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Personal care product use and lifestyle affect phthalate and DINCH metabolite levels in teenagers and young adults

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ABSTRACT

Humans are widely exposed to phthalates and their novel substitutes, and considering the negative health effects associated with some phthalates, it is crucial to understand population levels and exposure determinants. This study is focused on 300 urine samples from teenagers (aged 12–17) and 300 from young adults (aged 18–37) living in Czechia collected in 2019 and 2020 to assess 17 plasticizer metabolites as biomarkers of exposure. We identified widespread phthalate exposure in the study population. The diethyl phthalate metabolite monoethyl phthalate (MEP) and three di (2-ethylhexyl) phthalate metabolites were detected in the urine of >99% of study participants. The highest median concentrations were found for metabolites of low-molecular-weight (LMW) phthalates: mono-*n*-butyl phthalate (MnBP), monoisobutyl phthalate (MiBP) and MEP (60.7; 52.6 and 17.6 μg/L in young adults). 1,2-cyclohexanedicarboxylic acid diisononyl ester (DINCH) metabolites were present in 68.2% of the samples with a median of 1.24 μg/L for both cohorts. Concentrations of MnBP and MiBP were similar to other European populations, but 5–6 times higher than in populations in North America. We also observed large variability in phthalate exposures within the study population, with 2–3 orders of magnitude differences in urinary metabolites between high and low exposed individuals. The concentrations varied with season, gender, age, and lifestyle factors. A relationship was found between high levels of MEP and high overall use of personal care products (PCPs). Cluster analysis suggested that phthalate exposures depend on season and multiple lifestyle factors, like time spent indoors and use of PCPs, which combine to lead to the observed widespread presence of phthalate metabolites in both study populations. Participants who spent more time indoors, particularly noticeably during colder months, had higher levels of high-molecular weight phthalate metabolites, whereas participants with higher PCP use, particularly women, tended to have higher concentration of LMW phthalate metabolites.

Abbreviations: 5cx-MEPP, mono(2-ethyl-5-carboxy-pentyl) phthalate; 5OH-MEHP, mono(2-ethyl-5-hydroxy-hexyl) phthalate; 5oxo-MEHP, mono(2-ethyl-5-oxo-hexyl) phthalate; BzBP, benzyl butyl phthalate; BMI, body mass index; CELSPAC, Central European Longitudinal Studies of Parents and Children; cx-MINCH, cyclohexane-1,2-dicarboxylic acid-mono(carboxy-isooctyl) ester; cx-MiNP, 7-carboxy-(monomethyl-heptyl) phthalate; DEHP, di(2-ethylhexyl) phthalate; DEP, diethyl phthalate; DCHP, dicyclohexyl phthalate; DiBP, diisobutyl phthalate; DiDP, diisodecyl phthalate; DINCH, 1,2-cyclohexanedicarboxylic acid diisononyl ester; DiNP, diisononyl phthalate; DMP, Dimethyl phthalate; DnBP, di-*n*-butyl phthalate; DnOP, di-*n*-octyl phthalate; DPHP, bis(2-propylheptyl) phthalate; ELSPAC, European Longitudinal Study of Pregnancy and Childhood; EU, European Union; HBM, human biomonitoring; HMW, high-molecular weight; IQ, intelligence quotient; LMW, low-molecular weight; MBzP, monobenzyl phthalate; MEHP, mono(2-ethylhexyl) phthalate; MEP, monoethyl phthalate; MCHP, monocyclohexyl phthalate; MiBP, monoisobutyl phthalate; MiNP, monoisononyl phthalate; MMP, monomethyl phthalate; MnBP, mono-*n*-butyl phthalate; MnOP, mono-*n*-octyl phthalate; OH-MiDP, mono-hydroxy-isodecyl phthalate; OH-MINCH, cyclohexane-1,2-dicarboxylic acid-mono(hydroxyl-isononyl) ester; OH-MiNP, 7-hydroxy-(monomethyl-octyl) phthalate; oxo-MiDP, mono-oxo-isodecyl phthalate; oxo-MiNP, 7-oxo-(monomethyl-octyl) phthalate; PCPs, personal care products; PVC, polyvinylchloride.

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1. Introduction

Phthalate esters, commonly referred to as phthalates, are heavily used as plasticizers and solvents because of their durability and stability. Phthalates are high production volume chemicals (Heudorf et al., 2007) with worldwide production of around 5.5 million tonnes per year (Fréry et al., 2020). Phthalates with low molecular weight (LMW) and shorter chains (e.g. DMP, DEP, DBP and DiBP) are often used as solvents in personal care products (PCPs), cosmetics, and pharmaceuticals, and as plasticizers in non-polyvinyl chloride (PVC) products, including textiles, paints, adhesives, and food packaging. Phthalates with high molecular weight (HMW) and longer chains (e.g. DEHP, DiNP and DPHP) are mainly used as plasticizers in PVC products, including in medical devices and children's toys (Fréry et al., 2020; Koch and Angerer, 2012; Silano et al., 2019). Phthalates are not covalently bonded to plastic, therefore they can be emitted from consumer products and become widely distributed in humans and the environment (Husøy et al., 2019).

In humans, several phthalates are considered endocrine disruptors (WHO, 2013) associated with potential negative health effects. The effects on developmental and reproductive disorders, including neurobehavioral disorders and low IQ, respiratory problems or asthma and other allergic disorders have been reviewed elsewhere (Hlisníková et al., 2020; Katsikantami et al., 2016; Wang et al., 2019). Due to these adverse health effects, some phthalates have been legislatively banned in the EU (EC, 2009; REACH, 2006) and alternative non-aromatic, non-ortho-phthalate plasticizers are seeing increasing use (Lemke et al., 2021; Frederiksen et al., 2020; Lessmann et al., 2019). One of these plasticizers is DINCH (1,2-cyclohexanedicarboxylic acid diisononyl ester), which is used as a substitute for HMW phthalates, mainly DEHP and DiNP, particularly in sensitive products such as toys, food contact material and medical devices (Koch et al., 2013; Correia-Sá et al., 2017; Kasper-Sonnenberg et al., 2019). DINCH production is high (>10,000 t/year production and import to EU, 300,000 t/y global production), and DINCH is among the alternative plasticizers seeing substantial increases in use and exposure (Bui et al., 2016; Kasper-Sonnenberg et al., 2019). DINCH intake thresholds and hazard-based limit values suggest a lower hazard than for phthalates, however, there is particular concern about DINCH exposure in children, given its use as a replacement for HMW phthalates in toys, and evidence of exposure close to the tolerable daily intake (Bui et al., 2016).

