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1 Are nails a valuable non-invasive alternative for
2 estimating human exposure to phthalate esters ?

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31 **Abstract**

32 Most human biomonitoring studies conducted in the past year for assessing the human
33 exposure to phthalate esters (PEs) employed measurements of PE metabolites in urine.
34 Although urine is recognized as a valuable non-invasive matrix, it has also limitations
35 regarding the short time window for exposure.

36 Therefore, in this pilot study we aimed to assess the human exposure to seven PE
37 metabolites (including mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-oxohexyl)
38 phthalate (5-oxo-MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP), mono-
39 *n*-butyl phthalate (MnBP), mono-isobutyl phthalate (MiBP), monoethyl phthalate (MEP)
40 and mono-benzyl phthalate (MBzP)) using human nails. Paired nails and urine samples from
41 the same individuals were used for comparison. Median levels of specific PE metabolites
42 measured in nails and in spot urine of twenty Belgian individuals ranged from <LOQ_m
43 146 ng/g and from 0.2 to 6.7 ng/mL (creatinine adjusted), respectively. The major PE
44 metabolites found in nails were MEHP (average 146 ng/g), sum (MnBP, MiBP) (average
45 212 ng/g) and MEP (average 205 ng/g).

46 Significant correlations were achieved between different metabolites in nails and urine, i.e.,
47 MEHP levels in nails correlate well with sum (MnBP, MiBP) ($r = 0.73$, $p < 0.01$) and with
48 MBzP ($r = 0.52$, $p < 0.05$) levels in urine. Moderate correlations were observed between 5-
49 OH-MEHP and sum (MnBP, MiBP) ($r = 0.62$, $p < 0.01$) as well as with MEP ($r = 0.56$, $p <$
50 0.05) in both matrices. However, no significant correlation was observed for the same
51 metabolite measured in both matrices.

52 Based on participant questionnaires and after performing multivariate statistics, the relevant
53 parameters of exposure positively associated with PE metabolites in nails were the use of
54 hand care products, weight of the individuals and sport activity hours. Based on the detected
55 levels and aforementioned predictors, nails seem a valuable non-invasive matrix for
56 estimating human long-term exposure to DEP, DBnP and/or DIBP and DEHP.

57 **Keywords:** Human Biomonitoring, nails, urine, phthalate metabolites, long-term exposure
58

1. Introduction

For more than 50 years, phthalate esters (PEs) have been part of our daily life. Due to their outstanding chemical properties, PEs have been successfully incorporated mainly as excipients, emollients or plasticizers in diverse products, such as medical devices, clothing, car upholstery, food packaging, furniture, varnishes, or personal care products (e.g. hairsprays, shampoos, deodorants, nail polish)[1, 2]. As a result, their release to the environment or contact with humans is practically constant and unavoidable. The main known routes of human exposure are ingestion, dermal contact, inhalation or intravenous contact through medical devices [1, 3].

PEs can induce toxic effects (e.g. reproductive, developmental, and neurological, among other effects) on humans, rats and other animal species [4-7]. Additionally, recent restrictions were introduced for the toxic PEs (reproduction categories 1 and 2) such as DBP, BBzP and DEHP used in toys or personal care products [8, 9] and for DEHP or DBP in food contact materials (e.g. seals, caps) [1, 10]. Still, no restrictions were yet foreseen for other PEs pointed out as toxic to humans (e.g. short chain PEs) [10].

As a result, human biomonitoring (HBM) has gained a lot of attention, especially to identify exposure and to perform risk assessment. Thus, the detection of PEs in humans is mainly demonstrated by their primary and/or secondary metabolites (hydrolytic or oxidative). For PEs, the first detoxification step consists in the conversion of diesters into monoesters that can be transformed into even more bioactive substances, i.e. more toxic to humans [10].

PE metabolites are considered as valid biomarkers of internal exposure due to its good representativeness in our body and strong correlation with the parent diester. Until now, urine has been the matrix of choice for measuring PE metabolites, due to all advantages associated to the ease of sampling, stability, storage, and fast screening of (short term) exposure [3, 7, 11-16]. Within 24 h after exposure, most of the metabolized PEs are excreted. For example, 67 % of the DEHP (diethyl hexyl phthalate) is eliminated after 24 h via its oxidative metabolites (e.g. mono-(2-ethyl-5-carboxypentyl) phthalate (5-cx-MEPP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP), and mono-(2-ethyl-5-oxohexyl) phthalate (5-oxo-MEHP). Still, a small percentage (3.8 % of DEHP) can be eliminated after 48 h [10, 16].

