MiDP, OH-MiDP, oxo-MiDP and cx-MiNP expressed as DiDP
SDINCHm	molar sum of OH-MiNCP and cx-MiOP expressed as DINCH
SDEHTPm	molar sum of 5OH-MEHTP, 5oxo-MEHTP, 5cx-MEPTP and 2cx-MEHTP expressed as DEHTP

[a] All molar sums were converted to concentrations in ng/mL by multiplying with the molecular weight of their respective mother compound.

Table 3b
Included benzophenones, bisphenols and triclosan and limits of detection (LOD).

Chemical	Abbreviation	LOD (ng/mL)
Benzophenones		
Benzophenone	BP	0.06
Benzophenone-1	BP-1	0.01
Benzophenone-3	BP-3	0.03
4-Hydroxy-benzophenone	4-HBP	0.20
Bisphenols		
Bisphenol A	BPA	0.07
Bisphenol S	BPS	0.02
Other substances		
Triclosan	TCS	0.04

were summed and expressed as ΣDnBPm, ΣDiBPm, ΣDEHPm, ΣDnHxPm, ΣDnHpPm, ΣDiNPm, ΣDiDPm, ΣDINCHm and ΣDEHTPm respectively (Table 3a). We divided the boys into tertiles for all osmolality corrected chemical concentrations to categorise the boys into low-, medium-, and high exposure groups. The reasons we categorized exposure into tertile were that for many of the compounds 10–20% of the samples were <LOD. Also, with our limited study sample size a single outlier may be given too much weight in a linear model compared to using tertiles. Finally, analysing the data in tertiles allowed for observing also non-linear associations, which may be relevant for potential endocrine disrupting effects. For some compounds, more than 33% of the measurements were below LOD. In these cases, the low-exposure group consisted of all boys with concentrations below the LOD, and the remaining children were divided in two equally sized groups corresponding to the medium- and high-exposure infants.

2.4.4. Statistical analyses

Our main aim was to investigate the association between concurrent exposure to BBzP, DnBP, DiBP, DEHP, or BPA and reproductive hormone concentrations in boys (n = 36) during minipuberty using osmolality corrected urinary concentrations of BPA, MBzP, ΣDnBPm, ΣDiBPm, ΣDEHPm as exposure biomarkers. Secondly, we assessed the rest of the included chemicals (n = 14) and their associations with the included reproductive hormones in an exploratory approach (see supplementary tables).

Table 4
Osmolality corrected chemical concentrations (ng/mL) in 36 boys.

	N > LOD	% > LOD	Min	Percentiles		Max	Mean
				50	95		
Phthalate metabolites							
MMP	27	75	<LOD	0.64	16.50	34.55	2.09
MEP	36	100	0.95	2.78	34.24	52.37	6.35
MBzP	26	72.2	<LOD	0.31	3.70	7.29	0.68
∑DiBPm	36	100	1.01	6.10	59.90	85.85	13.09
∑DnBPm	36	100	0.84	5.06	20.07	52.09	6.83
∑DEHPm	36	100	0.267	3.88	11.92	28.48	4.86
∑DnHxPm	26	72.2	<LOD	0.24	14.97	16.10	1.70
∑DnHpPm	19	52.3	<LOD	0.01	0.53	1.01	0.08
∑DiNPm	33	91.7	<LOD	0.61	4.15	10.68	1.10
∑DiDPm	30	83.3	<LOD	0.10	0.84	1.00	0.20
∑DINCHm	30	83.3	<LOD	0.18	2.67	3.15	0.39
∑DEHTPm	36	100	0.81	2.78	11.83	26.05	3.81
Benzophenones, bisphenols and triclosan							
BP	18	50.0	<LOD	0.05	3.14	4.84	0.76
BP-1	23	63.9	<LOD	0.21	12.57	62.47	2.15
BP-3	34	94.4	<LOD	0.69	58.23	276.72	9.37
4-HBP	18	50.0	<LOD	0.10	1.27	1.46	0.34
BPA	31	86.1	<LOD	0.34	1.66	1.85	0.45
BPS	29	80.6	<LOD	0.05	0.24	0.24	0.07
TCS	28	77.8	<LOD	0.10	7.89	29.12	0.98

Associations between chemical exposure and hormone SD-scores were analysed by general linear models. Differences between the low-exposed group (reference group) and the medium- and high-exposed boys were investigated for each exposure biomarker using an ordinal variable corresponding to the first, second and third tertiles as a categorical variable in the model. Tests for trend were done by entering the ordinal variable for exposure tertiles in the model as a continuous variable. Our outcome variables were age- and sex-dependent SD-scores, so we did not adjust for age in our main analyses. We conducted sensitivity tests to adjust for gestational age and birth weight for all associations mentioned in the result section with no major changes in the estimates. We also conducted a sensitivity test for age at sample collection to adjust for changes in the chemical measures due to age differences in the three tertiles. Results are presented as the parameter estimate and its confidence interval (CI 95%) listed for each association. P-values ≤0.05 were considered significant. Finally, we also used the Benjamini-Hochberg method to adjust for multiple testing with a false discovery rate of 0.05. All statistical analyses have been calculated in the IBM SPSS statistics version 25.0.

3. Results

3.1. Associations between urinary concentrations of metabolites of the endocrine disrupting phthalates BBzP, DiBP, DnBP and DEHP, as well as BPA and serum concentrations of reproductive hormones during minipuberty in boys

3.1.1. BBzP, DiBP, DnBP and DEHP metabolites

Our analyses of associations between tertiles of urinary concentrations of MBzP, ΣDiBPm, ΣDnBPm or ΣDEHPm and SD-scores of serum concentrations of reproductive hormones in the 36 boys with time-matched samples resulted in eight significant results (Fig. 2 and Supplementary Table S1). Seven of these results were related to urinary concentrations of ΣDnBPm and ΣDEHPm (Fig. 2a and c).