Inhalation and dermal uptake is a relevant uptake route for the more volatile LMW phthalates, like DMP, DEP and DBP (Giovanoulis et al., 2018; Wormuth et al., 2006; Janjua et al., 2008; Lorber et al., 2017). Oral routes play the most important roles in total exposure to the HMW phthalates like DEHP and DiNP (Koch et al., 2013; Martínez et al., 2018; Correia-Sá et al., 2018). After exposure, phthalates in humans are rapidly metabolized by phase I and phase II reactions and excreted in both conjugated and free forms via urine (Silva et al., 2003), and partially also in feces (Domínguez-Romero and Scheringer, 2019). Thus, human biomonitoring typically focuses on phthalate metabolites in urine (Koch and Calafat, 2009; Calafat et al., 2013). More polar and LMW phthalates are hydrolysed to monoester forms and are eliminated mostly in free form and glucuronidated conjugates. In contrast, longer chain HMW phthalates (and DINCH) are further metabolized to secondary, alkyl chain oxidised metabolites (Fréry et al., 2020; Wittassek et al., 2011; Schütze et al., 2017).

Biomonitoring suggests that nearly all the European population is exposed to phthalates (Den Hond et al., 2015; Koppen et al., 2019). A recent study on phthalate metabolites in 140 Norwegian adults found 10 metabolites in 100% of the samples and all remaining phthalate and DINCH metabolites in 88–97% of the samples (Husøy et al., 2019). In a Slovenian study of 387 people, detection rates were 97–100% for all phthalate metabolites (Runkel et al., 2020). Several recent studies also point out the rather rapid changes in exposures to phthalates and their substitutes due to market changes and regulatory measures (Frederiksen et al., 2020; Apel et al., 2020; Lemke et al., 2021; Gyllenhammar et al., 2017). Human biomonitoring (HBM) is a useful and necessary tool to assess total exposure to phthalates in a timely and rapid manner, regardless of the route of exposure. In combination with other accompanying data (e.g. questionnaire data) HBM provides valuable information to help us better understand important exposure sources, as well as the health effects related to chemical exposure. HBM4EU is a joint EU project co-funded under Horizon 2020 to coordinate and advance human biomonitoring, involving 30 countries, the European Environment Agency and the European Commission. HBM under HBM4EU provides better evidence and understanding of the exposure of European citizens to chemicals, and the possible impact of chemical exposure on human health. In this study, we focus on plasticizer metabolites in urine samples from teenagers and young adults from the Czech Republic, collected as a part of the HBM4EU project.

2. Materials and methods

2.1. Sample information

Three hundred urine samples were obtained from 12 to 17-year-old teenagers residing in the South Moravian region of Czechia, identified as the "CELSPAC: Teenagers cohort" (TAC) (Table 1). The CELSPAC: Teenagers study was approved by the Research Ethics Committee of Masaryk University, Czech Republic (Ref. No: EKV-2019-046, dated May 27, 2019). Most urine was collected in 2019, between October and December; one urine sample was collected in January 2020. An additional 300 urine samples were obtained from participants from 18 to 37 years of age, also from South Moravia, Czechia, identified as the "CELSPAC: Young Adults cohort" (YAC) (Table 1). CELSPAC: Young Adults represents a follow-up study of longitudinal ELSPAC study in the Brno region of the Czech Republic (Piler et al., 2017) and follows ELSPAC children born in 1991 and 1992, their siblings, and spouses (the study is ongoing). The CELSPAC: Young Adults study was approved by the ELSPAC Ethics Committee (Ref. No: ELSPAC/EK/2/2019, dated March 13, 2019). YAC urine samples were collected in 2019, between March and December. All urine samples from the TAC and YAC cohorts were spot samples.

All study participants completed a questionnaire at the time of urine sample collection gathering information including age, gender, number of siblings, education and household income, time spent indoors and various parameters of the home environment, dietary information, smoking (active and passive), PCP usage and chronic illnesses. Details about the cohorts (age distribution, education and income, lifestyle variables) are given in the Supplementary Material, Figures S1-S5, Table S1.

Urine was analysed for creatinine concentration as well as for specific gravity (SG) and a wide set of chemical biomarkers, including phthalates and DINCH (presented here), as well as bisphenols, hydroxylated polycyclic aromatic hydrocarbons and metals (in preparation).

2.2. Chemicals and reagents

High purity (>97%) standards of 15 phthalate metabolites (MEP, MiBP, MBzP, MnBP, MCHP, MEHP (AccuStandard, USA), 5OH-MEHP, 5oxo-MEHP, 5cx-MEPP, (Toronto Research Chemicals, Canada), MnOP, OH-MiNP, cx-MiNP, oxo-MiNP, OH-MiDP and oxo-MiDP (Cambridge Isotope Laboratories, USA)), along with a creatinine standard solution, were purchased. OH-MINCH, cx-MINCH, OH-MINCH-d_4 and cx-MINCH-d_2 standards were provided by the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance - Institute of the Ruhr-University Bochum (IPA, Germany). High purity (>98%) standards of 15 isotopically-labelled phthalate metabolites (MEP-$^{13}C_4$, MiBP-$^{13}C_4$, MBzP-$^{13}C_4$, MnBP–$^{13}C_4$, MCHP-$^{13}C_4$, 5OH-MEHP-$^{13}C_4$, 5oxo-MEHP-$^{13}C_4$, 5cx-MEPP-$^{13}C_2$, OH-MiNP-$^{13}C_2$, cx-MiNP-$^{13}C_4$, oxo-MiNP-$^{13}C_2$, OH-MiDP-$^{13}C_2$, oxo-MiDP-$^{13}C_2$, (Cambridge Isotope Laboratories, USA), MEHP-d_4 and MnOP-d_4 (Chiron, Norway))

were purchased.

The following chemicals were used: a solution of β-glucuronidase (*E. coli*, Type IX-A 1–5MU/g), acetic acid, ammonium fluoride, ammonium acetate and Milli-Q water (Sigma-Aldrich, USA). The following solvents were purchased: acetonitrile and methanol for LC-MS (Biosolve Chimie, France), dimethylsulfoxide (DMSO, Sigma-Aldrich, USA).

2.3. Sample preparation procedure

Frozen aliquots were allowed to reach laboratory temperature, homogenized with a Wizard Advanced IR vortex mixer (Velp Scientifica, USA) and 500 μL of each sample were pipetted into a 96-well sample collection plate with 2 mL square wells (Waters, Prague). Then, 500 μL of a mixture containing β-glucuronidase solution (1000 units/mL) and isotopically-labelled standards (c = 10 ng/mL) in 0.1 M acetate buffer were added to the samples. The 96-well plate was covered with foil and samples were incubated using a sample concentrator (Miu Lab, China) for 120 min at 55 °C. After incubation, 50 μL of 1% acetic acid was added. Samples were then precleaned using 96-well plate SPE (Oasis HLB; 3 mL, 60 mg; Waters, Ireland), which was previously conditioned with 1 mL of methanol and activated with 0.1% acetic acid. Samples were passed through the SPE plate, then the wells were washed with 0.1% acetic acid and dried using a Laboport vacuum pump (KNF, Germany) for at least 30 s. Finally, the samples were eluted with 1.5 mL of acetone and collected into a new 96-well plate containing 10 μL of DMSO. After the clean-up step, samples were concentrated to 10 μL. Finally, 500 μL of 50% methanol were added, samples were covered with a foil, homogenized and kept in the fridge (4 °C) until analysis.

