Biomonitoring of fast-elimination endocrine disruptors – Results from a 6-month follow up on human volunteers with repeated urine and hair collection

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Biomonitoring of fast-elimination endocrine disruptors – Results from a 6-month follow up on human volunteers with repeated urine and hair collection

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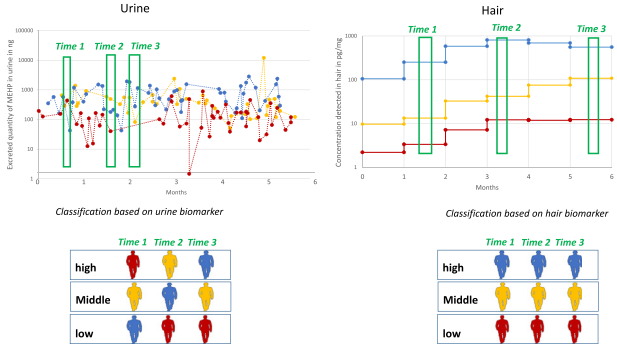
HIGHLIGHTS

- Hair and urine samples were repeatedly collected from 16 volunteers over 6 months.
- 16 phthalate metabolites, 4 bisphenols, 8 pesticides were tested in 94 hair and 805 urine samples.
- 19 biomarkers (in hair) and 24 (in urine) were detected in >50% of the samples.
- Biomarkers ICC ranged from 0.1 to 0.8 (10 above 0.4) in hair and from 0.09 to 0.51 in urine (2 above 0.4).
- No correlation between hair and urine was observed for most biomarkers.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: The assessment of human exposure to fast-elimination endocrine disruptors (ED) such as phthalates, bisphenols or pesticides is usually based on urinary biomarkers. The variability of biomarkers concentration, due to rapid elimination from the body combined with frequent exposure is however pointed out as a major limitation to exposure assessment. Other matrices such as hair, less sensitive to short-term variations in the exposure, have been proposed as possible alternatives. Nevertheless, no study compared the information obtained from hair and urine respectively in a follow-up allowing to assess biomarkers variability over time in these two matrices, and to compare the correlation between them.

Methods: In the present study, hair and urine samples were collected from 16 volunteers over a 6 months follow-up. All in all, 92 hair samples and 805 urines samples were collected and analyzed for the presence of 16 phthalate metabolites, 4 bisphenols and 8 pesticides/metabolites.

Abbreviations: GC–MS/MS, gas chromatography tandem mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S; MBzP, monobenzylphthalate; MEP, monoethylphthalate; MMP, monomethylphthalate; MEHP, mono(2-ethylhexyl)phthalate; 5-OH-MEHP, 5-OH-mono(2-ethylhexyl)phthalate; 5-oxo-MEHP, 5-oxo-mono(2-ethylhexyl)phthalate; 5-cx-MEPP, 5-carboxy-mono(2-ethylhexyl)phthalate; 2-cx-MMHP, mono(2-carboxymethylhexyl)phthalate; MINP, mono-iso-nonylphthalate; OH-MINP, mono(hydroxyisononyl)phthalate; cx-MINP, mono-carboxyisooctylphthalate; OH-MPHP, mono-hydroxypropylheptylphthalate; oxo-MPHP, mono-oxopropylheptylphthalate; MINCH, monoester mono-isononyl-cyclohexane-1,2-dicarboxylate; OH-MINCH, cyclohexane-1,2-dicarboxylicmonohydroxyisononylester; oxo-MINCH, cyclohexane-1,2-dicarboxylicmonooxoisononylester; cx-MINCH, cyclohexane-1,2-dicarboxylicmonocarboxyisooctylester; IMPy, 2-isopropyl-4-methyl-6-hydroxypyrimidine; 3Me4NP, 3-methyl-4-nitrophenol; PNP, para-nitrophenol; 3-PBA, 3-phenoxybenzoic acid; TCPy, 3,5,6-trichloro-2-pyridinol; DEP, diethyl phosphate.

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Hair
Urine
Endocrine disruptor
Biomarkers of exposure

Results: All the biomarkers analyzed were detected in at least one of the two matrices. 21 biomarkers were more frequently detected in hair, 6 in urine, and 1 was equivalent. Biomarkers intraclass correlation coefficients (ICC) ranged from 0.1 to 0.8 (ten above 0.4) in hair, and from 0.09 to 0.51 in urine (two above 0.4). The concentrations of biomarkers in hair and urine were significantly correlated for only one compound.
Conclusion: This study highlights the complexity of assessing exposure to fast-elimination ED and suggests considering with caution the specificity of the matrix in data interpretation. The results document the respective advantages and limitations of urine and hair, and provide new insight in the understanding of the information provided by these biological matrices and their relevance for the assessment of human exposure to fast elimination contaminants.
Capsule: 92 hair and 805 urine samples collected from 16 volunteers over 6 months, tested for phthalate metabolites, bisphenols and pesticides. 19 biomarkers (in hair) and 24 (in urine) were detected in >50% of the samples.

1. Introduction

An endocrine disruptor (ED) is defined as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" (WHO/IPCS, 2002). Although the first chemicals targeted as ED were mainly persistent pollutants such as PCBs, dioxins and DDT (Reiter et al., 1998), many other chemicals from different groups, including for instance non-persistent pesticides, phenols and phthalates, have since been included in the list of ED (Chemsec SIN LIST, 2020; TEDX The Endocrine Disruption Exchange, 2020; Beausoleil et al., 2018; Mnif et al., 2011; Ewence et al., 2015; Kabir et al., 2015; European Chemicals Agency, n.d.). The increasing awareness of ED ubiquitous presence and the possible consequences on human health has incited to consider ED a public health priority at the international level, and to intensify research in order to highlight human exposure to ED and better understand their effect (Reiter et al., 1998; Kabir et al., 2015; Kahn et al., 2020; Beausoleil et al., 2013; Heindel et al., 2015).

Among the different approaches adopted to assess exposure to pollutants, biomonitoring, consisting in the analysis of biomarkers of exposure (the pollutants themselves or their metabolites) directly in biological matrices collected from individuals, offers the advantage of providing information on the internal dose and integrating all the sources of exposure including unsuspected ones. For this purpose, urine has been historically the most used matrix for the biomonitoring of hydrophilic ED such as phenols, phthalates and some pesticides by targeting their urinary metabolites.

Nevertheless, several studies pointed out that the concentration of these pollutants (or their metabolites) in urine is highly variable on the short term. This high variability is due to both the rapid elimination of these chemicals (mainly in urine) after exposure, and the frequent re-exposure over time due to their presence in homes, materials, food, care product (Aylward et al., 2014; Giovanoulis et al., 2018; Faÿs et al., 2020). The successive rises and falls in biomarker concentration, possibly covering several orders of magnitude within one day, make a single urine sample irrelevant to assess chronic exposure, and represent the main limitation to classifying individuals according to their level of exposure based on urinary biomarkers (Faÿs et al., 2020).

In order to circumvent the limitation associated with urinary biomarker variability, two approaches can be taken. On the one hand, increasing the number of samples collected from each individual over a defined period is usually considered to improve the quality of exposure assessment over this period (Faÿs et al., 2020; Kissel et al., 2005; Xiao et al., 2014; Wielgomas, 2013). Samples can then be analyzed as pools to obtain an averaged value, or separately to capture biomarkers variability over time (Faÿs et al., 2020; Philippat and Calafat, 2021). The repeated collection of samples however increases the sampling workload, the effort requested from the participant, and the analytical work and cost if the samples are analyzed separately. Moreover, depending on the biomarker tested, the number of samples required to obtain a reliable global information on exposure may easily reach several tens (Faÿs et al., 2020). On the other hand, other matrices covering wider temporal windows than urine can be considered. For this purpose, hair is a promising candidate that has been extensively used in other contexts such as forensic and clinical toxicology for the determination of drugs of abuse or biomarkers of alcohol consumption, doping control, hormone analysis and heavy metals detection (Kintz et al., 2015; Appenzeller et al., 2007; Staufer and Yegles, 2016; Thieme and Anielski, 2015; Grova et al., 2020). More recently, several recent studies also validated its relevance for exposure assessment (Appenzeller et al., 2017; Duca et al., 2014a), and demonstrated the possibility to detect in this matrix various contaminants such as polycyclic aromatic hydrocarbons (PAH), pesticides from different classes and persistent organic pollutants (POPs) (Appenzeller and Tsatsakis, 2012; Peng et al., 2020a; Peng et al., 2020b; Palazzi et al., 2018). Incorporation of chemicals into hair is considered to occur mainly from blood, in living hair bulb cells (Chata et al., 2016; Pragst and Balikova, 2006), making the concentration of xenobiotic in hair a reliable surrogate of the level of exposure and the internal dose (Appenzeller et al., 2017). Nevertheless, although many different chemicals classes have been detected in hair, only very few studies focused on emerging contaminants such as phthalates and bisphenols. In particular, the chemicals most recently introduced (e.g. bisphenol B, DINCH) as replacement of previous ones submitted to use restriction (e.g. bisphenol A, DEHP) have not been tested in hair yet.