Boys in the middle ΣDnBPm tertile had significantly higher LH and AMH SD-scores [0.79 (0.04; 1.54) p = 0.04 and 0.91 (0.13; 1.68) p = 0.02, respectively] compared to boys in the lowest tertile. In addition,

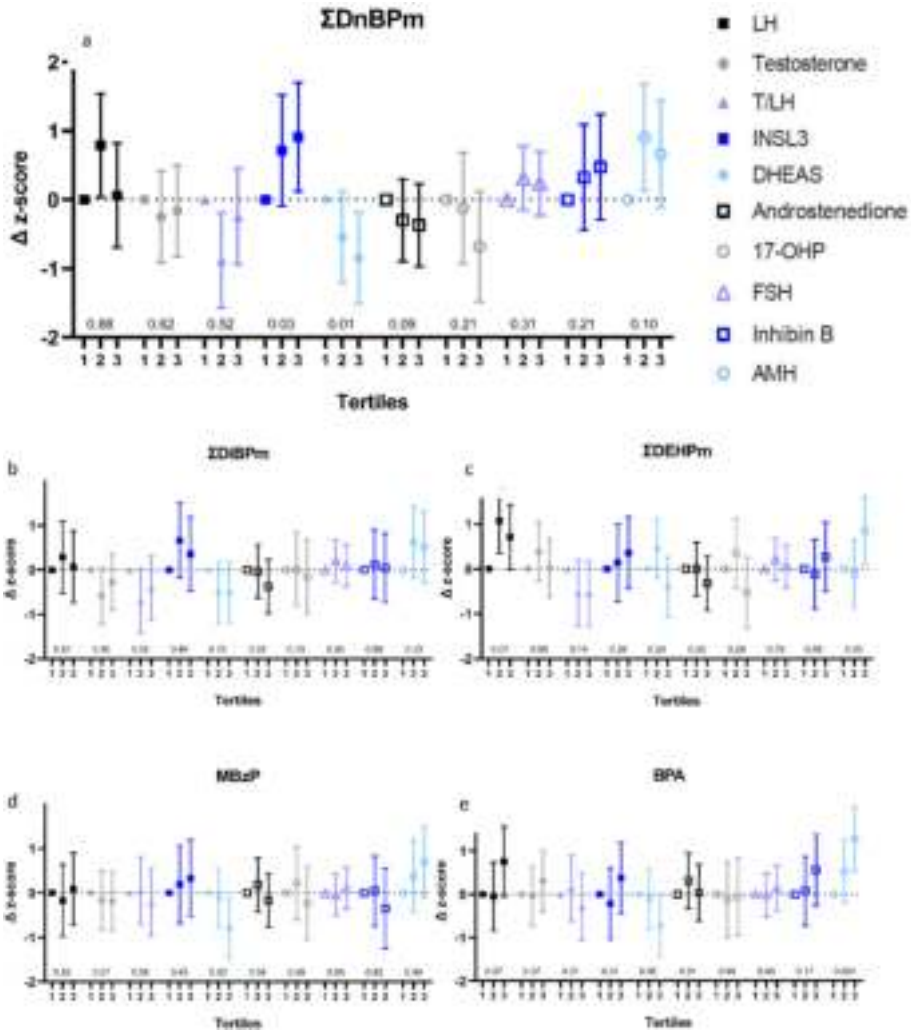

Fig. 2. Associations between reproductive hormones and ΣDnBPm, ΣDiBPm, ΣDEHPm, MBzP, and BPA in 36 boys

1: Reference group corresponding to the lowest tertile, 2: middle tertile, 3: highest tertile. Confidence intervals and estimates from a linear regression model. Z-score according to exposure of phthalate (tertiles) during minipuberty. Z-scores represent age- and sex-dependent z-scores. The chemical abbreviations are described in Tables 3a and 3b. P-trends for each outcome are indicated above the x-axis.

INSL3 SD-scores were significantly higher among boys in the highest ΣDnBPm tertile [0.91 (0.12; 1.70) p = 0.03] (Fig. 2a), and a significant positive trend (P-trend = 0.03) towards higher INSL3 SD-scores across ΣDnBPm tertiles was observed. Finally, ΣDnBPm was associated with a significantly lower DHEAS SD-score among boys in the highest tertile compared to those in the lowest tertile [−0.85 (−1.51; −0.18) p = 0.01] and lower T/LH ratio SD-score among boys in the middle tertile [−0.88 (−1.58; −0.19) p = 0.01]. Additional analyses for ΣDnBPm excretion were conducted on all 75 boys (i.e. including the 39 boys with urine and blood samples collected several days apart). These sub-analyses confirmed the main analyses, i.e. estimates generally pointed in the same direction, but except for DHEAS they were no longer statistically significant (Supplementary Table S2).

Our analysis of ΣDiBPm (Fig. 2b) excretion and associations with reproductive hormones did not result in any significant findings.

For the 36 boys, ΣDEHPm excretion was also significantly associated with higher LH and AMH serum concentration SD-scores. Thus, boys in the middle ΣDEHPm tertile had higher LH SD-scores [1.07 (0.35; 1.79) p = 0.01] and boys in the highest tertile had higher AMH SD-scores [0.85 (0.10; 1.61) p = 0.03] compared to boys in the lowest tertile (Fig. 2c). A significant positive trend of higher AMH SD-scores across increasing ΣDEHPm tertiles was found (p-trend = 0.03). These significant findings could not be replicated in sub-analyses including all 75 boys (Supplementary Table S2).

Urinary concentrations of MBzP in 36 boys was associated with lower DHEAS SD-scores among boys in the highest MBzP tertile [−0.80 (−1.46; −0.13) p = 0.02] as the only significant finding (Fig. 2d). In sub-analyses of all 75 boys, there were no significant findings (Supplementary Table S2).

Adjusting for age did not change the significant findings for MBzP, DiBP, DnBP and DEHP. However, when adjusting for multiple testing with the Benjamini-Hochberg method none of the observed associations remained significant (data not shown).

3.1.2. BPA

For the 36 boys with time-matched samples (i.e. chemicals and hormone concentrations were measured in respectively a urine and a blood sample collected the same day) we observed that boys in the highest BPA tertile had higher AMH, lower DHEAS and lower DHEAS/17OHP ratio SD-scores compared to boys in the lowest tertile [1.28 (0.54; 2.02) p = 0.001, −0.73 (−1.45;-0.01) p = 0.05, −0.69 (−1.39; 0.00) p = 0.05, respectively] (Fig. 2e). A significant P-trend was observed for all three outcomes (P = 0.001, P = 0.046 and P = 0.048, respectively). When adjusting for age at sampling in a sensitivity test, all associations were no longer significant and likewise when adjusting for multiple testing (data not shown). We further conducted sub-analyses on all 75 boys with no significant findings (Supplementary Table S2).