2.4. Liquid chromatography-mass spectrometry (LC-MS) conditions

The analytical method for phthalate metabolite determination was developed on the Agilent 1290 Infinity II HPLC system (Agilent Technologies, Germany) for separation. An Acquity UPLC BEH C18 (100 × 2.1 mm, 1.7 μm, Waters, Czechia) was used as an analytical column. The mobile phases were A: 0.1 mM ammonium fluoride in Milli-Q water and B: 0.1 mM ammonium fluoride in methanol. The gradient elution was set up as follows: mobile phase A (10%) for 7 min, followed by an immediate increase to 50% for the rest of the analysis. The injection volume was 5 μL and the flow rate was set to 250 μL/min.

Agilent QQQ 6495 (Agilent Technologies, Germany) was used for analyte detection, with an electrospray ionisation (ESI) source, in negative mode. Optimal parameters for phthalate metabolites are shown in Table S2. The isotopic dilution method was used for data quantification. A corresponding isotopically-labelled standard was used for each compound as the internal standard. MassHunter software was used to process the data from instrumental analysis.

2.5. Specific gravity

For the SG measurement, a "Pocket" Urine Specific Gravity Refractometer PAL-10 S (Atago, Japan) was used. First, 300 μl of Milli-Q water was pipetted to the refractometer to reach a value of 1.000. Then, the refractometer was wiped with a paper towel. The urine sample was vortexed and 300 μL were pipetted onto the refractometer. When the temperature stabilised, the value was deducted from the refractometer. The procedure was repeated for all the samples.

2.6. Quality assurance/quality control (QA/QC)

The methods used were developed and validated under the HBM4EU framework through the successful completion of four rounds of proficiency testing (Esteban López et al., 2021). We report 10 phthalate and 2 DINCH biomarkers that are quality assured under HBM4EU (both external and internal quality assurance) and 4 biomarkers under additional internal quality assurance.

External quality assurance: For external quality assurance we participated in all four rounds of the HBM4EU Proficiency testing (Esteban López et al., 2021) and received certificates for the following biomarkers: MEP, MBzP, MiBP, MnBP, MEHP, 5OH-MEHP, 5oxo-MEHP, 5cx-MEPP, OH-MiNP, cx-MiNP (Elbers and Mol, 2019a), OH-MINCH and cx-MINCH (Elbers and Mol, 2019b).

Internal quality assurance: Procedural blanks were prepared with Milli-Q water, approximately one blank for every ten urine samples. The samples were blank subtracted based on the average mass of metabolite found in the blanks (Table S3). LODs were defined as three times the standard deviation of the blank samples (Table S3). A calibration curve was prepared in the range of 0.01–100 ng/mL, containing 10 concentration points and showing linearity. QC samples were analysed approximately every ten samples. The average recoveries of QC samples ranged from 83 to 110% (except for MnBP with 124.1%) with RSD ranging from 5.1 to 16.5 (except for MEP and MnBP with 38.1 and 40.5% respectively).

2.7. Data adjustment

SG adjustment was applied to urine samples, since creatinine correction has a higher dependence on age (Carrieri et al., 2000), time of urine sampling, sex, activity, diet (Miller et al., 2004), muscularity, BMI, disease status (Wang et al., 2015) and seasonal variations (Pearson et al., 2009). Moreover, degradation studies suggest that after only 10 days, approximately 20% of creatinine is degraded via freezing (storage at −20 °C), while SG is not (Schneider et al., 2002). Considering all this, SG correction was deemed to be the more reliable correction for the TAC and YAC samples.

2.8. Data analysis

For the correlation analyses, only values > LOD were used. For the rest of the statistical analyses, values below LOD were substituted with LOD/$\sqrt{2}$. Nonparametric Spearman's correlation was used to determine the relationship among phthalate metabolites. To investigate seasonal variations, YAC samples were divided into two groups according to the sampling season. The cold season covered seven months from October to April, and the warm season covered five months from May to September. Basic statistical characteristics (detection frequency, median, minimal

Table 1
Profile of studied cohorts. YAC represents Young Adult cohort, TAC represents Teenage cohort, N_{mis} represents number of samples where we lack corresponding data from questionnaires.

		Young Adults (YAC) (n = 300)				Teenagers (TAC) (n = 300)		
		Median	10th90th	Minmax	N_{mis}	Median	10th90th	Minmax
Age (years)		27	26–28	18–37		13	12–15	12–17
Weight (kg)		72.6	56.5–92.5	47.3–117.3	2	Not collected		
Height (cm)		174.1	163.2–188.3	155.1–202.3	2	Not collected		
BMI (kg m^{-2})		23.3	19.9–28.7	17.3–39.2	2	Not collected		
Gender	female	155; 52%				124; 42%		
	male	145; 48%				176; 58%		

and maximal value with 5th and 95th percentiles) are shown in Table S4 for YAC and in Table S5 for TAC. Table S6 refers to number of samples and medians for categories of the exposure determinants used for non-parametric testing.

Linear regression was used to explore the relationship between phthalate metabolites and multiple exposure factors (gender, age, season of sample collection, time indoors, and sum of all PCP products used). Only metabolites with more than 75% of samples above the LOD for each cohort were used for regression analysis (MEP, MiBP, MBzP, MnBP, 5OH-MEHP, 5cx-MEPP, OH-MiNP). The regression analysis used gender and season as categorical predictors; age, time spent indoors, and sum of PCPs were used as continuous predictors. Use of individual PCPs could not be incorporated as predictors in the regression models, due to the small sample sizes in some groups stratified by e.g., cohort/age, gender and season. Metabolite concentrations were log-transformed, and outliers were excluded prior to analysis. The normal distribution of the residuals was checked by using histograms and the Kolmogorov-Smirnov test. Regressions for each exposure factor and metabolite were adjusted for other factors to explain the main effects of each factor. Another regression model for each metabolite was computed with all factors and their interactions to determine the maximum explained variability (coefficient of determination, R^2) in phthalate concentrations by the given factors. Factor interactions were added to the model because we assume that one factor can influence the effect of another factor (e.g. indoors and season) on the dependent variable (phthalate metabolite concentrations).