Nevertheless, there are also disadvantages associated to the use of urine, namely related to the creatinine content (e.g. highly dependent on the time collection) [17].

In this sense, is important to explore alternative matrices and more recently few studies have shown the potential of other non-invasive matrices (e.g. hair or saliva) [3, 18, 19] to assess the human exposure to PEs, integrating larger detection windows of exposure. Particularly, five major DEHP metabolites were determined in human hair by Chang et al. [20], considering that MEHP is a relevant metabolite of internal exposure (levels in hair ranged from 15 to 96 ng/g). Yet, in previous HBM studies using urine, MEHP was not considered an important biomarker of human internal exposure [16].

As far as we know, the use of other non-invasive samples, instead of urine, has not been widely explored in HBM for internal exposure assessment to PEs, therefore a lack of knowledge in this field still exists.

Thus, the aim of the present pilot study was to demonstrate the feasibility of nails as an alternative matrix for assessing the human exposure on PEs. Measurement of nails has a multitude of advantages: ease of sampling in different populations (adults, babies, elderly and/or sick people) and without restriction of collection at any time of the day; sampling of sufficiently low amounts (few mg per analysis); reproducibility of measurements over time and translation of a long exposure period (weeks to months). In the present study, the aim was to determine the levels of seven PE metabolites, including the mono-(2-ethylhexyl) phthalate, mono(2-ethyl-5-oxohexyl) phthalate, mono(2-ethyl-5-hydroxyhexyl) phthalate, mono-*n*-butyl phthalate, mono-isobutyl phthalate, monoethyl phthalate and mono-benzyl phthalate, in paired nails and urine samples collected from twenty Belgian individuals. A new approach for correcting the dilution effects of PE metabolites based on the individual and on the average of creatinine levels measured in all individuals is proposed.

Statistical analysis was performed for better understanding of the results in both matrices and in order to describe the relative importance of each matrix in human exposure to PEs. Participant questionnaires, univariate and multivariate statistical analysis was performed in order to discriminate (personal and environmental) variables that could explain the levels in nails.

2. Materials and Methods

2.1 Standards and chemicals

Seven PE metabolites, including MEHP (99.9 %), MiBP (97.8 %), MnBP (97.4 %), MBzP (99.8 %), MEP (100 %) (supplied by Accustandard Inc., Connecticut, USA) and two MEHP oxidative metabolites (5-oxo-MEHP (>95 %) and 5-OH-MEHP (>96.5 %) were

provided by BCP instruments (Irigny, France)) were investigated. Mass-labelled internal standard (IS) solutions for all metabolites ($^{13}\text{C}_4$ -MEHP, $^{13}\text{C}_4$ -5-oxo-MEHP, $^{13}\text{C}_4$ -5-OH-MEHP, d_4 -MiBP, $^{13}\text{C}_4$ -MnBP, $^{13}\text{C}_4$ -MBzP and $^{13}\text{C}_4$ -MEP) (95 %) were supplied by Cambridge Isotope Laboratories (Andover, USA) All solutions (stock, working and spike solutions) were prepared on a weight basis in acetonitrile or in ultra-pure water (i.e. only spike solutions).

The organic solvents, such as trichloroethylene, nitric acid (65 %) and the acetic acid (100 %) were obtained from Merck (Darmstadt, Germany). The organic solvents, such as acetone, acetonitrile (ACN) and methanol (MeOH) were of UPLC grade (Fisher Scientific, Loughborough, UK). Trifluoroacetic acid (TFA, 99 %) was supplied by Sigma-Aldrich (Steinheim, Germany). Ammonium acetate buffer (NH_4Ac) was prepared by dissolving 1.93 g of ammonium acetate (99.99 %, Sigma-Aldrich, Diegem, Belgium) in 100 mL ultra-pure water and acidifying the solution drop by drop with acetic acid until reach pH 6.5. The enzyme β -glucuronidase (E. coli K12) was supplied by Roche Applied Sciences (Mannheim, Germany). The creatinine urinary colorimetric kit was supplied by Sanbio (The Netherlands). The 96-well plate spectrophotometer for creatinine measurements was provided by Tecan Bio M200 PRO (Mechelen, Belgium). The ultra-pure water was provided by the Advantage A10 system (Millipore S.A, Overijse, Belgium).

2.2 Nails and urine collection

Finger nail and urine samples were collected among twenty volunteers at our institute (VITO). The sampling had a duration of three weeks during the second trimester of 2015. All participants were duly informed about the purpose of this study giving their consent to participate (Ethical approval register N.° B300201316329).