In parallel, due to differences in the temporal window covered by urine (hours) and hair (weeks to months) respectively, and different mechanisms of incorporation of the biomarkers of exposure in these matrices, the results obtained from each of them might not be directly comparable. The choice of the matrix used for exposure assessment is thus not trivial, and the nature and quality of the information provided by each of these matrices might be significantly different, particularly for fast-elimination chemicals. Nevertheless, only very few studies compared the results obtained with different types of specimens collected from the same individuals (Wang et al., 2018; Kim et al., 2019; Hernandez et al., 2019; Kokkinaki et al., 2014; Hardy et al., 2021). These studies only focused on a limited number of chemicals from the same class, and relied on a single sample of each matrix per individual, with rather limited relevance for fast-elimination chemicals. Research is thus still needed to understand which information can be provided by each matrix respectively, in the framework of repeated samplings over time allowing to assess the high variability in exposure and internal dose which characterizes fast-elimination endocrine disruptors.

In order to fill this gap, hair and urine samples were collected from 16 volunteers over a 6 months-follow up and analyzed for 29 biomarkers of exposure to fast-elimination pollutants including phthalates, bisphenols and pesticides from different classes. In a first step, the variability observed for these biomarkers in urine on this population has been presented in a previous article (Faÿs et al., 2020). More specifically, we demonstrated that a single urine sample fails to provide reliable information on exposure, and that increasing the number of urine samples only slightly improved the quality of exposure assessment. In the

present article, an analytical method based on liquid chromatography tandem mass spectrometry (LC-MS/MS) and gas chromatography tandem mass spectrometry (GC–MS/MS) was specifically developed and validated to analyse several biomarkers that had never been analyzed in hair until now. The results obtained from the analysis of each matrix were compared regarding biomarkers concentration and frequency of detection, to better understand which information could be obtained on exposure variability and chronicity, and how individuals could be classified according to their level of exposure extrapolated from biomarkers concentration in hair and urine.

2. Material & methods

2.1. Studied population and sample collection

Sixteen volunteers (8 women, 8 men) aged from 22 to 71 years old (average 40) were recruited for a 6 months follow-up, as previously described by Faÿs et al. (2020). Briefly, during this period, urine samples were randomly collected 1–3 times per week and each void was weighed in order to calculate the total quantity excreted per urination for each chemical. The number of urine samples collected from each participant ranged from 43 to 57. Overall, 805 urine samples (including 25% as first day voids, 6% collected in the morning, 30% in the afternoon and 34% in the evening) were collected. Over the same period, hair strands were also sampled at the end of each month and only the first proximal centimeter (close to scalp) was later analyzed, to represent the month before sampling. In total, 92 hair samples were analyzed, with a number of samples ranging from 4 to 6 per subject. After collection, hair samples were stored in aluminum paper at −80 °C in the lab freezer until analysis. The date of sampling, participant code and length of the strand were written both on the aluminum paper and on a paper form. A mark was also applied on the aluminum paper to indicate which part of the strand was the closest to the scalp and the foil was folded to immobilize the strand of hair until analysis.

Population characteristics were previously detailed in Faÿs et al. (2020) and presented in supplemental data (Supplemental Data, Table S1). This study was approved by the National Committee of Ethic and Research of Luxembourg (CNER, approval number 201601/04).

2.2. Biomarkers selection

Since the present study was focused on exposure assessment but did not target biological effects associated with specific ED or ED class, different families were included in the list of target biomarkers: bisphenols, phthalates and pesticides, which are among the most intensely investigated currently regarding their possible effects on human. The list of target biomarkers was defined in agreement with the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), who was partner of the project. For pesticide metabolites, we took advantage of previous research conducted by our team and others, in which we could demonstrated that the biomarkers could actually be detected in both urine and hair (Hardy et al., 2021; Hardy et al., 2015a; Beranger et al., 2018a). For bisphenols and phthalates, we included metabolites of both "classical" compounds that have already been submitted to restriction measures (e.g. Bisphenol A and DEHP) but for which important amount of data is available in the literature regarding exposure and pharmacokinetics, and newly introduced chemicals (e.g. bisphenol B, F, metabolites of DINCH) that were mainly introduced to replace the previous ones. In parallel, the final list of biomarkers was also limited by the analytical constraints associated with multi-residue methods. Although the initial list included a higher number of biomarkers, many had to be removed because the method did not allow reaching sufficient sensitivity. Namely, pentachlorophenol, Cl_2CA (cis-3-(2,2dichlorovinyl)-2,2-dimethylcyclopropane-carboxylic acid, metabolite of permethrin), DETP (di-ethyl thiophosphate, metabolite of organophosphate pesticides) and BADGE (Bisphenol A diglycidyl ether) were included in the initial method but removed because they were not compatible with the final analytical method. The final list of biomarkers is presented in Table 1.

2.3. Preparation and measurement equipment

A New Brunswick G25 orbital incubator shaker and an IKA Vortex Genius 3 were used to perform agitation steps. Centrifugations of samples were done with a Sigma 4-16KS centrifuge. Hair pulverization was conducted with a MM200 ball mill from Retsch. The heating chamber used for derivatization reaction was provided by Binder.

The analyses were performed with two instruments: an Agilent 7890 gas chromatograph system equipped with a HP-5MS capillary column (30 m, 0.25 mm I.D., 0.25 μm film thickness) coupled to an Agilent 7000A triple quadrupole mass spectrometer operating in negative chemical ionization mode; and a Waters Acquity UPLC H-Class ultra-high performance liquid chromatograph equipped with a BEH C18 column (100 mm, 2.1 mm ID, 1.7 μm particle size) combined with a Waters Xevo TQ-S tandem mass spectrometer.

2.4. Urine analysis

The protocol and LC-MS/MS parameters used for the analysis of the urine samples were fully detailed in Faÿs et al. (2020). Briefly, urine samples (1 mL) were deconjugated with 250 μL of β-glucuronidase solution in ammonium acetate buffer (10 mM, pH 6.7) at 37 °C overnight under agitation. 667 μL of aqueous acetic acid solution were then added to stop deconjugation. A solid phase extraction was performed with Waters OASIS HLB 6 cm3 150 mg columns and compounds were eluted with 6 mL of methanol before evaporation to dryness under nitrogen stream at 37 °C. Samples were reconstituted in 100 μL of acetonitrile before injection. Phthalate metabolites, pesticides and bisphenol S were analyzed directly after extraction with LC-MS/MS while bisphenols A, B and F were derivatized with 1-methylimidazole-2-sulfonyl chloride to enhance their detection in LC-MS/MS.

2.5. Hair analysis

2.5.1. Hair sample preparation protocol

In order to remove external contaminants from hair surface, the strand of hair was decontaminated according to the previously published procedure (Duca et al., 2014b) including a first washing of 5 min with 5% SDS aqueous solution and a second one with methanol during 5 min. After that, the lock of hair was dried at ambient temperature with a paper tissue and then pulverized for 5 min at 25 Hz with a ball mill.

An aliquot of 50 mg of hair powder was weighed in a 4-mL screw cap glass vial containing 10 μL of internal standard solution. Hair powder was then extracted with 1 mL of acetonitrile/HCl 0.6 M in water (80:20, v/v) under overnight agitation at 40 °C. After centrifugation of the sample at 5000 rpm for 15 min, the supernatant was collected in a 5-mL glass tube. The hair powder residue was rinsed by an additional 1 mL of extraction solvent, a vortex and a centrifugation. Thereafter, the supernatant was combined with the first extract obtained. The resulting extract was then evaporated to dryness under nitrogen flow and reconstituted in 1 mL of 4% NH_4OH aqueous solution. The extract was loaded onto a Waters Oasis® MAX cartridge without vacuum, washed with 2 mL of 4% NH_4OH aqueous solution, and the cartridge was dried with vacuum for 5 min. Elution was conducted with 2 mL of methanol.