To supplement the key factors identified by the regression analysis, individual exposure factors were also examined, both individually, and stratified by age, season, and gender. The non-parametric Mann-Whitney *U* test was used to test for differences in concentrations of phthalate metabolites with questionnaire parameters with two categories such as gender, season (cold vs. warm), smoking (YES/NO), redecoration and renovation made at home in the last two years (YES/NO), drinking beverages from plastic bottles (YES/NO) and for the use of some PCPs (Table S7). Tests were also performed for each combination of factors.

Due to inhomogeneity of the variances and non-equal sample sizes in some categories of PCPs, nonparametric Kruskal-Wallis ANOVA was used to test for differences in concentrations of phthalate metabolites between multiple categories of frequencies of PCP use. To remove the influence of other factors, participants were first stratified by age (TAC vs YAC), gender, and season before testing of PCPs. For some frequencies of PCP use stratified by gender, age and season, the number of samples for testing was insufficient, due to the nature of the PCP use (e.g. eye make-up or nail polish is used mainly by women). The PCP use categories "never" and "sometimes" for shampoo and deodorant were combined because very few participants did not use these products at all. Tests were performed when the number of samples in each category was greater than 20 (Table S7) and only for metabolites with more than 75% samples above the LOD for each cohort (MEP, MiBP, MBzP, MnBP, 5OH-MEHP, 5cx-MEPP, OH-MiNP).

Finally, two cluster analyses were completed as an explanatory technique for cumulative phthalate exposure and cumulative PCP use:

i) First, Ward's hierarchical clustering method with the squared Euclidean distance was used as an explanatory technique to create clusters of participants with similar phthalate exposure. Metabolite concentrations were log-transformed and standardized (*z*-score) prior to cluster analysis. The cluster analysis provides insight into the total cumulative exposure of all metabolites. Specifically, participants with similar concentrations of phthalate metabolites (e.g. participants with high/low levels of all metabolites) were clustered. Then it was determined how the exposure factors (age, gender, season, time spent indoors, PCPs) were distributed among these clusters. Differences in cumulative concentrations of LMW, HMW phthalate metabolites and DINCH between clusters were tested for by the Kruskal-Wallis ANOVA.

ii) In the second case, cluster analysis was used to create clusters of participants with similar use of personal care products. Frequencies of use of individual PCPs were expressed as number of days per week and clustered using Ward's hierarchical clustering method with the squared Euclidean distance. In contrast to the Kruskal-Wallis ANOVA, which tests differences for each PCP separately, the cluster analysis shows the common use of all PCPs and complements the results from the regression analysis where individual PCPs and their interactions could not be included. This analysis was conducted for the YAC and TAC cohorts separately. The distribution of gender and season was determined for each cluster and differences in the concentrations of each metabolite in the clusters were tested using Kruskal-Wallis ANOVA.

3. Results and discussion

Twelve of the 17 targeted phthalate metabolites were broadly detected in TAC and YAC. The most frequently detected compounds were MEP, 5oxo-MEHP and 5cx-MEPP, detected in >99% of the YAC samples and in all TAC samples, and OH-MINP and 5OH-MEHP were often detected (>98%) in both cohorts. The detection frequencies, medians, geometric means, and ranges are shown in Table 2. Other statistical characteristics (minimum, maximum, 5th and 95th percentiles) for both cohorts are shown in Tables S4 and S5.

In the YAC, the highest median concentration was found for MnBP, followed by MiBP and MEP (60.6; 52.6 and 17.6 μg/L, respectively). All DEHP metabolites, OH-MiNP, oxo-MiNP and OH-MINCH had medians between 1.34 and 7.92 μg/L, while the other metabolites were detected with median concentrations under 1.01 μg/L (Table 2). In the TAC, the highest median concentrations were also found for MnBP, followed by MEP and MiBP (45.5; 38.1 and 28.8 μg/L, respectively). MBzP, all DEHP metabolites, the secondary DiNP metabolites and OH-MINCH were detected with the medians in the range of 1.71–13.9 μg/L. Despite the use of DINCH as a replacement for DEHP in sensitive applications (Bui et al., 2016), the DINCH metabolites are detected at lower levels and with lower detection frequency than DEHP metabolites. The other metabolites were detected in median concentrations below 1 μg/L (Table 2). MEP, MiBP, MnBP, MBzP, 5OH-MEHP, 5cx-MEPP, OH-MiNP, cx-MiNP, OH-MiDP and OH-MINCH (marked with [a] in Table 2) were selected as priority metabolites for further statistical analysis based on their appropriateness as biomarkers of the parent compounds and detection frequency. Correlations between individual phthalate metabolites were calculated to examine associations between specific phthalate metabolites and common sources. The strongest correlations were found between metabolites of the same parent compound (e.g., DEHP metabolites 5OH-MEHP, 5oxo-MEHP and 5cx-MEPP; and DINCH metabolites OH-MINCH, cx-MINCH) (Figure S6).

3.1. Determinants of phthalate exposure

The regression analysis on all data (YAC + TAC, $N = 600$) for MiBP, MEP, MBzP, MnBP, 5cx-MEPP, 5OH-MEHP, OH-MINP show the contributions of both individual factors and their interactions to metabolite concentration (Table S8a,b).

Regression analysis highlighted the differences between exposures to low and high molecular weight phthalates. Gender, sampling season, and age were significant factors for HMW phthalate metabolites 5cx-MEPP, 5OH-MEHP, OH-MINP, with explained variability around 10%. The only significant interaction for HMW metabolites were gender and age for 5cx-MEPP, time indoors, and age for 5OH-MEHP and time indoors and season for OH-MINP, but these interactions did not significantly increase R^2 (Table S8). For the LMW phthalate metabolites MiBP, MEP, MBzP, the main significant factors were similar – gender and age – with an explained variability between 10% and 16%, but the significant interaction of age and gender with total PCP use increased the explained variability of MiBP and MEP to 18% and 25%, respectively. This

Table 2
LODs, detection frequencies and median concentrations (μg/L, SG adjusted) with range (min-max) and geometric means of phthalate metabolites for both cohorts.