On the last day of the sampling, the participants filled a short survey recording their personal and lifestyle information during the past three weeks. Information included the gender, body weight, use of nail polish and hand creams, time spent in transports/car and at home, frequency of sport exercise, habits of eating with hands (e.g. snacks, chips, fruit), eating pre-packed food, heating/storing food in plastic containers (frequency), habits of ventilating the house. At that time, the volunteers also collected a first morning urine sample. In the end, in total twenty nails and sixteen urine samples were collected by the participants.

Before the analysis, all glassware was washed and heated in the oven at 450°C (overnight) to remove possible organic contamination. Afterwards, the materials were stored and wrapped in aluminium foil. The use (and contact) of plastic materials was minimized (septa and caps of vials were covered with aluminum foil prior to use to avoid dust contamination). The polypropylene bottles used for urine collection were prior rinsed with HNO₃ solution (10 %, v/v) followed by methanol.

Before the extraction, the nails were rinsed twice with acetone in order to remove residues of nail polish. Then the samples were grinded and homogenized in a mixer mill (30 min, 20 Hz).

2.3 PE metabolites extraction from nails and urine

Thirty mg of powdered nails were weighed into glass vials and spiked with IS (typically 5 ng). Then, the PE metabolites were extracted according to a new microextraction method (US-DLLME) [21]. Briefly, 2 mL of TFA:MeOH (2M) was added to the vials and the analytes were extracted using ultrasound for 2h at 45°C. The extract mixture containing the target compounds (dispersion solvent) and 180 µL of trichloroethylene (extraction solvent) were dispersed in 1 mL of water placed in a conical vial. For separation of the organic phase (µL), the samples were centrifuged (6 min at 3500 rpm). The final extract was evaporated until dryness and the residue was resuspended in 100 µL of H₂O:ACN (75:25; v/v).

The same metabolites were determined also in urine samples following a method previously developed and validated in our institute [22]. Briefly, the urine samples were thawed at room temperature. Then, the urine (1 mL) was spiked with IS (typically 5 ng) and 250 µL of NH₄Ac buffer containing 5 µL of β-glucuronidase (0.7 U/mL urine) was added. The mixture was incubated during 90 min at a controlled temperature of 37°C. Afterwards, an aliquot of 200 µL was transferred to glass vials (300 µL) and 10 µL was injected into the UPLC system.

Creatinine was measured via the Cayman's colorimetric assay doing duplicate analysis [23]. The renal clearance of the analytes in urine was afterwards corrected by the creatinine adjustment following a new calculation approach (results reported in ng/mL in Equation 1).

Additionally to ensure the quality control (QC) of the measurements, each analytical batch also included a set of procedural blanks (enzymatically treated in case of urine), as well as the injection of solvent blanks and calibration standards every ten samples. After extraction, the levels detection in the procedural blanks were subtracted from those detected

in the samples. The method limit of quantification (LOQ_m) was defined as 3 times the standard deviation (SD) of the PE metabolite's concentration that could be measured in procedural blanks (99 % confidence level). If the analytes were not detected in the blanks, thus the LOQ was defined as the LOQ_i (based on $10\times$ S/N of the lowest calibration standard).

2.4 Analytical method

The instrumental analysis was performed by Ultra Performance Liquid Chromatography (UPLC) coupled to tandem mass spectrometer (Quattro Premier XE Micromass) from Waters (Milford, MA, USA). The chromatographic separation was performed on an Acquity UPLC BEH phenyl column (100 mm x 2.1 mm I.D; 1.7 μ m particle size) with a Van Guard Acquity UPLC BEH C_{18} pre-column (5 mm x 2.1 mm, 1.7 μ m). The column oven temperature was set at 40 °C.

Optimal chromatographic conditions were achieved using a gradient elution program: water (solvent A) and ACN (solvent B), both acidified with 0.1 % acetic acid (v/v). The gradient elution program was as follows: 0-0.5 min: 95 % A; 0.5-8 min: 95-10 % A; 8-8.5 min: 10-95 % A; 8.5-10 min: equilibration of the column. The mobile phase was discarded in the first 3 min to avoid interference of proteins or other hydrophilic substances with the analyte's ionization. The flow rate was 0.4 mL/min and the injection volume was 10 μ L. The total run time was 10.5 min.