The extract was then evaporated to dryness and reconstituted with 100 μL of 0.1% formic acid in water/acetonitrile (80:20, v/v).

An aliquot of 20 μL was transferred in another vial, evaporated to dryness and derivatized at 60 °C for 5 min after addition of 50 μL of

Table 1
Concentration levels (pg/mg) of phthalate metabolites, bisphenols and pesticide biomarkers in hair samples from the 6-month follow-up of 16 volunteers.

Target biomarker (corresponding parent)	LOD (pg/mg)	% of detection	Mean	Min	P25	Median	P75	Max
Phthalate metabolites								
MMP (DMP)	0.13	48	6.9	–	–	<LOD	1.6	62.3
MEP (DEP)	0.5	87	857.2	<LOD	2.5	7.9	31.6	18,120
MBzP (BBzP)	0.03	88	0.7	<LOD	0.16	0.4	0.68	5.8
MEHP (DEHP)	0.74	98	50.2	<LOD	5.4	12.1	26.6	692
5-oxo-MEHP (DEHP)	0.004	97	0.28	<LOD	0.05	0.11	0.22	6.74
5-OH-MEHP (DEHP)	0.01	90	0.28	<LOD	0.03	0.1	0.21	1.84
5-cx-MEPP (DEHP)	0.02	67	0.46	–	<LOD	0.06	0.13	8.2
2-cx-MMHP (DEHP)	NA	0	nd	nd	nd	nd	nd	nd
OH-MPHP (DPHP)	0.19	3	0.43	–	–	–	<LOD	0.72
oxo-MPHP (DPHP)	0.004	51	0.07	–	<LOD	0.004	0.017	0.86
MiNP (DiNP)	NA	0	nd	nd	nd	nd	nd	nd
OH-MINP (DiNP)	0.24	8	1.11	–	–	–	<LOD	2.88
cx-MINP (DiNP)	0.01	46	1.52	–	–	<LOD	0.05	35.6
MINCH (DINCH)	0.03	97	1.04	<LOD	0.25	0.49	0.97	27.7
OH-MINCH (DINCH)	0.32	2	0.38	–	–	–	<LOD	0.44
cx-MINCH (DINCH)	NA	0	nd	nd	nd	nd	nd	nd
oxo-MINCH (DINCH)	0.18	2	0.39	–	–	–	<LOD	0.6
Bisphenols								
Bisphenol S[a]	0.16	98	31.3	<LOD	2.3	5.7	15.5	458
Bisphenol F[a]	0.05	52	0.42	–	<LOD	0.05	0.1	2.99
Bisphenol A[a]	0.85	76	22.5	<LOD	0.86	6.4	17.2	95.8
Bisphenol B[a]	0.01	19	1.72	–	–	–	<LOD	16.2
Pesticides/metabolites								
PNP (parathion)	0.17	97	3.3	<LOD	1.3	2.8	4	16.9
TCPy (chlorpirifos)	0.01	63	0.83	–	<LOD	0.04	0.16	22.3
3-PBA (pyrethroids)	0.03	94	0.6	<LOD	0.13	0.27	0.64	7.5
IMPy (diazinon)	0.02	71	0.2	–	<LOD	0.12	0.16	1.35
3Me4NP (fenitrothion)	0.01	90	0.33	<LOD	0.07	0.18	0.29	1.38
Diflufenican[a]	0.004	61	0.19	<LOD	<LOD	0.014	0.03	4.9
Fipronil[a]	0.01	78	12.2	<LOD	0.007	0.05	0.18	175
Fipronil sulfone (fipronil)	0.04	100	3.2	0.04	0.14	0.25	0.63	76

[a] The biomarker is the parent.

bicarbonate buffer 100 mM at pH 10.5 and 50 μL of internal standard at 10 mg/mL in acetone. After reaction, 100 μL of water was added and a volume of 1 μL was injected for analysis of bisphenols by LC-MS/MS.

Another aliquot of 30 μL was transferred in a 5 mL glass tube, evaporated to dryness and derivatized at 80 °C for 30 min with 100 μL of PFBBr/acetonitrile (1:3, v/v) and 1 mL of acetonitrile. After another evaporation to dryness, the extract was reconstituted with 20 μL of ethyl acetate and 2 μL was injected in GC–MS/MS to determine TCPy and 3-PBA.

The remaining extract was used to quantify all the other compounds by LC-MS/MS (volume injected: 10 μL).

2.5.2. LC-MS/MS and GC–MS/MS parameters

After sample preparation, extract was split into 3 sub-extracts: two were analyzed by LC-MS/MS, one injected after SPE and the other one was submitted to derivatization with IS, and the 3rd one was analyzed by GC–MS/MS.

2.5.2.1. LC-MS/MS. For all types of analysis, the mobiles phases used for separation were: water +0.1% of formic acid (mobile phase A) and acetonitrile (mobile phase D). The column temperature and sample compartment were maintained at a temperature of 40 °C and 10 °C respectively. The flow rate applied for all methods was set at 0.35 mL/min.

The solvent gradient applied for the determination of compounds present in the first extract was the following: initial composition at 98% of A, linear increase of D to 65% until 3.7 min, then linear increase to 95% of D from 3.7 to 8 min, composition hold during 2 min and back to initial composition for next injection during 4 min.

The gradient used for bisphenols determination in the IS derivatized extract was defined as follows: initial composition at 98% of A, linear increase of D to 40% until 1 min, then linear increase to 95% of D from 1 to 6 min, composition hold during 2 min and back to initial composition for next injection during 4 min.

The tune parameters set for MS/MS acquisition were the same for all types of injection: capillary voltage at 2 kV, source and desolvation temperatures at 150 °C and 650 °C respectively, cone gas flow at 150 L/h, desolvation gas flow at 1200 L/h, collision gas flow at 0.15 mL/min and nebulizer set at 6 bar.

The MRM transitions scanned for detection of compounds of interest are detailed in the Table S2. The validation data obtained for hair analysis are presented in the Table S3 (Supplemental Data).

2.5.2.2. GC–MS/MS. The parameters used for GC–MS/MS analysis of the third sub-extract in which pesticide metabolites were extracted were already detailed in a previously published study (Hardy et al., 2015b). In brief, 2 μL of the third sub-extract reconstituted in ethyl acetate were injected into the GC system with the pulsed splitless injection mode at a pulse pressure of 35 psi. The injector, transfer line and MS source temperatures were set at 260 °C, 250 °C and 150 °C respectively. Helium was used as carrier gas at a flow of 1.2 mL/min. Inside the MS/MS system, helium, nitrogen and methane were used for quench gas, collision gas and CI reagent gas respectively. The temperature program of the GC oven was defined as follows: set at 60 °C for 1 min, increase by 20 °C/min to reach 180 °C, held for 1 min, rise at 4 °C/min to 240 °C then at 60 °C/min to 300 °C, final temperature maintained during 11 min plus 4 min more with backflush.

The MS/MS parameters used for detection are detailed in the Supplemental Data S4. The results from the method validation for hair determination are detailed in the Table S3 (Supplemental Data).

2.6. Data analysis

2.6.1. Intraclass coefficient and variability

Intraclass correlation coefficient (ICC) was first used by Shrout and Fleiss (1979). Briefly, ICC represents the portion of the total variability explained by difference between individuals, assuming that only between-individuals variability (BV) and within-individuals variability (WV) composed the total variance. BV and WV were here calculated using "Excel". Formulas used for ICC calculation are detailed in Faÿs et al. (2020). All the samples (for both urine and hair) collected over the 6 months-follow up were used for the calculation of the variability and ICC.

To assess the dispersion around the mean, the coefficient of variation (CV) was computed for each subjects and each biomarker in hair and urine (Fig. 1). CV was calculated by dividing the standard deviation of a population by the mean, and then by multiplying by 100 to obtain a percentage.

2.6.2. Correlation

A bootstrap-based algorithm was developed to address the influence of the number of urinary samples on the correlation between hair and urine samples (Fig. 2). In order to limit the bias due to non-detects replacement, only the 14 biomarkers (7 phthalates, 2 bisphenols and 5 pesticides) with a detection rate above 60% in both matrices were considered in the algorithm. Data were log-transformed before the launch of the algorithm and for each biomarker, non-detects values were replaced by the LOD divided by 2.