			YAC			TAC		
Parent phthalate	Phthalate metabolite	LOD (μg/L)	Detection frequency (%)	Median (minmax)	Geo. Mean	Detection frequency (%)	Median (minmax)	Geo. Mean
DEP	MEP [a]	0.20	99.0	17.5 (0.02–860)	22.0	100	38.1 (3.04–704)	42.2
DiBP/DnBP	MiBP [a]	0.07	99.6	52.6 (0.07–446)	64.0	97.3	28.8 (0.03–486)	26.1
BzBP	MBzP [a]	0.10	75.6	0.69 (0.10–25.3)	0.74	94.6	1.74 (0.07–112)	1.85
DnBP/BzBP	MnBP [a]	0.18	96.3	60.6 (0.18–664)	60.5	91.0	45.5 (0.08–303)	28.8
DCHP	MCHP	0.20	0	0.20 (0.20–0.20)	0.18	0.3	0.14 (0.08–1.07)	0.15
DEHP	MEHP	0.18	93.6	2.29 (0.18–40.1)	2.57	93.6	2.14 (0.11–63.6)	2.09
DEHP	5OH-MEHP [a]	0.07	99.6	7.85 (0.07–150)	9.30	99.3	13.9 (0.03–679)	14.2
DEHP	5oxo-MEHP	0.07	99.3	3.65 (0.07–113)	4.43	100	5.56 (1.33–74.5)	6.00
DEHP	5cx-MEPP [a]	0.07	99.6	5.04 (0.07–108)	6.02	100	7.52 (1.74–128)	8.32
DnOP	MnOP	0.20	0.3	0.20 (0.20–2.95)	0.18	3.3	0.14 (0.08–6.63)	0.16
DiNP	OH-MiNP [a]	0.30	98.3	7.92 (0.30–1649)	11.1	98.3	11.4 (0.16–169)	12.1
DiNP	cx-MiNP [a]	0.20	49.6	0.20 (0.20–105)	0.48	100	5.34 (0.96–503)	6.19
DiNP	oxo-MiNP	0.20	92.0	1.99 (0.20–111)	2.59	97.3	2.75 (0.16–228)	3.01
DiDP	OH-MiDP [a]	0.20	31.6	0.20 (0.20–102)	0.37	52.3	0.55 (0.09–13.4)	0.48
DiDP	oxo-MiDP	0.10	41.6	0.10 (0.10–60.4)	0.26	55.0	0.39 (0.05–14.2)	0.30
DINCH	OH-MINCH [a]	0.30	61.3	1.34 (0.30–952)	1.67	75.0	1.71 (0.15–494)	1.76
DINCH	cx-MINCH	0.25	39.6	0.25 (0.25–277)	0.74	51.0	0.76 (0.10–204)	0.80

[a] Indicates a metabolite was selected as priority biomarker for further analysis.

suggests the greater importance of PCPs use for LMW phthalate exposure (Table S8). We found no relationship between the factors and MnBP concentration. However, an important outcome of this is the limited explanatory power of these selected factors for phthalate exposure. Thus, the majority of the variance in phthalate metabolite levels remains unexplained by these factors.

We evaluated the impact of key determinants in greater detail: age, season, gender, time indoors and PCP use on phthalate metabolite levels to identify key determinants of phthalate exposure in the teenager and young adult cohorts.

3.1.1. Age

A limited relationship between age as a continuous variable and HMW metabolites was observed (Table S8), but when age was treated as a categorical variable (YAC vs. TAC) a few clear differences were apparent (Fig. 1, Table S9). Notably, MiBP was significantly higher in YAC ($p < 0.05$), with median concentrations two times higher than in TAC. MnBP was also detected in higher concentrations ($p < 0.1$) in YAC (60.65 μg/L compared to 45.47 μg/L in TAC). On the other hand, statistically significant differences were found for MEP, MBzP and 5OH-MEHP with median concentrations approximately two times higher in TAC; and the rest of the secondary DEHP metabolites were also detected in higher concentrations in TAC (Table 2), but these differences were not statistically significant. These differences may be due to lifestyle differences between teenagers and young adults, and are discussed further below.

3.1.2. Season

Analysis of the season of sample collection was stratified by cohort and gender, and only performed on the YAC, as all TAC samples were collected in the cold season. The results of the non-parametric tests showed that in YAC, statistically significant differences were found for 5OH-MEHP and OH-MiNP with 20% and 100% higher concentrations, respectively, in urine samples collected in the warm season ($p < 0.05$), and concentrations of cx-MiNP were also 50% higher ($p < 0.1$) (Tables S6 and S9). This may indicate a link between levels of phthalates in the ambient environment and human exposures (Pilka et al., 2015). Phthalates are found in outdoor air in higher concentrations in warmer months (Pilka et al., 2015; Puklová et al., 2019), attributed to increases in both primary and secondary emissions at higher temperatures

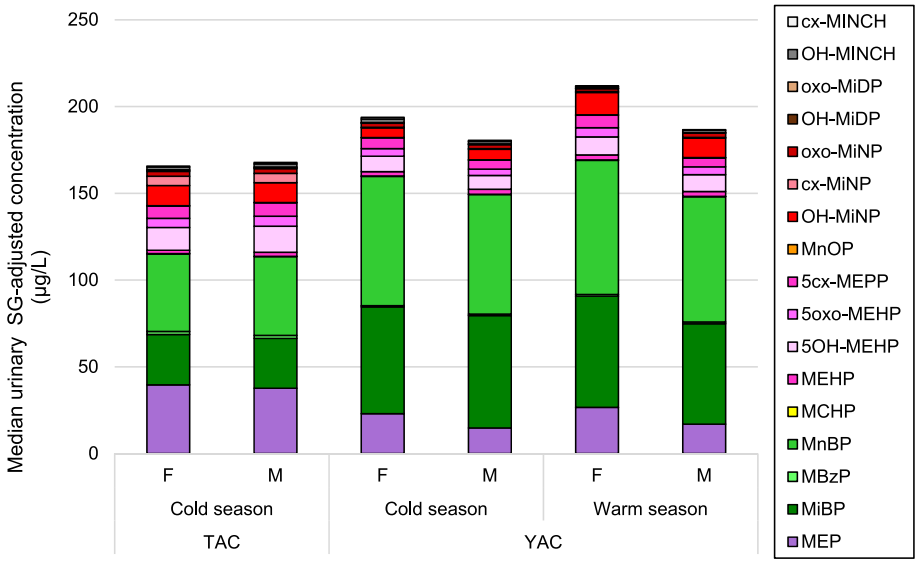

Fig. 1. Median concentration of phthalate metabolites in both cohorts. M represents males, F represents females.

(Vasiljevic et al., 2021). Higher emissions, and resulting higher environmental concentrations suggest the potential for greater human exposure in warm seasons, reflected in urinary metabolites (Puklová et al., 2019).

3.1.3. Gender

The results of the non-parametric tests show statistically significant gender differences in three phthalate metabolites (MEP, 5oxo-MEHP and 5cx-MEPP; Figure S7) in YAC, and no gender differences for any metabolite in TAC (Table S9). There are greater gender differences in lifestyles in the YAC cohort compared to the TAC: teenagers typically spend weekdays in the same location (school) regardless of gender, while there is greater differentiation in adult occupations by gender, and PCP use in teenagers is similar across males and females, but differs significantly by gender for young adults (Figures S4 and S5).