The UPLC system was coupled to the mass spectrometer (MS) interfaced with an electrospray ionisation source operating in negative ionization mode (ESI-). The cone voltage was set at 25 V. The collision cell energy varied between 14 and 20 eV (Table SI-1 in Supplementary Information). The dwell time varied between 0.05 and 0.25 sec. The electrospray source block and the desolvation temperature were 120 °C and 350 °C, respectively. The argon collision gas flow was kept constant at 0.25 mL/min. The cone and desolvation nitrogen gas flow were set at 50 L/h and 800 L/h, respectively. The characteristic precursor and daughter ions that were selected for detection of PE metabolites in multiple reaction monitoring mode (MRM) are presented in the Supplementary Information. The daughter ions resulting from the MRM transitions were used for the quantification.

2.5 Statistical analysis

All statistical analyses were performed using SPSS (SPSS for Windows, version 22.0, SPSS, Chicago, IL). Data on urine (ng/mL) and nails (ng/g) was used for statistical analysis. The data was normalized (Z-scores) before the correlation analysis (Pearson), linear regression (scatter plots) and multiple linear regression analysis. Moreover, before the statistical analysis, all values below the LOQ_m were transformed to LOQ_m/√2 [24]. Linear regression analysis was conducted to investigate correlations between analytes.

The non-parametric Mann–Whitney U test was performed to determine if significant differences in the (non-log transformed) levels of PE metabolites in nails exist between groups/categories (gender, weight, habits of eating with hands, eating pre-packed food, storing/heating food in plastic containers, use of hand care products, use of nails polish, time spent inside a transport/car, daily ventilation of the house or doing sport activity).

Afterwards, multiple linear regression analysis was conducted to study the association between all predictors and levels of PE metabolites in nails. The unstandardized (b), standardized beta (β), 95 % confidence intervals (95 % CI), p-values and adjusted R² were presented.

3. Results and Discussion

3.1 Assessment of human exposure to PE metabolites in nails and urine collected from a Belgian sub-population

This study used a Belgian sub-population (i.e., 20 individuals) to provide insight into human exposure to phthalate esters through their primary (MEHP, MEP, MBzP, MnBP and MiBP) and secondary PE metabolites (5-OH-MEHP and 5-oxo-MEHP). To this extent, paired urine and nail samples were used. With regard to improve comparability of the results between matrices, the levels in urine were adjusted to the creatinine content (i.e. and to compensate dilution of the target analytes among all individuals), using a new correction approach (Equation 1):

$$\text{Corrected concentration} \left(\frac{\text{ng}}{\text{mL}} \right) = \frac{\text{Conc} \left(\frac{\text{ng}}{\text{mL}} \right)}{\text{Creatinine level} \left(\frac{\mu\text{g}}{\text{g}} \right)} \times \text{Average creatinine} \left(\frac{\mu\text{g}}{\text{g}} \right)$$

(Equation 1)

This approach provides the correction of the analytes concentrations in urine taking into account the individual creatinine content (creatinine ranged between 20 to 285 mg/dL) and the average creatinine levels measured in healthy volunteers, while results remain expressed as ng/mL, instead of $\mu\text{g/g}$ creatinine (Table 1). In this sense, we believe that a better approximation of the excreted levels of PE metabolites is measured in this population, since only one urine spot was analyzed per participant. Also, due to the high inter-individual variability of the creatinine content, expressing the results as $\mu\text{g/g}$ creatinine may increase an artefact of the metabolite's levels, especially for the individuals who had a too high (e.g. $> 230 \text{ mg/dL}$) or too low (e.g. $< 30 \text{ mg/dL}$) creatinine content. In here, it is understandable that individuals with too high/low creatinine content should be excluded from the analysis (according to established WHO guidelines) [25]. Yet, this is usually done when large population are studied (> 1000 individuals) and there is a wide variation between the urinary levels measured, which was not the case in the present study ($< \text{LOQ}_m$ -28 ng/mL).

When measuring more urinary spots/day, intra-individual variability of creatinine content is reduced [26], therefore this approximation is not needed and the accuracy on reporting the results as $\mu\text{g/g}$ creatinine is much higher.

At the same time, it is known that the urine density is approximately the same as for water (i.e. can vary between 1.002 and 1.030), therefore the results reported as ng of metabolite per g or mL of matrix are similar in terms of comparing their units (analytical point of view). Since, we do not know if the exposure scenarios of these participants are comparable or not (i.e. between 24 h and 6 months ago), thus the only way to possibly compare and discuss the levels of the target compounds in two different matrices (ng/mL and ng/g), is by extrapolating that the exposure occurred at six months ago was constant.

Thus, the levels of PE metabolites in urine (ng/mL) and in nails (ng/g) are represented in Table 1.