For each biomarker, the "hair mean" (HM) was calculated for each volunteer using all available hair samples collected over 6 months, used as reference. Next, n urine samples per volunteer were randomly selected to calculate "urinary means" (UM). The correlation between UM and HM for the 16 volunteers was assessed using Spearman coefficient. The process was iterated 10,000 times per n, with n varying from 2 to 43 to limit the bias due to sample selection. The average Spearman coefficients over the 10,000 iterations were presented in Fig. 3.

The correlation between biomarker concentration in hair and in urine was also tested by taking each hair sample of 1 cm separately instead of calculating a mean concentration for all the samples (per subject). The value of urinary biomarker plotted against each hair sample was the mean amount of biomarker excreted in the urine samples collected over the period theoretically corresponding to hair growth, considering 1 cm per month: biomarker concentration in 1 cm hair segment (corresponding to 1 month) versus average concentration of urinary biomarker in all the urine samples collected over the same month.

3. Results

3.1. Detection rates and concentrations of biomarkers in hair

Eleven biomarkers (6 phthalates, 1 bisphenol and 4 pesticides) were detected in more than 80% of the hair samples, including 9 in 90% or more (Fig. 4, Table 1).

Concerning phthalates, the highest detection rate was observed for MEHP with 98%. Six metabolites were detected in ≥87% of the samples, 3 metabolites were detected in 48 to 68% of the samples, and 7 were detected in less than 9%. Three biomarkers (MINP, 2-cx-MMHP, cx-MINCH) were never detected in this work. The highest median concentrations were observed for MEHP (12.1 pg/mg) and MEP (7.9 pg/mg), and the maximal value was observed for MEP 18120 pg/mg.

For bisphenols, detection rates were 98%, 76%, 52% and 19% for bisphenol S, A, F and B respectively. The highest median value for bisphenols was observed for bisphenol A with 6.4 pg/mg, and the lowest for bisphenol F with 0.05 pg/mg. Since hair samples were extracted under highly acidic conditions, conjugated bisphenols (if present in hair) were hydrolyzed. The values presented here therefore correspond to total bisphenols.

For pesticides, only fipronil sulfone (metabolite of fipronil), was detected in all the samples. For the other ones, detection rate ranged from 61% (diflufenican) to 97% (PNP). Median concentration ranged from 0.04 pg/mg (TCP-y) to 2.8 pg/mg (PNP), with PNP ten times higher than the second highest values (0.27, 3-PBA).

3.2. Detection rates and concentrations of biomarkers in urine

Detection rates and concentrations of the urinary biomarkers analyzed in this study were fully detailed in a previous article (Faÿs et al., 2020) and are presented here in supplemental data (Tables S5 and S6). Briefly, the detection rates observed in the 805 urine samples ranged from 28% (bisphenol B) to 100% (MEP, MBzP, 5-oxo-MEHP, 5-OH-MEHP, 5-cx-MEPP and TCPy).

Twelve out of the 16 phthalate metabolites were detected in >90%. The amount of biomarker excreted per urination was highly variable, as shown with the example of MEHP which ranged from 1.5 to 139,499 ng. The highest median value was observed for MEP (11,028 ng), and the lowest for OH-MPHP (<0.8 ng).

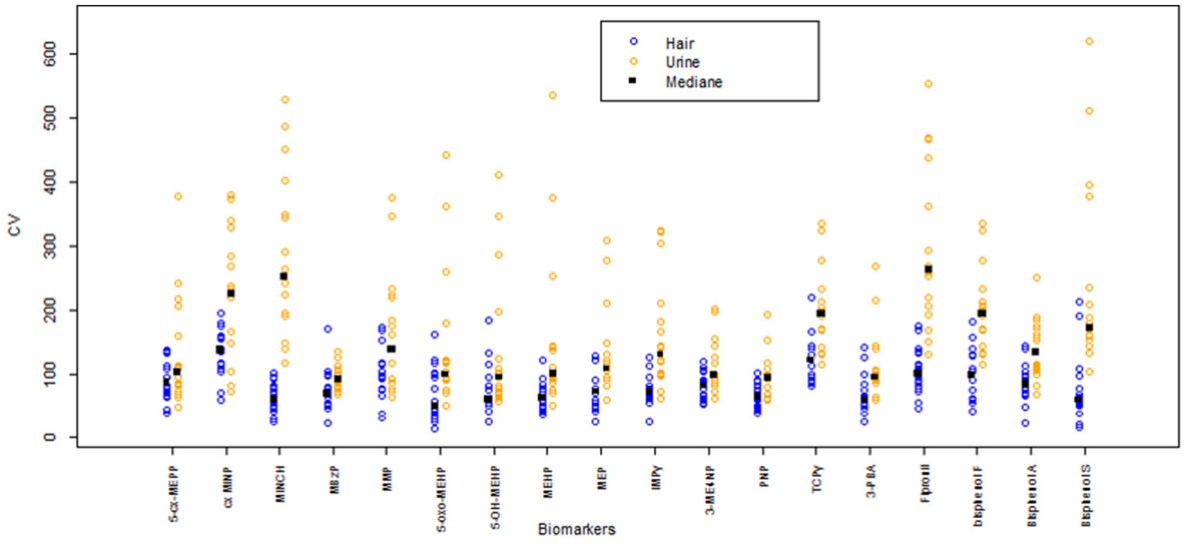

Fig. 1. Coefficients of variation calculated for the biomarkers detected in >45% in hair and in urine. Each circle represents a CV value for one subject.

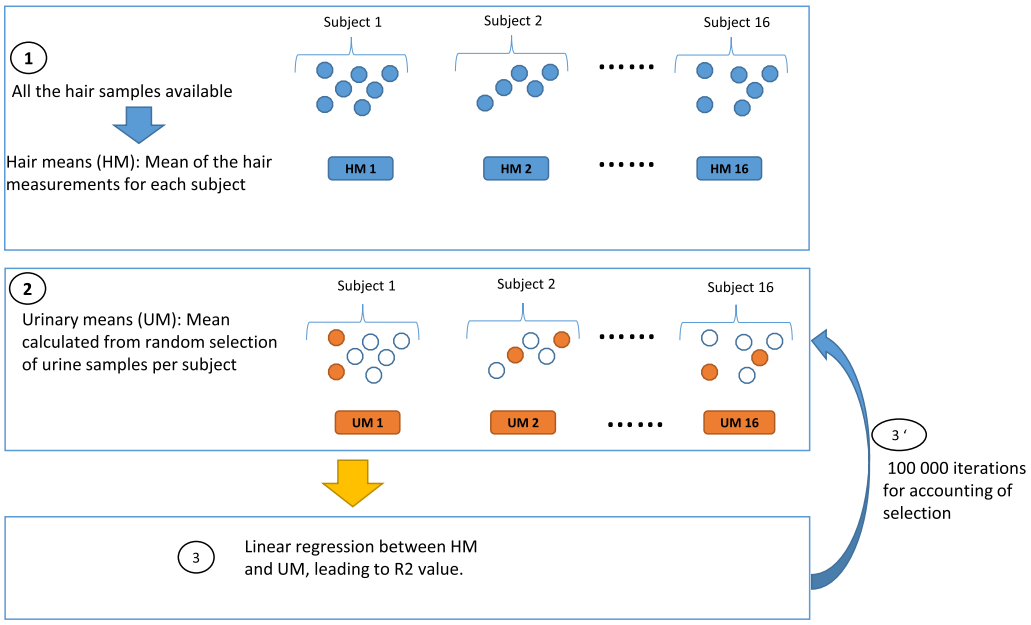

Fig. 2. Representation of the algorithm determining the correlation between hair and urine samples depending on the number of urine samples.

All the bisphenols were detected in >87% of the samples with the exception of bisphenol B (28%). The highest amount excreted in one urination was observed for bisphenol S (145,054 ng) and the highest median amount excreted in urine was observed for bisphenol A (360.5 ng). Since urine was hydrolyzed before analysis, these values correspond to total bisphenols.

For pesticides, the detection rates ranged from 21% (diflufenican) to 99% (PNP). The lowest median value was observed for fipronil and its metabolite fipronil sulfone (<0.1 ng) and the highest was observed for DEP (1611.3 ng).