3.2. Exploration of associations between urinary concentrations of other phthalate metabolites or phenols and serum concentrations of reproductive hormones during minipuberty in boys

For the remaining fourteen compounds present in more than 50% of the urine samples (MMP, MEP, ΣDnHxPm, ΣDnHpPm, ΣDiNPm, ΣDiDPm, ΣDINCHm, ΣDEHTPm, BP, BP-1, BP-3, 4-HBP, BPS and TCS; see Table 4) but for which less evidence of endocrine disrupting action is available, we tested associations with reproductive hormone concentrations in an exploratory approach. For the 36 boys with time-matched samples four statistically significant differences in outcomes between the lowest tertile (reference group) and the middle or highest tertiles were observed among the phthalate metabolites: being in the highest MEP tertile was associated with higher FSH SD-scores, being in the middle ΣDnHpPm and ΣDiDPm tertiles was associated with lower DHEAS SD-scores, and being in the middle ΣDEHTPm tertile was associated with lower INSL3 SD-scores (Supplementary Table S1). Furthermore, two statistically significant findings were observed for 4-HBP: being in the highest tertile was associated with lower SHBG SD-scores and being in the middle tertile was associated with higher 17-OHP SD-scores (Supplementary Table S3). These results did not persist when adjusting for multiple testing (data not shown).

4. Discussion

Associations between markers of exposure to endocrine disrupting phthalates and BPA and reproductive hormones during minipuberty.

In infant boys, higher urinary concentrations of ∑DnBPm were associated with concurrent higher LH, AMH and INSL3 SD-scores, and lower T/LH ratio, DHEAS and 17-OHP SD-scores. A similar pattern was present for urinary ∑DEHPm and BPA concentrations. When adjusting for multiple testing, these associations were no longer significant. However, while procedures to adjust for multiple testing reduce the likelihood of spurious findings, they also lower the statistical power, thereby greatly reducing the probability of detecting relevant effects. For a balanced trade-off between a lower probability of detecting a true effect when adjusting and a higher probability of a false positive when not adjusting, we have focused on the chemicals that showed (unadjusted) significant associations with at least two hormones. The reproductive hormones are inter-related through positive and negative feedback regulation. Thus, we consider associations affecting two or more hormones to be more likely to be true effects; especially if the direction of the associations fit with the known physiology of the studied hormones and mode of action/effects of the chemicals.

Urinary concentrations of phthalate metabolites observed in our relatively small study were comparable to those measured in two-months old infants in the recent French SEPAGES cohort (Philippat et al., 2021) indicating similar exposure pattern in the two study populations. DBP and DEHP are believed to exert anti-androgenic effects by affecting steroidogenesis and thus altering androgen concentrations (Borch et al., 2006; Thompson et al., 2004). Studies *in vitro* and *in utero* in rats have shown that exposure to the phthalates DBP and DEHP alters the expression of several genes crucial for steroidogenesis, including the gene encoding the steroidogenic acute regulatory (StAR) protein (Kariyazono et al., 2015; Traore et al., 2021; Wang et al., 2007). StAR regulates a rate-limiting step in steroidogenesis, specifically the transport of cholesterol to the inner mitochondrial membrane (Manna et al., 2016). In our study, we used urinary concentrations of phthalate metabolites as biomarkers of exposure to phthalates as no data of direct exposure to the parent compounds was available. The combined pattern of higher LH and INSL3 concentrations, lower DHEAS concentrations, a tendency towards lower 17-OHP concentrations and normal/low concentrations of testosterone and androstenedione observed among boys in the middle and highest ∑DnBPm tertiles (and partly among boys in the highest ∑DEHPm tertile) compared to the lowest tertile is consistent with an anti-androgenic effect of exposure to the mother compounds DBP and DEHP, possibly through an inhibition of StAR expression. We did not observe a decrease in testosterone, but we observed a significantly decreased testosterone/LH ratio among boys excreting higher ∑DnBPm concentrations than the reference group. Hypothetically, an initially decreased testosterone concentration would result in pituitary compensation with increased LH secretion due to the feedback mechanisms of the HPG hormone axis, which is active during minipuberty (Busch et al., 2022). An increased LH SD-score combined with a normal testosterone SD-score, as observed in our data, could indicate a degree of compensated hypogonadism among boys in the middle ∑DnBPm tertile. LH signaling upregulates both StAR and CYP17 expression and it may be speculated that increased LH signalling could thus counteract an inhibitory effect that DBP exposure may have on steroidogenesis and drive it towards testosterone production through upregulation of the StAR and CYP17 activity (Murayama et al., 2012). This scenario also fits with the observed lower DHEAS and 17-OHP SD-scores among the boys excreting the highest concentration of ∑DnBPm. DHEAS, together with the non-sulphated DHEA, are the most abundant steroid hormones in circulation (Prough et al., 2016). They are the main adrenal androgen precursors, although they themselves are biologically inactive (Turcu et al., 2020), and DHEAS/DHEA is thus a prohormone that has androgenic or estrogenic potential (Labrie et al., 2005). Likewise, it may be speculated that the concurrent significant higher INSL3 associated with increasing excretion of ∑DnBPm may be a side-effect of increased LH stimulation of the Leydig cells further indicating that particularly steroidogenesis, and not overall Leydig cell function, may be affected.

Knowledge on long-term reproductive consequences of early-life phthalate exposure is sparse, but gestational and pubertal exposure to low doses of DEHP were shown to impair sperm quality in adult mice (Dostalova et al., 2020). Moreover, a cross sectional human study suggests a negative impact on sperm count and volume in adulthood after maternal occupational exposure during pregnancy (Istvan et al., 2021). In our study, we have no information on the children's prenatal exposure, but hypothetically, postnatal exposures affecting the HPG-hormone axis during minipuberty may also have consequences for future reproductive health (Scheutz Henriksen et al., 2022). Additionally, infant postnatal urinary excretion may for some chemicals (or metabolites thereof) be a proxy of the mother's general exposure to the chemical, both during and after pregnancy as indicated by Frederiksen et al. which showed correlation between maternal and infant urinary excretion postnatally for several chemicals (Frederiksen et al., 2022a, 2022b). It may therefore be difficult to separate prenatal and postnatal exposure effects.