In urine, MEHP was the least detected metabolite (detection frequency 56%) with levels not exceeding 0.64 ng/mL. The secondary oxo- and hydroxy-MEHP metabolites were abundantly detected (94-100 % detection). This is due to the known fast metabolism of the primary metabolites (MEHP) into secondary metabolites (5-OH-MEHP and 5-oxo-MEHP), which in turn are quickly excreted through renal clearance. The median levels of secondary metabolites of DEHP ranged from 0.39 ng/mL to 0.46 ng/mL. Apart from the secondary MEHP metabolites, the major metabolites present in urine were the sum (MnBP, MiBP) (range from 2.2 to 28.7 ng/mL) and MEP (range from 0.9-10 ng/mL).

PE metabolites were detected in nails collected from the same sub-population at considerable high ng/g levels (Table 1). The major metabolites detected in nails were MEHP, sum (MnBP, MiBP) and MEP (100 % DF), followed by MBzP (60 % DF), 5-oxo-MEHP (25 % DF) and 5-OH-MEHP (15 % DF). These results are confirmed by our previous study (US-DLLME method validation) where the PE metabolites were measured in ten individuals [21].

To our knowledge, this is one of the first studies reporting the human exposure to PEs through their metabolites using nails and urine from the same individuals. Our findings suggest that the most relevant metabolites in nails are MEHP (average of 146 ng/g), sum (MnBP, MiBP) (averages of 212 ng/g) and MEP (205 ng/g). Also, in a previous (non-published) study involving the determination of PE metabolite in various urinary spots and nails, the authors confirmed that the oxidative DEHP metabolites were minor while MEP, MEHP and sum (MnBP, MiBP) were the major metabolites found in nails [26].

In Figure 1, the boxplots summarize the distribution of the total PE metabolite levels in nails and in urine for all individuals (boxplot A and B). Some outliers were noticed in both distributions, still a lower variability is obtained by expressing the total levels in nails than in urine (difference between the minimum values and the outliers was ~ 9 times *versus* 25 times in nails and urine, respectively).

3.2 Levels of PE metabolites in nails: a study comparison

The present study did not intend to be representative for the Belgian population (where usually the purpose is to investigate exposures in more than 200 individuals [27-29]). In contrast, due to the limited number of volunteers this is considered a pilot feasibility study using a novel microextraction method [21] and nails as an alternative matrix for assessing exposure to PEs [21]. Since this application is new, there is no published scientific literature for direct comparison of the levels in both matrices. Still, it is possible to compare these results with our (unpublished) data regarding the assessment of PE metabolites in multiple urine spots and in nails [26]. We have found that the major metabolites detected in morning urine were sum (MnBP, MiBP) and MEP (mean of 3 and 2.3 µg/g creatinine, respectively in all individuals) and in nails sum (MnBP, MiBP), MEP and MEHP (mean of 46, 31 and 41 ng/g, respectively in all individuals), showing that these results have a good agreement with those reported in this study. More, the MEHP oxidative metabolites (i.e. 5-OH-MEHP and 5-oxo-MEHP) were minor present in nails (although the detection

frequency was higher than 50 %) while in urine they were highly detected (100 %). Again, these results are in concordance with the findings in the present study.

3.3 Predictors of PE exposure in nails

Although it is impossible to specify the contribution of each source of exposure by the metabolite's levels in nails, we believe that there is a correlation between some particular routines that might explain the high bioaccumulation of certain compounds in nails. Despite the limited information reported in the questionnaires (number of questions and categories per question) and the low number of participants involved in this study, the questionnaires were still a useful tool revealing that in general these individuals had common daily routines (e.g. 60 % did not use hand cream; 90 % ate with hands, 40 % did 1-3h physical exercise/week, 70 % ventilate their house every day; 55 % spent more than 5h/week in transports/own car), suggesting a similar exposure profile.

Further, statistical analyses based on univariate and multiple linear regression analysis were performed in order to evaluate the association of each predictor with the metabolite's levels in nails and to tentatively identify the major (s) sources of intake.

3.3.1 Univariate model

Taking into account the results provided by the Mann-Whitney non-parametric rank test used for testing the association of environmental/personal predictors with the PE metabolites in nails, it is possible to observe that in overall there are no significant differences (95% CI) between the groups assigned per predictor, except for gender ($p=0.025$) where the differences between men and women were only observed for the MEHP (Table 2).