3.3. Biomarkers variability in hair and in urine

In hair, ICC values ranged from 0.1 (IMPy) to 0.8 (MEP) and ten biomarkers presented ICC values above 0.4, suggesting fair reliability according to Rosner (2015) (Fig. 5). For the phthalate metabolites, the highest ICC were observed for MEP with 0.8 in hair and 0.51 in urine, and the lowest for 5-cx-MEPP in hair (0.26) and for MINCH in urine (0.11). For pesticides, the lowest ICC values were 0.1 (IMPy) for hair and 0.16 (PNP) for urine, whereas the highest were calculated for Fipronil in hair (0.75) and in urine (0.35). For bisphenols, bisphenol F

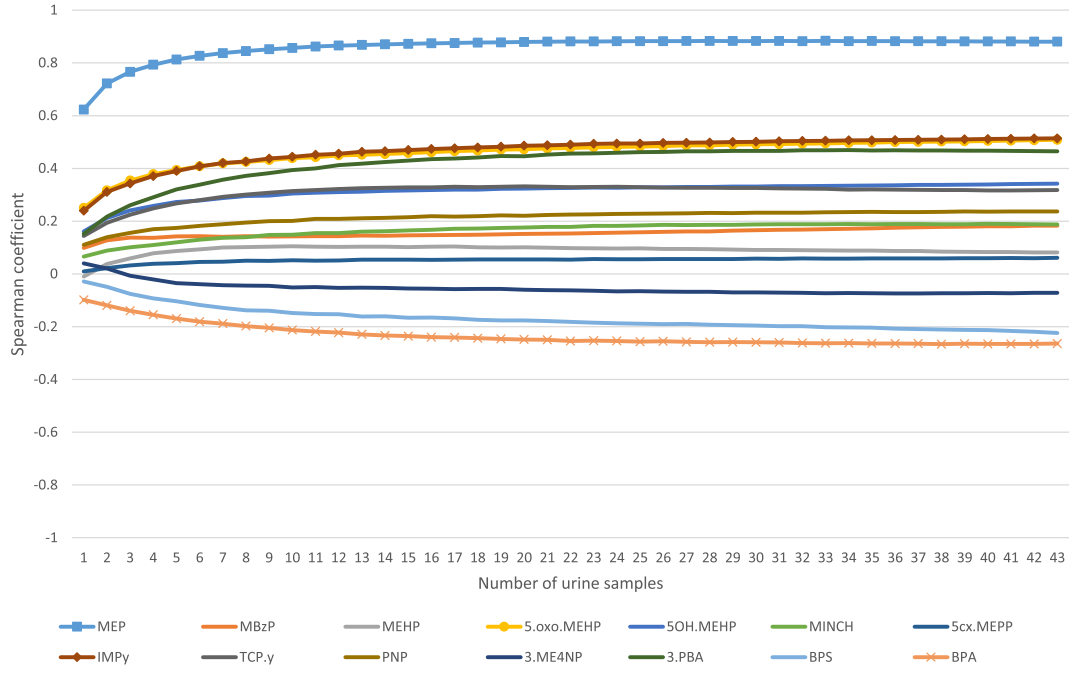

Fig. 3. Spearman correlation coefficient between the concentration of biomarkers in hair and in urine, depending on the number of urine samples considered in the calculation of the average concentration.

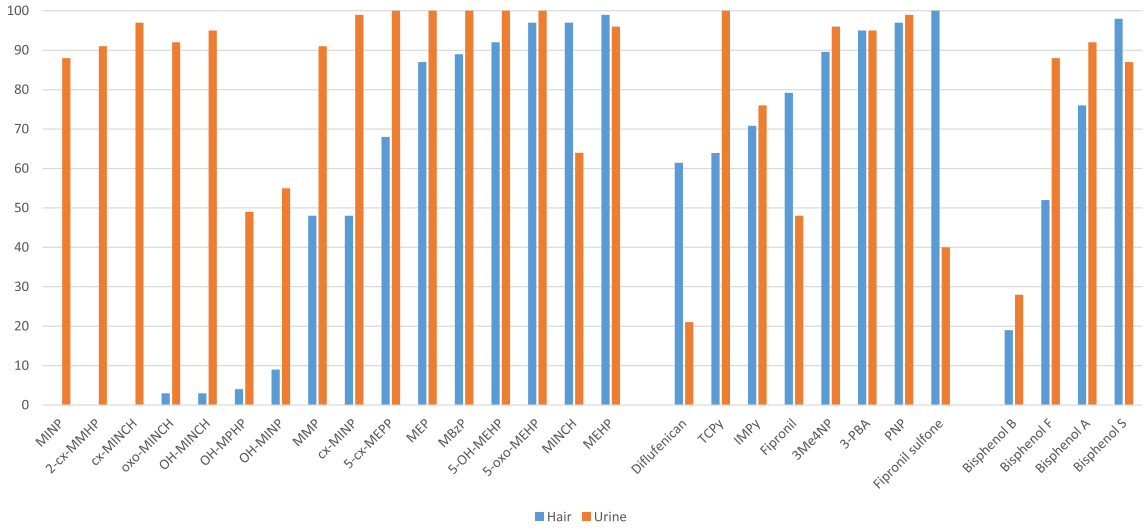

Fig. 4. Detection rate of the 29 biomarkers in urine and hair samples collected over 6 month follow-up.

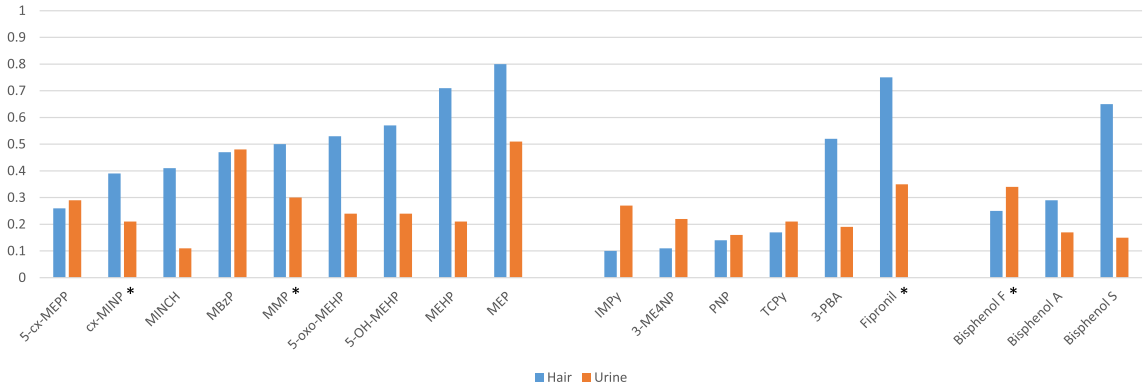

Fig. 5. Intraclass correlation coefficients (ICC) for biomarkers detected in >45% of the samples in hair and in urine. (*) indicates biomarkers detected in less than 60% of the samples.

presented the lowest ICC value in hair (0.25) and the highest in urine (0.34), while bisphenol S had the highest ICC value in hair (0.65) and the lowest in urine (0.15).

The coefficient of variation (CV) observed for one biomarker was different depending on the subject, but this difference was much more marked in urine than in hair (Fig. 1). For instance, the CV calculated for MINCH for the different subjects ranged from 23% to 102% in hair and from 117% to 530% in urine. For the phthalate metabolites, the highest CV was observed for cx-MINP (196%) in hair and for MEHP (535%) in urine, and the lowest value was 13% in hair (5-oxo-MEHP) and 49% in urine (5-cx-MEPP). For pesticides, TCPy presented the highest CV in hair (220%) and fipronil presented the highest CV in urine (535%), whereas the lowest values were obtained for IMPy (23%) in hair and PNP (62%) in urine. For the bisphenols, bisphenol S presented the highest CV in both hair (214%) and urine (621%), whereas the smallest CV was observed for bisphenol S in hair (16%) and for bisphenol A in urine (69%).

3.4. Correlation between hair and urine

When only one urine sample was considered, only MEP presented a significant correlation between its concentration in hair and in urine ($R_{Spearman} = 0.62$, p value $= 0.009$) (Fig. 3). The correlation increased to $R_{Spearman} = 0.88$ when MEP concentration in urine was based on the average of 43 samples.

For the other biomarkers, the correlation between the concentration in hair and in urine ranged from $R_{Spearman} = -0.098$ (bisphenol A) to 0.250 when only 1 urine sample was considered, and from −0.26 (for bisphenol A) to 0.51 (IMPy) when the 43 urine samples were used to calculate the average concentration. Nevertheless, this correlation was only significant (p value <0.05) for 2 biomarkers: 5-oxo-MEHP (for 36 samples and more) and IMPy (for 29 samples and more). For all the other biomarkers, the correlation between concentration in hair and in urine was never significant whatever the number of urine samples considered to calculate the average concentration.