Additionally, we found associations between BPA excretion and significantly decreased DHEAS and DHEAS/17-OHP ratio, while an elevated LH was borderline significant. BPA has been associated with numerous adverse health effects by interacting with, among others, the estrogen and androgen receptor, although it has been debated whether average human exposure levels are high enough to pose a health risk (Ma et al., 2019; Wang et al., 2017). While the tolerable daily intake (TDI) of BPA since 2015 have been estimated to 0.4 μg/kg/day by the European Food Safety Authorities (EFSA), new immunotoxic studies have led EFSA to propose a lower TDI of 0.04 ng/kg/day (EFSA, 2021). In addition, some epidemiological studies have linked BPA to poor semen quality following adult exposure, making Kortenkamp et al. suggest a TDI of 0.003 μg/kg/day for mixture risk assessment (Kortenkamp et al., 2022). The new proposed TDIs might be lower than what most European citizens experience, which would hypothetically make BPA the major contributor to the chemical cocktail effects on male reproductive development. BPA exposure has previously in adult men been associated with increased LH, testosterone and estradiol serum concentrations (Adoamnei et al., 2018; Lassen et al., 2014), while another study found negative associations between prenatal and/or childhood exposure to BPA and testosterone levels in the ages of 8–14 years (Ferguson et al., 2014). Our findings also suggest a positive association with LH. The decreased DHEAS further suggests an inhibition

of the steroidogenesis corresponding to an antiandrogenic effect with higher LH as a possible feedback mechanism to maintain testosterone levels. The BPA levels detected in our study were lower compared to previously reported levels in older children (Covaci et al., 2015; Frederiksen et al., 2014) which may be due to an observed declining trend in BPA exposure in the Danish population (Frederiksen et al., 2020) and/or to age differences in exposures.

Interestingly, we observed significantly higher AMH in boys with middle or highest levels of ∑DnBPm, ∑DEHPm and BPA urinary excretion. Produced by immature Sertoli cells in the foetal testis, AMH is especially important for the male genital differentiation during foetal life (Josso and Rey, 2020). Postnatally, AMH concentrations increase and peak at three months of age remaining high and somewhat stable until the abrupt pubertal decline (Aksglaede et al., 2010; Busch et al., 2022). However, the role of postnatal AMH in males is not clear (Aksglaede et al., 2018). Although basal AMH production occurs, FSH further increase AMH production through stimulation of the transcriptional activation of AMH genes and induction of Sertoli cell proliferation until downregulation occurs as the Sertoli cells differentiate at the onset of puberty through testosterone activation of the androgen receptor (Edelsztein et al., 2016; Lasala et al., 2004). Before puberty, the androgen receptor is not expressed in Sertoli cells, which explains the simultaneously high AMH and testosterone concentrations during minipuberty (Xu et al., 2019). Serum concentrations of AMH prepubertally is a biomarker of FSH action (Edelsztein et al., 2016), however, we did not observe an increase in FSH in our data. Another inducer of AMH was recently demonstrated in a study in which AMH expression was upregulated by estradiol in a prepubertal Sertoli cell line (Valeri et al., 2020). Estradiol was not measured in the boys included in our study due to a generally very low estradiol detection rate previously observed in infant boys even with a very sensitive analytical method (Frederiksen et al., 2020). However, LH hyperstimulation is thought to upregulate the transcription of aromatase (CYP19) in Leydig cells, at least in adults (Lardone et al., 2017; Valladares and Payne, 1979). This could potentially increase intra-testicular estradiol in these infants. Additionally, as already mentioned, BPA exposure has previously been associated with increased estradiol in adult men (Lassen et al., 2014).

The estimates for the differences in reproductive hormone concentrations among boy in the lowest tertile compared to boys in the middle and highest tertile were for the significant associations from 0.4 up to 1 SD, which can be considered a biologically significant difference. These differences could potentially last until adulthood, as a study recently showed that male reproductive hormone concentrations in infancy correlate well with concentrations seen in adulthood (Scheutz Henriksen et al., 2022).

4.1. Associations between chemicals and reproductive hormones: an explorative approach

Although our main focus in this sub-study was to investigate the EU-regulated phthalates DiBP, DnBP, DEHP, and BPA due to the amount of evidence of harmful endocrine disrupting effects, which exist for these compounds, we also assessed effects of other chemicals with endocrine disrupting potential but for which data on endocrine disruptive activity is less conclusive. The analyses of associations between the remaining included chemicals and reproductive hormones resulted in six significant associations, which did not remain significant after adjusting for multiple testing. Also, even before adjusting for multiple testing, these chemicals were only significantly associated with a single hormone. If the observed association represented a true biological effect, one might expect also other of the hormones to be affected as they are interlinked. Accordingly, the observed associations may be chance findings. However, the results might be useful in future meta-analyses and should be confirmed in larger populations.

4.2. Strengths and limitations

The number of human biomonitoring studies in healthy infants are very limited due to the challenge of collecting urine and blood from infants. Thus, regardless of its small size, our study on 36 boys with time-matched urine and blood samples adds important information to the scant available data on infant exposures. The broad selection of inter-related hormones measured including INSL3 and androgens, all measured by highly sensitive methods including mass spectrometry, the gold standard of hormone measurement, was a major strength of this study. Another strength included the well-described outcomes with individual sex and age-related SD-scores. We primarily focused on five chemicals that are known to be endocrine disrupting and reprotoxic and regulated in the EU due to the amount of evidence of their adverse effects and mode of action. Therefore, knowledge of hormone physiology and mechanisms/effects of the chemicals could be incorporated in the interpretation of our observations.

We were clearly limited by the relatively small number of boys with time-matched samples (n = 36). We limited our study to boys as our outcome variables were hormone SD-scores. Boys in this age group tend to stay longitudinally within the same tertile for most of the investigated hormones, as shown in (Busch et al., 2022), while girls are more likely to jump between tertiles over time (Ljubicic et al., 2022). In our study blood samples were collected over the day. Some diurnal variation in reproductive hormones exists in adults. However, information on diurnal variation in hormone levels in infants do not exist to our knowledge.

Another limitation of this study was the use of single spot urine to analyse non-persisting chemicals as for some of the metabolites studied, especially BPA, urinary concentrations are known to exhibit a large day-to-day variation in older children and adults (Frederiksen et al., 2013; Lassen et al., 2013). Within-person variation in urinary excretion of these chemicals may cause exposure misclassification, which can lead to over- or underestimation of effect estimates (Pollack et al., 2013). However, we do not know whether infants have the same within-person variability in the urinary excretion of the studied compounds. It could be speculated that within-person day-to-day variation may be lower in infants, who may present with a more stable daily routine compared to adolescents and adults.