At the same time, the low statistical significance found in this study for most of the predictors can be due to the low number of individuals ranked per group and/or low number of categories. Still, some predictors can be highlighted at 80 % CI instead ($p<0.2$), including: eating with hands ($p=0.126$) and time spent in a transport/car per day ($p=0.075$) are associated with increasing MEHP levels in nails. In particular, participants who ate less frequently with hands (1-2 times/day) and spent less hours in a transport/car (1-5h/day) have higher MEHP levels in nails, suggesting that these factors are not the ones that most contributed for increasing the MEHP levels. Also, these last predictors together with 'use of nails polish' ($p=0.126$) are associated with sum (MnBP, MiBP) levels. On contrary of what

we have observed for MEHP, the participants who had higher contact with food and spent more time in transport/car showed higher MnBP/MiBP median levels. In fact, DnBP and DiBP can be associated with food supplements [30] and auto interiors [31]. However the association of their metabolites with nail polish use is not understandable since the incorporation of the parent diesters in personal care products is not allowed [8].

Also the predictors ‘buy pre-packed food’ ($p=0.061$) and ‘ventilate the house’ ($p=0.121$) are associated with MBzP levels in nails. Although diet can be considered a source of BBzP, this is not seen as the major one. Since BBzP has been mostly incorporated in PVC products such as in vinyl floor tile, vinyl foams, and carpet backing [32], these are highlighted as predominant exposure sources to BBzP. Human are exposure to this PE at home (indoor) because BBzP can be leached out from PVC products (flooring and wall materials) for instance into dust, which has been considered a good vehicle of the BBzP uptake in humans [33, 34]. Thus, the house ventilation might help to increase BBzP levels in the surrounding environment and facilitate the exposure. Yet, the association between the external sources (e.g. dust, air) and the BBzP levels in nails is not yet addressed. Therefore, further studies need to be conducted in order to understand this association.

Finally the only predictor that might indicate any association with MEP levels in nails is ‘eating with hands’ ($p=0.168$). This can be due to the contact with specific food/drink products that might contain the DEP released from polyvinyl dichloride (PVDC) in food wrap film, for example [35]. More, its presence in food packaging materials and in the contacted food was already confirmed by other authors [36], therefore this source of DEP exposure is not unlikely. Nevertheless, the same association was not evidenced by the pre-packed food consumption, showing that the MEP levels in nails and its association with diverse (or specific) food items and food packaging needs to be more deeply investigated.

3.3.2 Multivariate regression analysis

Despite no significant differences (95% CI) explained by the predictors for each dependent variable (i.e. analyte) in the univariate models, the association of all predictors with the PE metabolites in nails (except for 5-OH-MEHP and 5-oxo-MEHP due to low % DF) was yet tested in a multiple linear regression analysis (Table 3). Since MEHP, sum (MnBP, MiBP) and MEP were the major metabolites in nails, the associations in a multivariate model were more significant than in the univariate analysis.

In the analysis, the independent variables (predictors) were added successively to the model (stepwise method), until there were no more significant contributions to the multiple regression. This approach was performed for all metabolites. In addition, all independent variables were forced to fit into the regression model (for MEP and MBzP), when in the stepwise method none of the predictors were significant for the model. In the end, some predictors seemed to contribute for explaining the MEP levels in nails, but not for MBzP.

In addition, due to the low number of individuals none of the groups (in univariate model) were discriminated, instead the global effect of a certain variable was studied. Based on the unstandardized and standardized coefficients (B and β , respectively), some predictors could be highlighted in each model.

3.3.2.1 DEHP exposure

The use of hand care products is positively associated with MEHP levels in nails ($\beta = 0.49$, $p = 0.04$), although there is only 19 % of the variance of this predictor which can be explained by the MEHP levels in nails, suggesting that this is not a major source of DEHP intake. Nevertheless, as reported by Koo et al. [2] DEHP can be incorporated in cosmetics and personal care products (e.g. perfume, nail polish, shampoo, etc.), although the incorporation of DEHP is no more allowed since 2009 (as well as for DBP and BBzP) [8], once it is considered a substance of very high concern which is classified as reproductive toxicant [37]. Yet, more recently the association of personal care products (e.g. bar soap) as predictor of DEHP exposure (i.e. through MEHP levels in urine) is still evidenced [38].

Also, diet can be also a source of DEHP due to the contamination of food/drink during processing, storage and transportation [39, 40]. Despite, it was suggested in the univariate possible associations (although not significant at 95 % CI) between MEHP levels and food, nevertheless this association did not persist in the multivariate model.

3.3.2.2 DBP exposure

For sum (MnBP, MiBP), there was a positive significant association with weight in the multivariate model ($\beta = 0.56$, $p = 0.02$). However, only 26 % of the variance on weight is explained by the sum (MnBP, MiBP) levels in nails. However in univariate no differences were found between groups (i.e. 50-70 Kg and >70 Kg) that could be explained by the gender effect (i.e. not significant either in univariate and multivariate analysis).