Considering each hair sample separately (against the urine samples collected over the corresponding period) instead of a mean value per subject did not improve the correlation between biomarkers concentration in hair vs urine (Fig. 6). The value of the R^2 ranged from <0.001 (BPS) to 0.53 (MEP) and the association was only significant for MEP. Removing the non-detects from the correlation analysis did not change the significance.

4. Discussion

The few studies investigating DEHP, DINCH and DPHP metabolites in hair were conducted on a limited number of subjects (Chang et al., 2013; Yin et al., 2019). Chang et al. (2013) detected MEHP, 5-oxo-MEHP and 5-OH-MEHP in all of the 10 hair samples they tested, which is in line with the high detection rates observed in the present

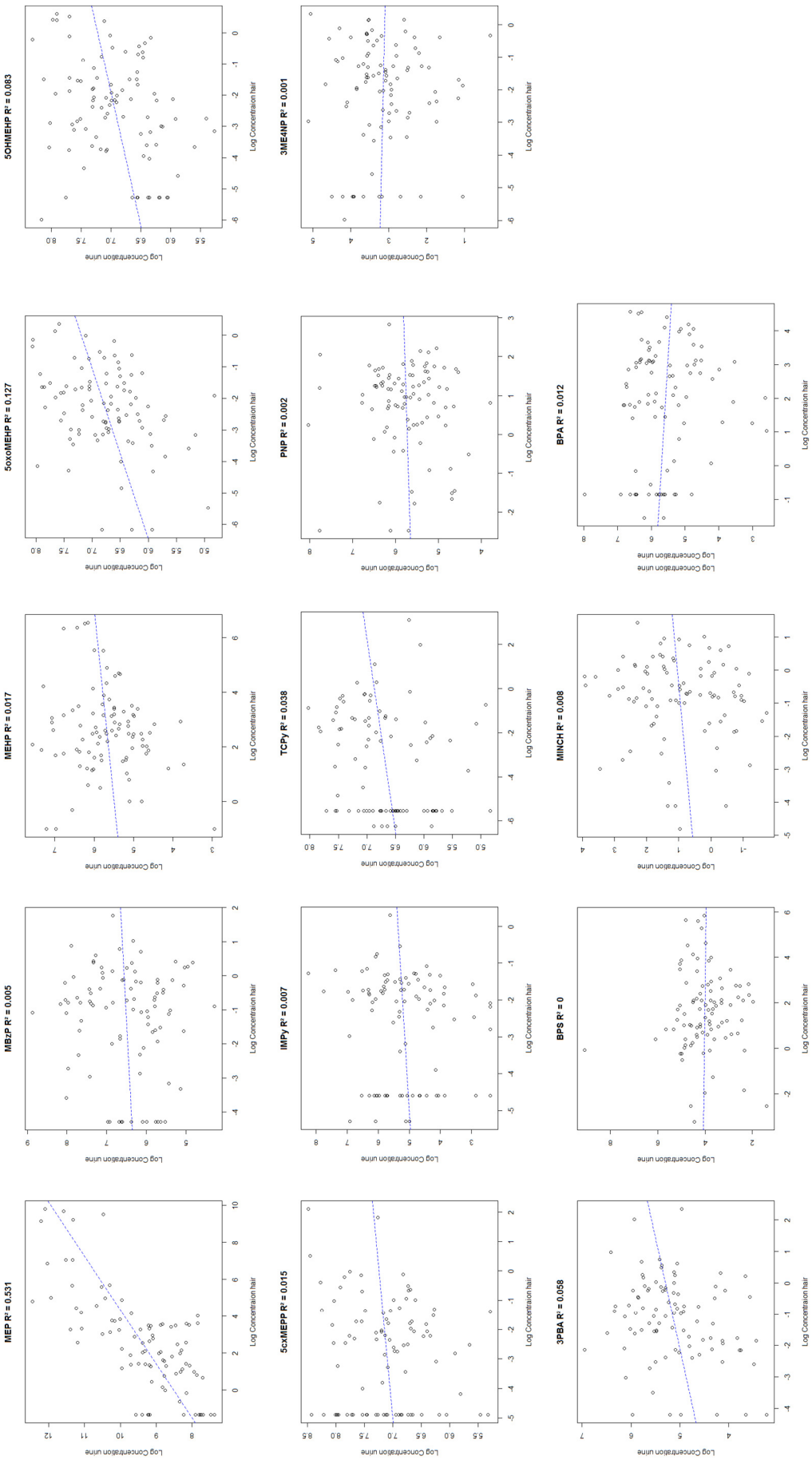

Fig. 6. Association between biomarker concentration in hair versus urine considering each hair sample separately ($n = 92$). Biomarker concentration in each 1 cm-hair segment (corresponding to 1 month) is plotted against the average amount of urinary biomarker excreted over the corresponding period.

study (98%, 97%, and 90% respectively). As in the present work, the detection rate of 5-cx-MEPP was also lower than the other metabolites of DEHP, and 2-cx-MMHP was never detected. However, the concentrations measured by Chang et al. were slightly higher than the ones observed here, suggesting a difference in DEHP exposure between the two groups tested. Concerning DPHP metabolites measured by Yin et al. (2019), the detection rate of OH-MPHP was slightly higher than in the present study (11% vs. 3%), and oxo-MPHP was never detected in their study while we observed this metabolite in 51% of the samples. The latter differences might be explained by differences in analytical sensitivity (0.5 pg/mg vs 0.004 in the present study). Among DINCH metabolites, even if the detection rate of MINCH was lower in the study of Yin et al. (2019) compared to the present work (78% vs. 97% here), the median concentration of MINCH (6 times higher than here) and the higher detection rate observed for OH-MINCH suggests a different level of exposure between the two groups. In the two studies, cx-MINCH was never detected, suggesting that this metabolite is minor in hair and would thus be less relevant.

To the best of our knowledge, bisphenol A (BPA) is the only bisphenol previously analyzed in hair in the literature. Two studies conducted on Spanish and Polish general populations (Martin et al., 2019; Nehring et al., 2017) found median concentrations in hair much higher than in the present study (195.1 pg/mg in Spain, 337.5 pg/mg in Poland, 6.4 pg/mg in the present work). However, two other works investigating BPA in hair from Greek adults (Karzi et al., 2018; Tzatzarakis et al., 2015) were more in line with our results since the median values presented ranged from <2.9 pg/mg to 13.8 pg/mg. Moreover, the geometric means reported in the latter publications were in agreement with the mean concentration observed here (Greece: 16.6–18.4 pg/mg vs. this study: 22.5 pg/mg). These findings could reflect differences in BPA exposure between the groups, although differences in methodology (decontamination procedures, extraction solvents, determination of free or total BPA for instance) cannot be excluded.

The detection rates of pesticide biomarkers in hair analyzed in the present study (61% to 100%) were similar to rates reported in previous studies conducted on the French population (40–100%) (Beranger et al., 2018b; Iglesias-Gonzalez et al., 2020). The concentration levels observed here were however in the lower range compared to these previous studies. For instance, the concentrations of organophosphate metabolites reported by Beranger et al. (2018b), and Iglesias-Gonzalez et al. (2020) were 3 to 42 times higher than values observed in the present work. Similarly, 3-PBA, a pyrethroid metabolite, presented a median concentration of 0.27 pg/mg compared to 1.69–2.36 pg/mg in the previous studies. In like manner, the levels of diflufenican and fipronil were also lower than in the other studies on French women and children (e.g. fipronil median concentration value at 0.05 pg/mg while reaching 0.3 pg/mg to 1.62 pg/mg in the literature). These results suggest rather low exposure of the individuals studied in the present work compared to the previous studies, confirming that no specific exposure leading to extremely high exposure levels (such as occupation) concerned this population.

The detection rates and concentration levels of the biomarkers in the urine samples collected from the 16 subjects involved in the study were fully detailed in a previous article (Faÿs et al., 2020).

As observed on Fig. 4, eight out the 28 biomarkers presented less than 10% difference in the detection rate between hair and urine. For the remaining ones, 15 were more frequently detected in urine and 5 were more present in hair (MINCH, diflufenican, fipronil, fipronil sulfone and bisphenol S).