In the CPHminipub cohort we have previously observed a trend of older infants excreting higher concentrations of most investigated chemicals (Frederiksen et al., 2022a, 2022b). Here we included boys between 1 and 6 months of age and both boys that were exclusively breastfed, and boys introduced to other diets. Changes in diet is strongly associated with age during the first year of life, and it is not possible in our relatively small material to distinguish between changes in diet and children getting older and potentially experiencing more sources of exposure. We cannot exclude that this variation may have influenced our results, although we adjusted for age in sensitivity tests, which only changed results for BPA. Furthermore, we cannot exclude that other unmeasured confounders may be responsible for the observed associations. Parents participating in this cohort were typically well-educated and from affluent areas of Copenhagen, thus rendering them a selected population, which should be considered when extrapolating our results to the general population. Although the CPHminipub Study was a longitudinal study, this sub-study was cross-sectional and causality could therefore not be assessed.

5. Conclusion

In conclusion, our study indicates that environmental and/or dietary infant exposure to certain non-persisting endocrine disrupting chemicals may modify reproductive hormone concentrations during the important developmental period of minipuberty. In particular, exposure to the phthalates DnBP and DEHP as well as BPA seemed to be associated with altered reproductive hormone concentrations in infant boys. The

potential long-term consequences of an impact on hormone levels during minipuberty remain to be elucidated.

Declaration of competing interest

The authors have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2023.114166.

References

Adoamnei, E., Mendiola, J., Vela-Soria, F., Fernández, M.F., Olea, N., Jørgensen, N., Swan, S.H., Torres-Cantero, A.M., 2018. Urinary bisphenol A concentrations are associated with reproductive parameters in young men. Environ. Res. 161, 122–128. https://doi.org/10.1016/J.ENVRES.2017.11.002.

Aksglaede, L., Olesen, I.A., Carlsen, E., Petersen, J.H., Juul, A., Jørgensen, N., 2018. Serum concentration of anti-Müllerian hormone is not associated with semen quality. Andrology 6, 286–292. https://doi.org/10.1111/ANDR.12456.

Aksglaede, L., Sørensen, K., Boas, M., Mouritsen, A., Hagen, C.P., Jensen, R.B., Petersen, J.H., Linneberg, A., Andersson, A.M., Main, K.M., Skakkebæk, N.E., Juul, A., 2010. Changes in anti-Müllerian hormone (AMH) throughout the life span: a population-based study of 1027 healthy males from birth (cord blood) to the age of 69 years. J. Clin. Endocrinol. Metab. 95, 5357–5364. https://doi.org/10.1210/JC.2010-1207.

Albrethsen, J., Frederiksen, H., Andersson, A.M., Anand-Ivell, R., Nordkap, L., Bang, A. K., Jørgensen, N., Juul, A., 2018. Development and validation of a mass spectrometry-based assay for quantification of insulin-like factor 3 in human serum. Clin. Chem. Lab. Med. 56, 1913–1920. https://doi.org/10.1515/CCLM-2018-0171.

Becker, M., Hesse, V., 2020. Minipuberty: why does it happen? Horm. Res. Paediatr. 93, 76–84. https://doi.org/10.1159/000508329.

Berger, K.P., Kogut, K.R., Bradman, A., She, J., Gavin, Q., Zahedi, R., Parra, K.L., Harley, K.G., 2019. Personal care product use as a predictor of urinary concentrations of certain phthalates, parabens, and phenols in the HERMOSA study. J. Expo. Sci. Environ. Epidemiol. 29, 21. https://doi.org/10.1038/S41370-017-0003-Z.

Borch, J., Metzdorff, S.B., Vinggaard, A.M., Brokken, L., Dalgaard, M., 2006. Mechanisms underlying the anti-androgenic effects of diethylhexyl phthalate in fetal rat testis. Toxicology 223, 144–155. https://doi.org/10.1016/J.TOX.2006.03.015.

Bornehag, C.G., Carlstedt, F., Jönsson, B.A., Lindh, C.H., Jensen, T.K., Bodin, A., Jonsson, C., Janson, S., Swan, S.H., 2015. Prenatal phthalate exposures and anogenital distance in Swedish boys. Environ. Health Perspect. 123, 101–107. https://doi.org/10.1289/EHP.1408163.

Busch, A., Ljubicic, M., Upners, E., Fischer, M., Kolby, N., Eckert-Lind, C., Jespersen, K., Andersson, A., Frederiksen, H., Johannsen, T., Hegaard, H., Sharif, H., Hagen, C., Juul, A., 2021. Cohort profile: the COPENHAGEN Minipuberty Study-A longitudinal prospective cohort of healthy full-term infants and their parents. Paediatr. Perinat. Epidemiol. 35, 601–611. https://doi.org/10.1111/PPE.12777.

Busch, A., Ljubicic, M., Upners, E., Fischer, M., Raket, L., Frederiksen, H., Albrethsen, J., Johannsen, T., Hagen, C., Juul, A., 2022. Dynamic changes of reproductive hormones in male minipuberty: temporal dissociation of Leydig- and Sertoli-cell activity. J. Clin. Endocrinol. Metab. 187 (1), 135–142. https://doi.org/10.1210/clinem/dgac115.

Çiftçi, S., Yalçın, S.S., Samur, G., 2021. Bisphenol A exposure in exclusively breastfed infants and lactating women: an observational cross-sectional study. J. Clin. Res. Pediatr. Endocrinol. 13, 375–383. https://doi.org/10.4274/JCRPE.GALENOS.2020.2021.0305.

Covaci, A., Hond, E. Den, Geens, T., Govarts, E., Koppen, G., Frederiksen, H., Knudsen, L. E., Mørck, T.A., Gutleb, A.C., Guignard, C., Cocco, E., Horvat, M., Heath, E., Kosjek, T., Mazej, D., Tratnik, J.S., Castaño, A., Esteban, M., Cutanda, F., Ramos, J.J., Berglund, M., Larsson, K., Jönsson, B.A.G., Biot, P., Casteleyn, L., Joas, R., Joas, A., Bloemen, L., Sepai, O., Exley, K., Schoeters, G., Angerer, J., Kolossa-Gehring, M., Fiddicke, U., Aerts, D., Koch, H.M., 2015. Urinary BPA measurements in children and mothers from six European member states: overall results and determinants of exposure. Environ. Res. 141, 77–85. https://doi.org/10.1016/J.ENVRES.2014.08.008.

Dostalova, P., Zatecka, E., Ded, L., Elzeinova, F., Valaskova, E., Kubatova, A., Korenkova, V., Langerova, L., Komrskova, K., Peknicova, J., 2020. Gestational and pubertal exposure to low dose of di-(2-ethylhexyl) phthalate impairs sperm quality in adult mice. Reprod. Toxicol. 96, 175–184. https://doi.org/10.1016/J.REPROTOX.2020.06.014.