Previously, in Dirtu et al. study [41], no associations were established between obesity indicators (e.g. weight, BMI, etc.) and PE metabolites in urine. Still, the association of weight (and gender) with DnBP/DiBP metabolites needs to be further analyzed considering a larger population (where possibly more weight categories or individuals per/weight category are included).

3.3.2.3 DEP exposure

In our study, the only variable associated with personal care products in the multivariate model was MEHP (and sum(MnBP, MiBP) in the univariate), while MEP is mostly associated with sports activity and not surprisingly with cosmetics (negative association in the multivariate statistics). Surprisingly the positive association with ‘sport hours/week’ is the highest and with statistical significance at 95% CI ($\beta=1.28$, $p=0.02$). To our knowledge, sport activity may change the release of diverse hormones (e.g. testosterone, cortisol) [42]. At the same time, most of the PEs are associated to endocrine disrupting effects, except DEP which nothing has been proven yet. Therefore, this association with DEP must be better understood.

Similarly, the variables ‘weight’ and ‘hours spent in transport/car’ have relatively high standardized coefficients ($\beta = 0.75$ and $\beta = -0.79$, respectively) at statistical significance ($p > 0.05$). Nevertheless, a positive association is found for weight while for ‘hours spent in transport/car’ is the opposite (i.e. negative). Thus, the results suggested that the participants with higher weight (independently of the gender) have high MEP levels in nails. However, in Dirtu et al. study [41], levels of MEP were higher (but in urine) in individuals who had follow a 3 month weight loss program.

All variables together can explain 45 % of the variance, showing that some of these predictors are highly associated with an increase/decrease of MEP levels in nails.

In overall, it is important to consider that people are exposed daily to many products containing more than one diester in its composition [43], thus apportioning one exclusive source of exposure for each metabolite is unrealistic and impossible. More, the identification of the major intake route(s) and source(s) is hampered by many intra- and inter-individual factors, such as the high variability between the analyte levels in the same individual or between individuals that were exposed to the same parent PE. Also differences in exposures to specific PEs (duration and frequency) via specific lifestyle habits (including

indoor and outdoor contact) or distinctive excretion rates and metabolism between nails and urine play an important role on exposure assessment and its variability.

3.4 Correlations between the metabolites in nails and urine

Table 4 lists the Pearson correlation factors (r) between the levels in nails and urine.

In nails, MBzP is moderately correlated with MEHP ($r=0.57$, $p<0.01$), therefore may reflect similar source of exposure, such as their use in food packaging and/or in household products [44].

Also, a moderate correlation was observed for the major metabolites detected in nails, i.e. MEP and sum (MnBP, MiBP) ($r=0.51$, $p<0.05$). Previously in the univariate models, the use of nail polish showed a possible association with DBP exposure (even if DnBP and DiBP have been already phase out [8]), while for DEP, the contact with food (eat with hands) was the most contributor. Nevertheless, the correlation of their metabolites in nails might suggest that in part they were provided by similar sources.

As far as we know, DnBP can be also used in enteric coatings of medications and food supplements and sometimes, DiBP is used as substitute for DnBP [30]. Nevertheless, we do not know in what extend DnBP and/or DiBP are incorporated in various food products but based on the lack of association in the multivariate results it seems that: 1) they are not widely used or 2) the participants had minor contact with them via food products (and their contact materials), otherwise this association could be evidenced. More, since DBP incorporation in cosmetics is not allowed, thus the probability of DEP and DBP metabolites coming from this source is considered very low.

A moderate correlation was also found between MEHP and 5-OH-MEHP ($r=0.66$, $p<0.01$) (see also Figure SI-2 in Supplementary information). Due to the low detection frequency (15 %) of 5-OH-MEHP in nails, this association response is mainly due to the high MEHP exposure levels. Here, it is possible to observe that even with high 5-OH-MEHP excretion (thought urine, 94 % detection), nails give an indication of a DEHP long-term exposure.

In urine, statistically significant correlations were observed between MEHP and 5-oxo-MEHP ($r=0.68$, $p<0.01$), 5-oxo-MEHP and 5-OH-MEHP ($r=0.81$, $p<0.01$), MBzP and sum (MnBP, MiBP) ($r=0.76$, $p<0.01$), MEP and sum (MnBP, MiBP) ($r=0.60$, $p<0.01$) and finally between MEP and MBzP ($r=0.67$, $p<0.01$). As expected, the oxidative metabolites correlate well among themselves and better than with the MEHP [3, 27, 28]. In urine, sum (MnBP,

MiBP) is highly correlated with MBzP, suggesting that MnBP that is a metabolite of both DnBP and BBzP. The MBzP that is an exclusive metabolite of BBzP, can be a result of similar intake source (e.g. PVC flooring, wall coverings, among others) [45].