In YAC, MEP had significantly higher concentrations in females, and this difference was more significant in the cold season ($p < 0.05$ in cold season, $p < 0.1$ in whole year) (Table S9). Two DEHP metabolites (5oxo-MEHP and 5cx-MEPP) also showed significantly higher concentrations in females (Table S9). Gender-dependent differences in oxidative DEHP metabolism have been identified (Tait et al., 2020), which could lead to differences between male and female concentrations of urinary metabolites (Koch et al., 2017). Higher MEP levels in women have been noted in other studies (Runkel et al., 2020; Wormuth et al., 2006) attributed to gender differences in PCP use. This suggests an association between higher MEP levels and frequency and amount of PCP use, which the gender differences are indirectly indicating.

3.2. Lifestyle determinants of phthalates exposure

The seasonal and gender-related differences, while in some part attributable to differences in environmental levels and physiological factors, are largely due to the lifestyle differences that manifest across seasons and between different populations. The key lifestyle aspects which are hypothesized to be related to phthalate exposure are time spent indoors, indoor activities such as cleaning, use of personal care products containing phthalates, and diet. We examined the individual influence of individual exposure determinants, excluding diet, as well as possible combined effects. Diet was excluded because we had limited information on dietary patterns that would be directly relevant to phthalate exposure. We only examined a link between phthalate metabolite levels and frequency of drinking from plastic bottles, and for this variable no relationships were found.

3.2.1. Exposures via the indoor environment

When comparing urinary metabolite concentrations with self-reported time spent indoors, we did not find a statistically significant relationship between the time spent indoors and individual phthalate metabolite for either YAC or TAC. Higher MEP, MBzP, 5oxo-MEHP, 5OH-MEHP, 5cx-MEPP, OH-MiNP and cx-MiNP concentrations were observed in TAC samples, indicating higher exposures to DEP, DEHP and DiNP. This may be related to longer times spent indoors by teenagers (with a median of 14 h, compared to 12 h for YAC, Figure S3). Moreover, this is similar to the effect of inclusion of time spent indoors in the regression model, where interaction of time spent indoors with age for 5OH-MEHP and with season for OH-MINP was statistically significant but did not lead to a significant increase in explained variability (Table S8). Urinary levels of MBzP and MEP have been positively correlated with indoor levels of BzBP and DEP in several previous studies, suggesting exposure via indoor air and dust as important routes of exposure to these phthalates (Adibi et al., 2008; Bekö et al., 2013; Langer et al., 2014); however, we note that these studies did not identify indoor sources as important for HMW phthalates.

No statistically significant differences were found between any metabolites and renovations/redecorations done in the past 2 years.

3.2.2. Exposures via PCP use

Participants reported frequency of PCP use per week in the four weeks prior to sample collection. The specific PCPs identified in questionnaires were shampoo, scented products (deodorants and perfumes), lotions, nail polish, make-up/foundation creams, lip balms and eye make-up. We tested differences in phthalate metabolite concentrations for different frequencies separately stratified by age (YAC vs TAC), gender and season to remove the influence of these factors. In TAC, we found statistically significantly higher levels of MEP for both genders in participants reporting using deodorant compared with those "never" or "once a week" using them (Table S7), as well as an increasing trend with increasing frequency of use (Fig. 2), which agrees with reports of high levels of DEP in perfumes (Guo and Kannan, 2013) and a previous study comparing urinary metabolites with PCP use (Nassan et al., 2017). In the YAC cohort, no differences in MEP concentrations were found when scented products were used because there is likely overlap with the use of other PCPs (see below).

Associations with nail polish use were observed in YAC in both the stratified and overall analyses. Women reporting use of nail polish had statistically significant higher levels of 5cx-MEPP and OH-MINP in the cold season than those reporting no use (Table S7), and when including all YAC (male and female), participants reporting "never" using nail polish had lower levels of 5cx-MEPP compared with those using nail polish four to six times per week (Figure S8). However, due to the low number of participants who used nail polish at a high frequency (7 times per week, $n = 3$), the significance of the highest use category could not be statistically tested. Nail polish use presents a documented pathway for dermal uptake of chemicals, as noted by Mendelsohn et al. (2016), and while exposure to DEHP is not typically associated with PCP use, some nail polishes have been also identified to contain DEHP (Guo and Kannan, 2013; Young et al., 2018). Moreover, nail polish is a long-term PCP exposure route, as nail polishes typically stay on the nails for days to weeks, meaning that the spot samples collected for this study may better capture this exposure source, in contrast to rinse-off PCP products. In the TAC cohort, no difference was observed in females who reported use vs. no use of nail polish (Table S7), likely because almost all participants used nail polish with a very low frequency (Figure S5A).

While the analysis comparing the use of individual products was able to identify some relationships (notably with deodorant and nail polish use), many of the product categories represent relatively small potential exposures (e.g., lip balm, eye shadow), which independently may not lead to substantial increases in phthalate exposures. Moreover, we do not know which brands of products were used by study participants, and there may be high variability in the phthalate content of products on the market, e.g., only some nail polishes contain phthalates (Guo and Kannan, 2013; Young et al., 2018). However, we hypothesized that by clustering the study participants according to patterns of PCP use, we could discern the influence of overall PCP use on human exposure to

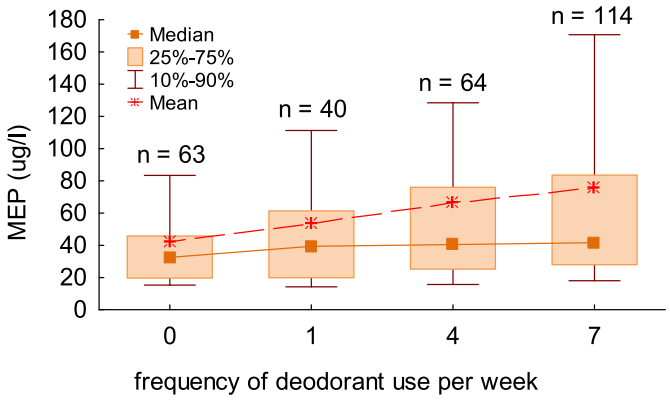

Fig. 2. Difference in MEP concentration for different frequency (times per week) of deodorant use in the TAC cohort (teenagers).

phthalates. Regression analysis shows that added interaction with the total PCP use increased the explained variability of MEP concentrations by 9%. In the case of TAC, the clusters do not provide any additional insight into PCP use patterns, mainly due to the similarity of behaviour among teenagers and low use of make-up products. However, for YAC, clear patterns of PCP use and associated exposures are visible (Figure S9). YAC data were separated into seven clusters according to PCP use, with cluster A associated with the least overall PCP use, and cluster D-G associated with frequent PCP use across most categories. Clusters A, B, and C were dominated by use of only shampoo and scented products, and included mainly male subjects, while clusters D-G included mainly female subjects and had notably higher uses of additional PCPs like lotion and decorative cosmetic products. This pattern of higher overall use of PCPs, mainly decorative cosmetic products, is connected with higher urinary concentrations of MEP (Figure S9) and presents a possible explanation for the higher levels of MEP noted in females (Table S7). However, there is no statistically significant difference in MEP concentrations between clusters, mainly due to the high variance in concentrations.