ECHA Candidate List [WWW Document], 2019. URL. https://echa.europa.eu/candidate-list-table. (Accessed 28 February 2022). accessed.

Edelsztein, N.Y., Grinspon, R.P., Schteingart, H.F., Rey, R.A., 2016. Anti-Müllerian hormone as a marker of steroid and gonadotropin action in the testis of children and adolescents with disorders of the gonadal axis. Int. J. Pediatr. Endocrinol. https://doi.org/10.1186/S13633-016-0038-2, 2016.

EFSA, 2021. Bisphenol A: EFSA draft opinion proposes lowering the tolerable daily intake | EFSA [WWW Document]. URL. https://www.efsa.europa.eu/en/news/bisphenol-efsa-draft-opinion-proposes-lowering-tolerable-daily-intake. (Accessed 26 August 2022). accessed.

Enangue Njembele, A.N., Tremblay, J.J., 2021. Mechanisms of MEHP inhibitory action and analysis of potential replacement plasticizers on Leydig cell steroidogenesis. Int. J. Mol. Sci. 22 https://doi.org/10.3390/IJMS222111456.

Ferguson, K.K., Peterson, K.E., Lee, J.M., Mercado-García, A., Blank-Goldenberg, C., Téllez-Rojo, M.M., Meeker, J.D., 2014. Prenatal and peripubertal phthalates and bisphenol A in relation to sex hormones and puberty in boys. Reprod. Toxicol. 47, 70–76. https://doi.org/10.1016/J.REPROTOX.2014.06.002.

Foster, P.M.D., Gray, E., Leffers, H., Skakkebæk, N.E., 2006. Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. Int. J. Androl. 29, 140–147. https://doi.org/10.1111/J.1365-2605.2005.00563.X.

Frederiksen, H., Jensen, T.K., Jørgensen, N., Kyhl, H.B., Husby, S., Skakkebæk, N.E., Main, K.M., Juul, A., Andersson, A.M., 2014. Human urinary excretion of non-persistent environmental chemicals: an overview of Danish data collected between 2006 and 2012. Reproduction 147, 555–565. https://doi.org/10.1530/REP-13-0522.

Frederiksen, H., Johannsen, T.H., Andersen, S.E., Albrethsen, J., Landersoe, S.K., Petersen, J.H., Andersen, A.N., Vestergaard, E.T., Schorring, M.E., Linneberg, A., Main, K.M., Andersson, A.M., Juul, A., 2020. Sex-specific estrogen levels and reference intervals from infancy to late adulthood determined by LC-MS/MS. J. Clin. Endocrinol. Metab. 105 https://doi.org/10.1210/CLINEM/DGZ196.

Frederiksen, H., Kranich, S.K., Jørgensen, N., Taboureau, O., Petersen, J.H., Andersson, A.M., 2013. Temporal variability in urinary phthalate metabolite excretion based on spot, morning, and 24-h urine samples: considerations for epidemiological studies. Environ. Sci. Technol. 47, 958–967. https://doi.org/10.1021/es303640b.

Frederiksen, H., Ljubicic, M.L., Upners, E.N., Fischer, M.B., Busch, A.S., Hagen, C.P., Juul, A., Andersson, A.-M., 2022a. Benzophenones, bisphenols and other polychlorinated/phenolic substances in Danish infants and their parents - including longitudinal assessments before and after introduction to mixed diet. Environ. Int. 169, 107532 https://doi.org/10.1016/J.ENVINT.2022.107532.

Frederiksen, H., Nielsen, O., Skakkebaek, N., Juul, A., Andersson, A., 2017. UV filters analyzed by isotope diluted TurboFlow-LC-MS/MS in urine from Danish children and adolescents. Int. J. Hyg Environ. Health 220, 244–253. https://doi.org/10.1016/J.IJHEH.2016.08.005.

Frederiksen, H., Upners, E.N., Ljubicic, M.L., Fischer, M.B., Busch, A.S., Hagen, C.P., Juul, A., Andersson, A.-M., 2022b. Exposure to 15 phthalates and two substitutes (DEHTP and DINCH) assessed in trios of infants and their parents as well as longitudinally in infants exclusively breastfed and after the introduction of a mixed diet. Environ. Int. 161, 107107 https://doi.org/10.1016/j.envint.2022.107107.

Frederiksen Nielsen, O., Koch, H., Skakkebaek, N., Juul, A., Jørgensen, N., Andersson, A., 2020. Changes in urinary excretion of phthalates, phthalate substitutes, bisphenols and other polychlorinated and phenolic substances in young Danish men; 2009-2017. Int. J. Hyg Environ. Health 223, 93–105. https://doi.org/10.1016/J.IJHEH.2019.10.002.

Howdeshell, K.L., Hotchkiss, A.K., Gray, L.E., 2017. Cumulative effects of antiandrogenic chemical mixtures and their relevance to human health risk assessment. Int. J. Hyg Environ. Health 220, 179–188. https://doi.org/10.1016/J.IJHEH.2016.11.007.

Istvan, M., Rahban, R., Dananche, B., Senn, A., Stettler, E., Multigner, L., Nef, S., Garlantézec, R., 2021. Maternal occupational exposure to endocrine-disrupting chemicals during pregnancy and semen parameters in adulthood: results of a nationwide cross-sectional study among Swiss conscripts. Hum. Reprod. 36, 1948–1958. https://doi.org/10.1093/HUMREP/DEAB034.

Jensen, T.K., Frederiksen, H., Kyhl, H.B., Lassen, T.H., Swan, S.H., Bornehag, C.G., Skakkebaek, N.E., Main, K.M., Lind, D.V., Husby, S., Andersson, A.M., 2016. Prenatal exposure to phthalates and anogenital distance in male infants from a low-exposed