The few studies comparing the detection rates between urine and hair samples collected from the same individuals reported higher detection frequency in hair (Wang et al., 2018; Kim et al., 2019; Hernandez et al., 2019; Kokkinaki et al., 2014; Hardy et al., 2021). Similarly, a study conducted on rats under controlled exposure to a mixture of pesticides from different chemical classes also reported that the number of compounds detected in hair was higher than in urine (Appenzeller et al., 2017).

The difference in biomarker detection between the two matrices can first be explained by the different time window covered by each of them. On the one hand, most chemicals are transferred in urine within a few hours after exposure, and their concentration may become undetectable rapidly after exposure stops. Urine is therefore representative of the few hours before sampling only, and the time of sample collection has therefore a direct impact on the chemicals present in this matrix. On the other hand, hair may contain the chemicals that have been present in the body (even for a short time) over a period of weeks to months, depending of the length of the hair strand. This wide window of detection highly increases the chances to cover the time of exposure and to detect the corresponding biomarkers, explaining the higher detection rates observed for some biomarkers in hair compared to urine. In the present work, the high detection rates observed for most biomarkers in urine (e.g. most phthalate metabolites, TCPy, 3Me4NP, 3-PBA, PNP, and most bisphenols) despite their fast elimination, strongly suggest a frequent exposure of the subjects to these chemicals. In counterpart, the relatively low detection rates observed for other biomarkers such as diflufenican and bisphenol B would suggest less frequent exposure.

Physicochemical properties may also influence the transfer of chemicals into urine and hair. As previously demonstrated, chemicals are mainly incorporated into hair from blood (Chata et al., 2016). The very low detection rate of some biomarkers (e.g. MINP, 2cx-MMHP, cx-MINCH, oxo-MINCH) in hair, despite their frequent presence in urine, might be explained by a very fast transfer of hydrophilic chemicals from blood to urine after their formation. This rapid transfer would lead to insufficient residence time and concentration of the biomarkers in blood to allow their incorporation in hair at detectable levels. This hypothesis is in line with pharmacokinetic data reported for DiNP metabolites in rat plasma. After oral administration (750 mg/kg/day), the concentration of MiNP in plasma was immediately decreasing, whereas the maximal concentration of the oxidized metabolites cx-MiNP and OH-MiNP was only reached after 2 h (Clewell et al., 2013), allowing better incorporation from blood into hair. Moreover, the concentration of cx-MiNP in plasma was much higher than the one of OH-MiNP, which is in line with the rates of detection respectively observed for these biomarkers in hair in the present work. Similarly, although MEHP is not the major metabolite of DEHP in urine compared to its oxidized metabolites (Koch and Angerer, 2007), MEHP however presents the highest concentration in plasma following exposure (Lorber et al., 2010), which would explain its higher frequency of detection in hair.

In parallel, differences in biomarker detection rates between hair and urine due to differences in the analytical methods between the two matrices cannot be excluded.

On top of demonstrating exposure, one of the main goals of biomonitoring consists in classifying individuals according to their level of exposure to pollutants. Classification of the individuals is then used to investigate possible association with health outcome or to identify determinants of exposure. Level of exposure (or internal dose) is extrapolated from biomarkers concentration in the biological samples analyzed. Consequently, variability in biomarker concentration in samples repeatedly collected from an individual is considered a key indication of the reliability of exposure assessment: high concentration variability is associated with poor reliability of the classification (Faÿs et al., 2020). For this purpose, intraclass correlation coefficient (ICC) is considered a good indicator of biomarker concentration variability and robustness of the classification of the subjects. A biomarker presenting ICC value ≥0.75 is interpreted as allowing classification with excellent reliability, 0.4 ≤ ICC ≤ 0.75 allows fair to good reliability, whereas biomarkers with ICC values <0.4 suggest that classification of the individuals based on biomarker concentration is poorly reliable (Rosner, 2015). Therefore, ICC values calculated for the same biomarker in hair and in urine respectively represent an interesting approach to compare the information obtained from each matrix. In the present study, most biomarkers presented much lower ICC value in urine than in hair (Fig. 5).

Actually, ICC values in urine were all below 0.4 except for MEP and MBzP, indicating higher intra-individual than inter-individual variability and therefore unreliable classification (Faÿs et al., 2020). In counterpart, eight biomarkers presented ICC values in hair between 0.5 and 0.8, indicating robust discrimination of the individuals according to biomarker concentration in hair. An illustration of the influence of biomarkers concentration variability on individual classification is presented in Fig. 7, with the example of MEHP, which presented ICC values of 0.21 in urine and 0.71 in hair respectively. In urine, the high variability in MEHP concentration results in different classification of the subjects depending on the time point considered. On the contrary, the classification of the subjects based on MEHP in hair remains unchanged whatever the time point.

In order to investigate the link between the information obtained from hair and from urine respectively, the correlation between biomarker concentrations in the two matrices was calculated. As observed in the few studies comparing paired hair and urine samples, poor correlation was expected when considering one sample of each matrix per individual because of the different time window covered by hair (months) and urine (hours) respectively (Hernandez et al., 2019; Kokkinaki et al., 2014; Hardy et al., 2021). Nevertheless, it is generally considered that increasing the number of urine samples collected from an individual enables to calculate a mean concentration more representative of the average/chronic level of exposure (Faÿs et al., 2020; Kissel et al., 2005; Xiao et al., 2014; Wielgomas, 2013). An algorithm was therefore developed to determine to what extent increasing the number of urine samples would improve the correlation between urine and hair. As expected, the concentration of biomarkers in hair was poorly correlated with the corresponding concentration in urine when only one urine sample was considered (Fig. 3). Surprisingly, increasing the number of urine samples to get an averaged value of the amount of biomarkers excreted per urination only had a very limited influence on the correlation between the two matrices. Actually, only MEP concentration presented significant correlation between urine and hair whatever the number of urine samples considered. Considering a scale of 1 month (each 1 cm hair-segment against the urine samples collected over the corresponding period) instead of the total follow-up (6 months) did not improve the association between biomarkers in hair and in urine (Fig. 6).

The lack of correlation between urine and hair can probably be partially explained by the high variability of biomarkers in urine. For nine biomarkers, the intra-individual variability in urine was above 100% in more than half of the subjects (Fig. 1). For 12 biomarkers, many subjects even reached variability values between 200 and 621%. In counterpart, the variability of biomarkers concentration in hair was much lower. The median intra-individual variability was below 80% for 11 biomarkers, and only 3 subjects presented variability above 200% for one of the biomarkers (2 for TCPy and 1 for bisphenol S respectively). As a result, the value used for a biomarker concentration in urine cannot be considered representative of the "real" average concentration, contrary to the concentration of the same biomarker in hair, and this lack of representativeness of urinary biomarkers concentration is likely to negatively impact the correlation between urine and hair.

In parallel, contrary to hair that covers the entirety of the follow-up period with no "missing window", each urination only represents a small part of the total volume excreted over the 6 months. Considering an average number of 8 urination per day for normal individuals (Society, I.C, 2015), the number of 43 to 60 urine samples collected during this study only represents 3–4% of the total amount of urine excreted over the 6 months-follow-up. The missing 97% of the information concerning urine, compared to the 100% available for hair can also explain the lack of correlation between the two matrices.

Finally, the present study suggests that urine and hair provide different (and possibly complementary) information. With the present study design, the results showed that information obtained from urine is limited to the short-term but fails to be representative of the chronic exposure even with a high number of samples. As observed with the example of MEHP on 6 of the participants (Fig. 8), the concentration in hair provides much more stable information over time than the corresponding biomarker in urine. Urine can however possibly capture exposure peaks, provided that samples are collected at the right time. As observed here, several subjects presented some peaks in urinary MEHP, reaching values several orders of magnitude higher than the "normal" fluctuation (Fig. 8). Such peaks suggest higher exposure episodes that could induce different effect than more stable exposure with equivalent total dose.

5. Limitations of the study

The limited number of subjects ($n = 16$) involved in the present study does not allow to investigate the possible influence of parameters such as age or demographic data on biomarker variability. Nevertheless, the workload and effort requested from the participant associated with repeated sample collection represented an important limitation, since about a thousand biological samples were collected over this six-month and required one year for their analysis with up-to-date methods. It thus does not seem realistic to conduct similar study on larger cohorts of hundreds or thousands of subjects, with large age distribution and better representativeness of general population.