3.2.3. *Patterns in overall phthalate exposure*

In view of the complex factors leading to phthalate exposures, we combined all phthalate metabolite data (YAC + TAC, $N = 600$) to evaluate the overall pattern of phthalate metabolites and exposure determinants. We hypothesize that the cumulative effect of different lifestyles and behaviours drives phthalate exposures, rather than individual products or activities.

Cluster analysis was selected as an additional multivariate technique to provide insight into patterns of exposure and exposure determinants. Cluster analysis was used to group participants who had similar metabolite concentrations from the lowest (A) to the highest (H) according to the median metabolite concentrations in the cluster (Fig. 3). These clusters had significant differences in metabolites associated with LMW phthalates, HMW phthalates and DINCH, with Cluster H notably higher in HMW phthalate and DINCH metabolites, and Cluster G having substantially higher levels of LMW phthalate metabolites (Figure S10; Table S10). The lifestyle variables associated with each cluster were then summarized. However, it is important to note that it is not possible to strictly divide phthalate metabolites according to their source, in this case, either from PCPs or from building materials/indoor products. We note that the cluster with the highest phthalate metabolite levels (Cluster H in Fig. 3) contains mainly cold season samples from men with an average age of 19 years with high PCP use, especially of products like shampoo and lotion which have larger application quantities, and among the highest time spent indoors. Additionally, this is the only cluster where a higher concentration of the alternative plasticizer metabolite was observed: Cluster H has a much higher contribution of OH-MINCH to the total plasticizer metabolites than any other cluster. Use of DINCH as a DEHP and DINP replacement is primarily in sensitive applications such as food contact materials, medical devices and toys (Bui et al., 2016). The reasons for the predominance of OH-MINCH in Cluster H are not fully clear; it may be due to behaviours associated with increased time indoors, as well as food consumption habits which were not well captured by the determinants of exposures available.

In contrast, a low exposure cluster (Cluster C) has low use of the PCPs with large application quantities, and the lowest time spent indoors. Similarly, clusters C and E, where participants declare lower time spent indoors, have smaller contributions of HMW phthalate metabolites. Additionally, clusters A and B have low levels and were largely all collected in cold seasons, which corresponds with the identification of low environmental levels and resulting low exposures during colder months. In some cases, the relation between the metabolite levels and

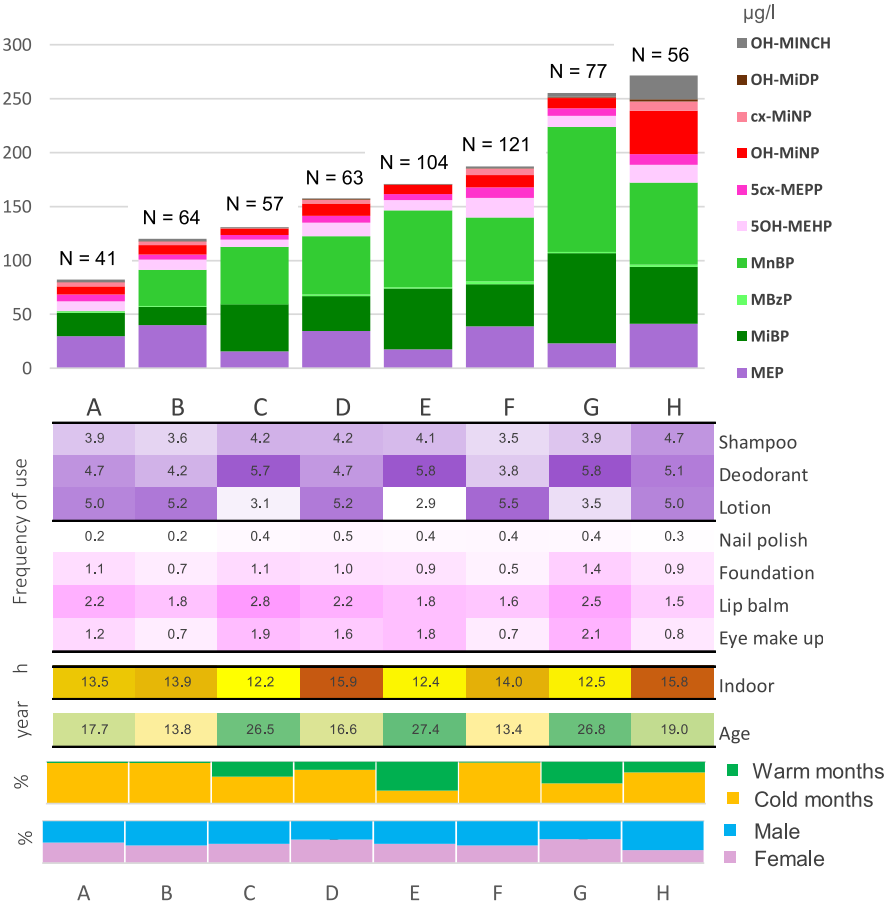

Fig. 3. Cluster analysis of metabolite concentrations in relation to lifestyle factors relating to phthalate exposure. For the metabolites, median concentrations in µg/L are given for each cluster. LMW metabolites which are often connected with PCP use are shown in tones of purple and green, HMW metabolites which are often considered "indoor exposure" biomarkers from consumer products and building materials are shown in tones of red and pink. Alternative plasticizers metabolites are shown in tones of grey. For lifestyle factors, the values indicate average PCP weekly use frequency (two-level scale – more frequently used in tones of purple, less frequently used in tones of pink), average hours per day spent indoors, and average age. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the lifestyle factors is unclear, e.g., phthalate metabolites are high in cluster G, but that cluster has relatively low median use of PCPs, and low time spent indoors.

The results of our cluster analysis support our hypothesis that phthalate metabolite concentrations relate to a combination of separate lifestyle factors affecting phthalate exposure, rather than one dominant exposure source. Some part of this disconnect may be because we are omitting a major phthalate exposure pathway: dietary ingestion. We tested whether there was a relationship between phthalate metabolites and consumption of drinks in plastic bottles, and found no relationship, but beyond this, no factors related to dietary exposure were tested. However, dietary ingestion is known to be important for many phthalates, particularly the HMW phthalates (Giovanoulis et al., 2018; Wormuth et al., 2006). Nevertheless, cluster analysis presents a useful technique to profile the complexity of exposure factors and interrelations between variables such as age, gender, and season with PCP use and behaviours.

3.3. Comparison with other studies

We compared the median urinary concentrations of phthalates in teenagers and young adults in our study with values reported in the literature for these age groups. Specifically, median phthalate metabolite concentrations from our study were compared with data from Germany (GerES V study, 2015–2017) (Schwedler et al., 2020a, 2020b), Canada (Canadian Health Measures Survey, cycle 5, 2016–2017) (Health Canada, 2019) and USA (NHANES, 2015–2016) (CDC, 2019).