Danish cohort (2010-2012). Environ. Health Perspect. 124, 1107–1113. https://doi.org/10.1289/EHP.1509870.
Josso, N., Rey, R.A., 2020. What does AMH tell us in pediatric disorders of sex development? Front. Endocrinol. 11 https://doi.org/10.3389/FENDO.2020.00619.
Kariyazono, Y., Taura, J., Hattori, Y., Ishii, Y., Narimatsu, S., Fujimura, M., Takeda, T., Yamada, H., 2015. Effect of in utero exposure to endocrine disruptors on fetal steroidogenesis governed by the pituitary-gonad axis: a study in rats using different ways of administration. J. Toxicol. Sci. 40, 909–916. https://doi.org/10.2131/JTS.40.909.
Kortenkamp, A., Martin, O., Ermler, S., Baig, A., Scholze, M., 2022a. Bisphenol A and declining semen quality: a systematic review to support the derivation of a reference dose for mixture risk assessments. Int. J. Hyg Environ. Health 241, 113942. https://doi.org/10.1016/J.IJHEH.2022.113942.
Kortenkamp, A., Martin, O., Ermler, S., Baig, A., Scholze, M., 2022b. Bisphenol A and declining semen quality: a systematic review to support the derivation of a reference dose for mixture risk assessments. Int. J. Hyg Environ. Health 241, 113942. https://doi.org/10.1016/J.IJHEH.2022.113942.
Kuiri-Hänninen, T., Sankilampi, U., Dunkel, L., 2014. Activation of the hypothalamic-pituitary-gonadal axis in infancy: minipuberty. Horm. Res. Paediatr. 82, 73–80. https://doi.org/10.1159/000362414.
Labrie, F., Luu-The, V., Bélanger, A., Lin, S.X., Simard, J., Pelletier, G., Labrie, C., 2005. Is dehydroepiandrosterone a hormone? J. Endocrinol. 187, 169–196. https://doi.org/10.1677/JOE.1.06264.
Lardone, M.C., Argandoña, F., Flórez, M., Parada-Bustamante, A., Ebensperger, M., Palma, C., Piottante, A., Castro, A., 2017. Overexpression of CYP19A1 aromatase in Leydig cells is associated with steroidogenic dysfunction in subjects with Sertoli cell-only syndrome. Andrology 5, 41–48. https://doi.org/10.1111/ANDR.12289.
Lasala, C., Carré-Eusèbe, D., Picard, J.Y., Rey, R., 2004. Subcellular and molecular mechanisms regulating anti-Müllerian hormone gene expression in mammalian and nonmammalian species. DNA Cell Biol. 23, 572–585. https://doi.org/10.1089/DNA.2004.23.572.
Lassen, T.H., Frederiksen, H., Jensen, T.K., Petersen, J.H., Joensen, U.N., Main, K.M., Skakkebaek, N.E., Juul, A., Jørgensen, N., Andersson, A.M., 2014. Urinary bisphenol A levels in young men: association with reproductive hormones and semen quality. Environ. Health Perspect. 122, 478–484. https://doi.org/10.1289/EHP.1307309.
Lassen, T.H., Frederiksen, H., Jensen, T.K., Petersen, J.H., Main, K.M., Skakkebæk, N.E., Jørgensen, N., Kranich, S.K., Andersson, A.M., 2013. Temporal variability in urinary excretion of bisphenol A and seven other phenols in spot, morning, and 24-h urine samples. Environ. Res. 126, 164–170. https://doi.org/10.1016/j.envres.2013.07.001.
Ljubicic, M.L., Busch, A.S., Upners, E.N., Fischer, M.B., Petersen, J.H., Raket, L.L., Frederiksen, H., Johannsen, T.H., Juul, A., Hagen, C.P., 2022. A biphasic pattern of reproductive hormones in healthy female infants: the COPENHAGEN minipuberty study. J. Clin. Endocrinol. Metab. 107, 2598–2605. https://doi.org/10.1210/CLINEM/DGAC363.
Lucaccioni, L., Trevisani, V., Boncompagni, A., Marrozzini, L., Berardi, A., Iughetti, L., 2021. Minipuberty: looking back to understand moving forward. Front. Pediatr. 8 https://doi.org/10.3389/FPED.2020.612235.
Ma, Y., Liu, H., Wu, J., Yuan, L., Wang, Y., Du, X., Wang, R., Marwa, P.W., Petlulu, P., Chen, X., Zhang, H., 2019. The adverse health effects of bisphenol A and related toxicity mechanisms. Environ. Res. 176 https://doi.org/10.1016/J.ENVRES.2019.108575.
Mackay, D., Hughes, D.M., Romano, M.L., Bonnell, M., 2014. The role of persistence in chemical evaluations. Integrated Environ. Assess. Manag. 10, 588–594. https://doi.org/10.1002/IEAM.1545.
Main, K.M., Mortensen, G.K., Kaleva, M.M., Boisen, K.A., Damgaard, I.N., Chellakooty, M., Schmidt, I.M., Suomi, A.M., Virtanen, H.E., Petersen, J.H., Andersson, A.M., Toppari, J., Skakkebæk, N.E., 2006. Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. Environ. Health Perspect. 114, 270–276. https://doi.org/10.1289/EHP.8075.
Manna, P.R., Stetson, C.L., Slominski, A.T., Pruitt, K., 2016. Role of the steroidogenic acute regulatory protein in health and disease. Endocrine 51, 7–21. https://doi.org/10.1007/S12020-015-0715-6.
Minatoya, M., Kishi, R., 2021. A review of recent studies on bisphenol A and phthalate exposures and child neurodevelopment A review of recent studies on bisphenol A and phthalate exposures. Environ. Res. Public Heal 18. https://doi.org/10.3390/ijerph18073585.
Muerköster, A.P., Frederiksen, H., Juul, A., Andersson, A.M., Jensen, R.C., Glintborg, D., Kyhl, H.B., Andersen, M.S., Timmermann, C.A.G., Jensen, T.K., 2020. Maternal phthalate exposure associated with decreased testosterone/LH ratio in male offspring during mini-puberty. Odense Child Cohort. Environ. Int. 144 https://doi.org/10.1016/J.ENVINT.2020.106025.
Murayama, C., Miyazaki, H., Miyamoto, A., Shimizu, T., 2012. Luteinizing hormone (LH) regulates production of androstenedione and progesterone via control of histone acetylation of StAR and CYP17 promoters in ovarian theca cells. Mol. Cell. Endocrinol. 350, 1–9. https://doi.org/10.1016/J.MCE.2011.11.014.
Namat, A., Xia, W., Xiong, C., Xu, S., Wu, C., Wang, A., Li, Y., Wu, Y., Li, J., 2021. Association of BPA exposure during pregnancy with risk of preterm birth and changes in gestational age: a meta-analysis and systematic review. Ecotoxicol. Environ. Saf. 220 https://doi.org/10.1016/J.ECOENV.2021.112400.