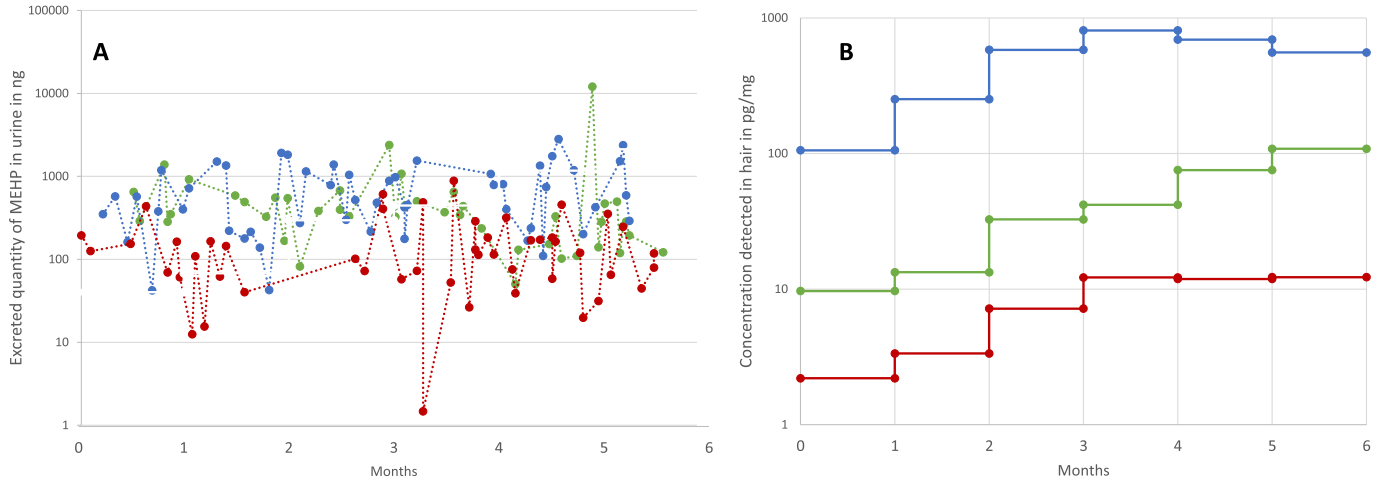

Fig. 7. Concentration of MEHP in urine (A) and hair (B) samples of three subjects over the 6 months follow-up.

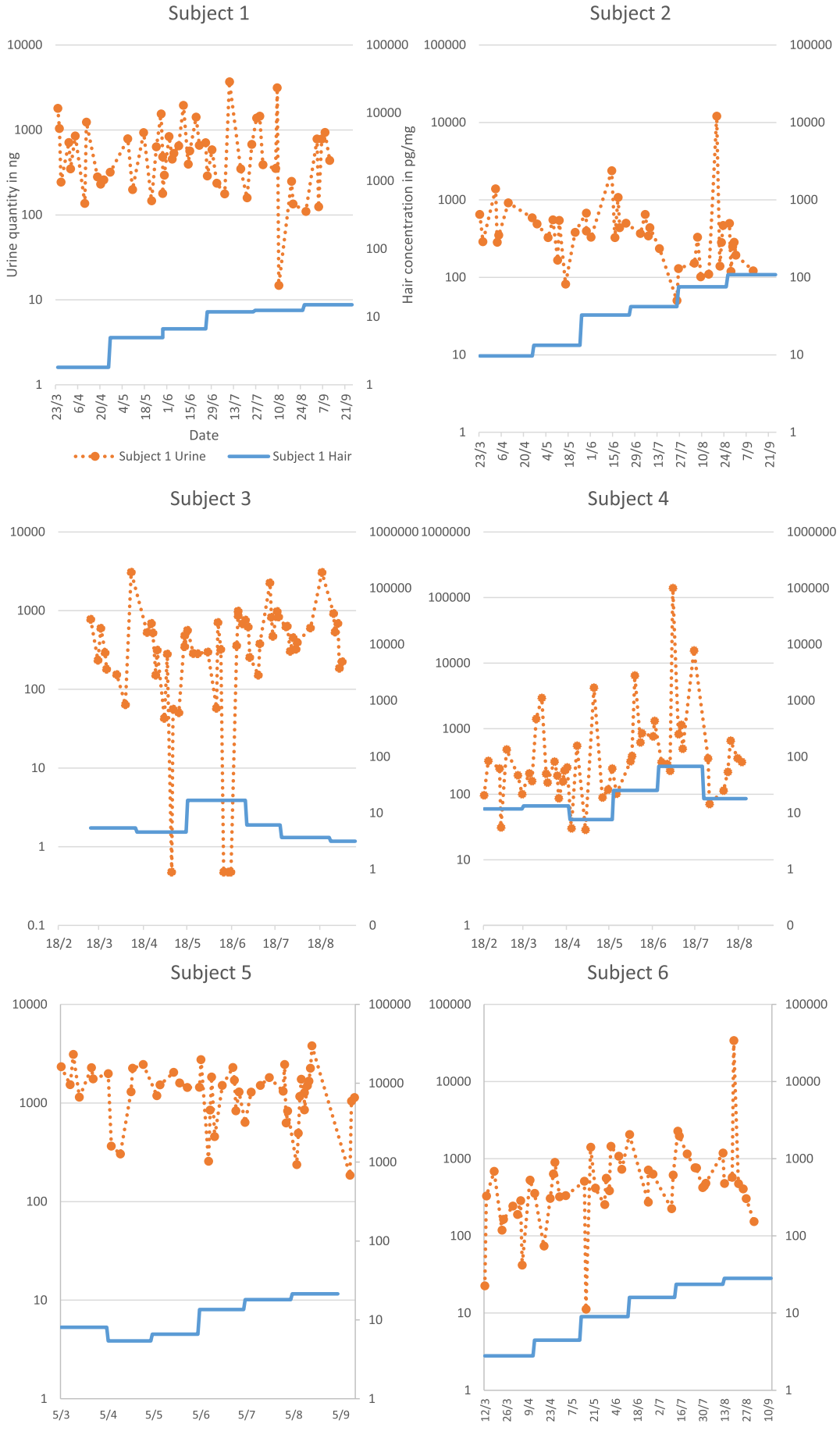

Fig. 8. MEHP in urine (dotted line) and in hair (plain line) samples collected over the 6 months for 6 of the participants.

The results and conclusions presented here are therefore valid for the population under study, which was however well balanced regarding gender and age, and did not present extreme values of biomarker detection frequency and concentration that would suggest considering it as an unusual group regarding exposure.

It also has to be acknowledged that to better understand which information can be obtain from different matrices respectively regarding exposure assessment, the combination of several different studies, with different design, conducted on different populations and targeting different biomarkers will be necessary. The present work therefore only represents a contribution to the total information that will be necessary to improve future biomonitoring strategies for the assessment of human exposure to pollutants, and particularly fast-elimination ED.

Finally, since the present study was not focused on the determinants of exposure, no analysis was conducted on environmental matrices or food.

6. Conclusion

The present study is the first one analyzing fast elimination endocrine disruptors from different chemical families in hair and urine samples collected from the same individuals over a follow-up. Moreover, we provide here the first values of concentration in hair for several phthalate metabolites and bisphenols.

Although most biomarkers were more frequently detected in urine than in hair, the variability of their concentration was much lower in hair. The lack of correlation in biomarker concentration between hair and urine illustrates the complexity of biomonitoring and suggests considering with caution the specificity of the matrix in data interpretation. Hair provides a more stable information than urine, allowing a more robust classification of the individuals according to their chronic level of exposure. In parallel, urine can capture peaks in the exposure, provided that samples are collected at the right time.

Highlighting the respective advantages and limitations of urine and hair, the present study provides new insight in the understanding of the information provided by these biological matrices and in their relevance for the assessment of human exposure to fast elimination contaminants.

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CRediT authorship contribution statement

François Fäys: Conceptualization, Methodology, Software, Investigation, Writing – original draft, Formal analysis. **Emilie M. Hardy:** Investigation, Writing – review & editing, Methodology. **Paul Palazzi:** Investigation, Writing – review & editing. **Serge Haan:** Writing – review & editing. **Claire Beausoleil:** Writing – review & editing, Project administration. **Brice M.R. Appenzeller:** Supervision, Writing – original draft, Conceptualization, Funding acquisition, Project administration.

Declaration of competing interest

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

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