Marked differences between countries were found for MnBP and MiBP concentrations, which were around 5–6 times higher in young adults in our study than the values reported in adults from Canada and USA. In teenagers, median MnBP and MiBP concentrations were also generally higher in our study than the values reported in Canada, USA, and Germany (Fig. 4, Table S11). However, data from other studies from Czechia (Puklová et al., 2019) or other European countries (Slovakia (Pilka et al., 2015) and Denmark (Søeborg et al., 2012)) show median MnBP and MiBP concentrations more similar to those from Czechia than to those from North America (Frederiksen et al., 2010). OH-MiNP concentrations in young adults and teenagers in our study were around 15 times higher than the values reported for the same age groups in Canada, with smaller differences (2-fold) in OH-MiNP levels between Czech and German teenagers. Conversely, the median MBzP concentration in Czech young adults was around 5–6 times lower than values reported for this metabolite in Canada and the USA. DINCH metabolites are reported in all regions at similar levels.

With the general similarities in development, climate and lifestyles between Europe and North America, we attribute the differences in specific metabolites to differences in food composition; in Europe, the major sources of DEHP, DBP, DiBP and BzBP in adults is food or diet in general (Wormuth et al., 2006). Moreover, differences in PCP use, chemical legislation and building practices between North America and the EU, as well as within the EU, can also lead to differences in phthalates exposure of the general population between countries (Runkel et al., 2020). Some smaller contribution may come from differences in the identified cohort populations, e.g., teenagers are defined as 11–17 years old in Czechia, 14–17 years old in Germany, and 12–19 years old in Canada and USA.

3.4. Study limitations

Samples for both cohorts were taken in different seasons. In some cases, there are indications that self-reported data may not be correct, e.g., implausible time spent indoors. A few participants declared an average time spent indoors less than 8 h (sleep included), which seems, particularly in the case of teenagers in cold/winter months, not probable. Therefore, these values were excluded from the statistical analysis. Moreover, questionnaires asked about products used in the previous four weeks, which may not correspond directly to metabolites detected in urine samples. Diet was excluded because we had limited information on dietary patterns that would be directly relevant to phthalate exposure. Another limitation is that urine samples were spot samples collected at different times of day, with insufficient information about the timing of sample collection to allow further grouping. Values of SG were measured after one freeze-thaw cycle; the ideal measurement would be on fresh urine samples (Pearson et al., 2009). Additionally, we cannot distinguish the origin of some metabolites, particularly if the parent phthalate is DiDP or DPHP (Gries et al., 2012; Koch et al., 2017).

4. Conclusions

We identified widespread exposure to phthalate esters in the Czech population based on the quantification of phthalate metabolites in spot urine samples from teenagers and young adults. While some relationships were identified between individual exposure determinants (use of certain PCPs, season, gender) and specific phthalate metabolites, in general, the individual factors did not show a strong relationship with phthalate metabolites. We hypothesize that phthalate exposures relate

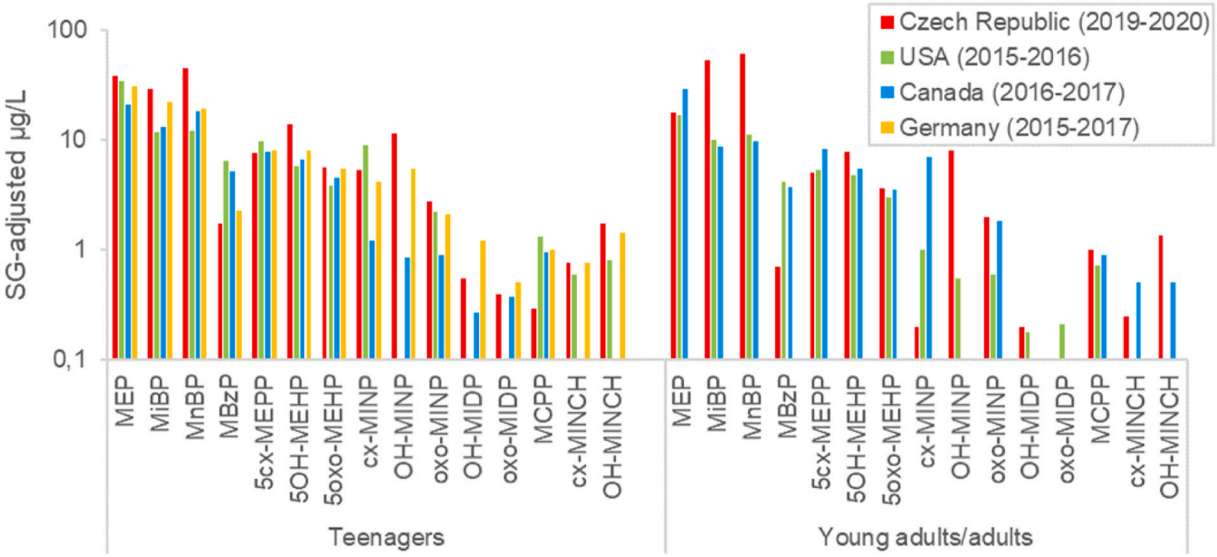

Fig. 4. Comparison of urinary concentrations of phthalate metabolites in teenagers and young adults from Czechia, Germany, Canada and USA, logarithmic scale.

to a combination of separate lifestyle factors, rather than one dominant exposure source. We tested this through cluster analyses, identifying the profiles of study participants with higher levels of phthalate metabolites. Participants who spent more time indoors, particularly noticeable during colder months, had higher levels of HMW phthalate metabolites, whereas participants with higher PCP use, particularly women, tended to have higher concentrations of LMW phthalate metabolites. We conclude that while phthalate exposure is ubiquitous, it is also very variable, according to age, gender, time spent indoors, food, and lifestyle in general.

Credit author statement

Petra Stuchlík Fišerová; – Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing; **Lisa Melymuk** – Investigation, Writing – review & editing, Supervision; **Klára Komprdová**; – Data curation, Visualization; Writing – review & editing; **Elena Domínguez-Romero** – Investigation, Writing – review & editing; **Martin Scheringer** – Writing – review & editing; **Jiří Kohoutek** – Methodology, Formal analysis; **Petra Přibylová**; – Resources, Supervision; **Lenka Andrýsková**; – Resources, Supervision; **Pavel Piler** – Resources, Data curation; **Holger M. Koch** – Validation, Writing – review & editing; **Martin Zvonař**; – Supervision, Funding acquisition, Resources; **Marta Esteban-López** – Supervision, Validation, Writing – review & editing; **Argelia Castaño** – Supervision, Validation, Writing – review & editing; **Jana Klánová**; – Resources, Funding acquisition, Supervision, Writing – review & editing.

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Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.113675.

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