Philippat, C., Rolland, M., Lyon-Caen, S., Pin, I., Sakhi, A.K., Sabaredzovic, A., Thomsen, C., Slama, R., 2021. Pre- and early post-natal exposure to phthalates and DINCH in a new type of mother-child cohort relying on within-subject pools of repeated urine samples. Environ. Pollut. 287 https://doi.org/10.1016/J.ENVPOL.2021.117650.
Pollack, A.Z., Perkins, N.J., Mumford, S.L., Ye, A., Schisterman, E.F., 2013. Correlated biomarker measurement error: an important threat to inference in environmental epidemiology. Am. J. Epidemiol. 177, 84–92. https://doi.org/10.1093/AJE/KWS209.
Prough, R.A., Clark, B.J., Klinge, C.M., 2016. Novel mechanisms for DHEA action. J. Mol. Endocrinol. 56, R139–R155. https://doi.org/10.1530/JME-16-0013.
Radke, E.G., Braun, J.M., Meeker, J.D., Cooper, G.S., 2018. Phthalate exposure and male reproductive outcomes: a systematic review of the human epidemiological evidence. Environ. Int. 121, 764–793. https://doi.org/10.1016/J.ENVINT.2018.07.029.
Ren, X., Zhang, T., Chen, X., Wei, X., Tian, Y., Li, G., Zhang, X., Zhang, W., You, Z., Wang, S., Qin, C., 2020. Early-life exposure to bisphenol A and reproductive-related outcomes in rodent models: a systematic review and meta-analysis. Aging (Albany. NY) 12, 18099–18126. https://doi.org/10.18632/AGING.103620.
Runkel, A.A., Mazej, D., Snoj Tratnik, J., Tkalec, Ž., Kosjek, T., Horvat, M., 2022. Exposure of men and lactating women to environmental phenols, phthalates, and DINCH. Chemosphere 286. https://doi.org/10.1016/J.CHEMOSPHERE.2021.131858.
Sánchez-Garrido, M.A., García-Galiano, D., Tena-Sempere, M., 2022. Early programming of reproductive health and fertility: novel neuroendocrine mechanisms and implications in reproductive medicine. Hum. Reprod. Update. https://doi.org/10.1093/HUMUPD/DMAC005.
Scheutz Henriksen, L., Petersen, J.H., Skakkebæk, N.E., Jørgensen, N., Virtanen, H.E., Priskorn, L., Juul, A., Toppari, J., Main, K.M., 2022. Serum testosterone levels in three-month-old boys predict their semen quality as young adults. J. Clin. Endocrinol. Metab. https://doi.org/10.1210/CLINEM/DGAC173.
Sharpe, R.M., 2020. Androgens and the masculinization programming window: human-rodent differences. Biochem. Soc. Trans. 48, 1725–1735. https://doi.org/10.1042/BST20200200.
Søeborg, T., Frederiksen, H., Johannsen, T.H., Andersson, A.M., Juul, A., 2017. Isotope-dilution TurboFlow-LC-MS/MS method for simultaneous quantification of ten steroid metabolites in serum. Clin. Chim. Acta 468, 180–186. https://doi.org/10.1016/J.CCA.2017.03.002.
Swan, S.H., Sathyanarayana, S., Barrett, E.S., Janssen, S., Liu, F., Nguyen, R.H.N., Redmon, J.B., 2015. First trimester phthalate exposure and anogenital distance in newborns. Hum. Reprod. 30, 963–972. https://doi.org/10.1093/HUMREP/DEU363.
Thompson, C.J., Ross, S.M., Gaido, K.W., 2004. Di(n-butyl) phthalate impairs cholesterol transport and steroidogenesis in the fetal rat testis through a rapid and reversible mechanism. Endocrinology 145, 1227–1237. https://doi.org/10.1210/EN.2003-1475.
Traore, K., More, P., Adla, A., Dogbey, G., Papadopoulos, V., Zirkin, B., 2021. MEHP induces alteration of mitochondrial function and inhibition of steroid biosynthesis in MA-10 mouse tumor Leydig cells. Toxicology 463. https://doi.org/10.1016/J.TOX.2021.152985.
Turcu, A.F., Rege, J., Auchus, R.J., Rainey, W.E., 2020. 11-Oxygenated androgens in health and disease. Nat. Rev. Endocrinol. 16, 284–296. https://doi.org/10.1038/S41574-020-0336-X.
Valeri, C., Lovaisa, M.M., Racine, C., Edelsztein, N.Y., Riggio, M., Giulianelli, S., Venara, M., Bedecarrás, P., Ballerini, M.G., di Clemente, N., Lamb, C.A., Schteingart, H.F., Rey, R.A., 2020. Molecular mechanisms underlying AMH elevation in hyperoestrogenic states in males. Sci. Rep. 10 https://doi.org/10.1038/S41598-020-71675-7.
Valladares, L.E., Payne, A.H., 1979. Acute stimulation of aromatization in Leydig cells by human chorionic gonadotropin in vitro. Proc. Natl. Acad. Sci. U.S.A. 76, 4460. https://doi.org/10.1073/PNAS.76.9.4460.
Voinescu, G.C., Shoemaker, M., Moore, H., Khanna, R., Nolph, K.D., 2002. The relationship between urine osmolality and specific gravity. Am. J. Med. Sci. 323, 39–42. https://doi.org/10.1097/00000441-200201000-00007.
Wang, H., Ding, Z., Shi, Q.M., Ge, X., Wang, H.X., Li, M.X., Chen, G., Wang, Q., Ju, Q., Zhang, J.P., Zhang, M.R., Xu, L.C., 2017. Anti-androgenic mechanisms of Bisphenol A involve androgen receptor signaling pathway. Toxicology 387, 10–16. https://doi.org/10.1016/J.TOX.2017.06.007.
Wang, Y.B., Song, L., Cui, L.B., Hong, X., Zhang, Z.D., Wang, X.R., 2007. Monobutyl phthalate inhibits steroidogenesis by downregulating steroidogenic acute regulatory protein expression in mouse Leydig tumor cells (MLTC-1). J. Toxicol. Environ. Health 70, 947–955. https://doi.org/10.1080/15287390701290717.
Xu, H.Y., Zhang, H.X., Xiao, Z., Qiao, J., Li, R., 2019. Regulation of anti-Müllerian hormone (AMH) in males and the associations of serum AMH with the disorders of male fertility. Asian J. Androl. 21, 109–114. https://doi.org/10.4103/AJA.AJA_83_18.
Yilmaz, B., Terekeci, H., Sandal, S., Kelestimur, F., 2019. Endocrine disrupting chemicals: exposure, effects on human health, mechanism of action, models for testing and strategies for prevention, 2019 Rev. Endocr. Metab. Disord. 127–147. https://doi.org/10.1007/S11154-019-09521-Z, 211 21.