The MEP is moderately correlated with MBzP and sum (MnBP, MiBP) ($r=0.6-0.67$) but weakly correlated with the other metabolites (see Figure SI-3 in Supplementary information). These results are in agreement with Dewalque et al. [28] suggesting that Belgian individuals are exposed to different sorts of phthalates although by similar routes of exposure. On the contrary, these results are contradictory and against those presented by other authors [14, 46, 47] that stated that correlations between MEP and other metabolites were weak, suggesting that the parent compound, i.e. DEP, mainly used as solvent or carrier in product formulations, is incorporated in plasticized products together with other diesters primarily used as plasticizers.

Upon the possible sources of human exposure and based on urinary data, several authors indicate that for DEHP diet is the major source while for DBP and DEP other sources (e.g. cosmetics, medication) might have a higher contribution [14, 15, 44, 48, 49].

Between matrices, significant correlations were present for the sum (MnBP, MiBP) and 5-oxo-MEHP ($r=0.37$) and the sum (MnBP, MiBP) with 5-OH-MEHP ($r=0.62$, $p<0.01$), although there was a low detection frequency of the oxidative DEHP metabolites in nails.

Also, good correlation was observed between MEHP in nails and sum (MnBP, MiBP) in urine ($r=0.73$, $p<0.01$). In fact, both MEHP and DBP metabolites were the major metabolites in nails, but not in urine, suggesting that this association between matrices is probably favored by their exposure levels in nails.

Moderate correlations were observed between urine and nails for MBzP and MEHP ($r=0.52$, $p<0.05$) and MEP and 5-OH-MEHP ($r=0.56$, $p<0.05$). Firstly, there are evidences that nails are the major contributor for explaining the MBzP and MEHP association, once a high correlation was found in nails and not in urine ($r=0.57$ vs $r=0.02$, respectively). Secondly, either urine or nails by themselves do not correlate MEP with 5-OH-MEHP levels, thus the intake sources are certainly distinct. Once 5-OH-MEHP was only detected in 15 % of the nail samples, therefore its association to any studied predictor was not performed.

The exposure assessment based on nails or urine will however lead to different conclusions as the compound profiles (levels, % detection) differs significantly between the two matrices. In Figure 2, the total levels of the metabolites determined in both matrices

were compared, showing that correlation is lacking ($R^2 = 0.221$). More, this is confirmed by the complete absence of correlation between the same measured compound in the two matrices, which is highlighted in the scatter plots (Figure 3). Hence, the major metabolites detected in nails (i.e. MEHP, MEP and the sum (MnBP, MiBP)) did not correlate among themselves in urine, confirming that the exposure response translated by urine and/or nails is unique and not interchangeable (and assuming that the exposure to the parent PEs did not change in about 6 months of exposure).

Overall, the primary and secondary DEHP metabolites (MEHP, 5-OH-MEHP and 5-oxo-MEHP) were better correlated among themselves in urine than in nails (except, for the correlation between 5-oxo-MEHP and MEHP ($r=0.68$, $p<0.01$), which can be explained by the higher levels of 5-oxo-MEHP in nails of individual 6, 11 and 19). This can be explained by the physico-chemical properties of the chemicals. The more polar oxo- and hydroxyl-compounds have a significantly shorter circulation time in the body before renal clearance, leading to a lower accumulation potential in the nail and relatively high concentrations in urine, while less polar metabolites circulate longer, resulting in higher levels in nails. As a result, DEHP oxidative metabolites are highly detected and more often in urine than in nails (94-100 % detection in urine vs. 15-25 % detection in nails).

3.5 Study Limitations

The small number of individuals ($n=20$) and the collection of only one spot (morning) urine per participant imposes limitations to the present pilot study. Thus, we agree that either a higher number of volunteers and more nails and urine spots (ideally more than one urine spot/day) collected over a longer period (>15 days), would translate a better exposure trend and give a better significance of the predictors (environmental or personal contributors) in the statistical univariate and/or multivariate analysis.

Taking into account that six months are necessary for the nail to completely grow out, a sequential nail sampling over a longer period of time (e.g. 1 to 6 months) could unravel the variation in exposure reflected by different nail segments in each participant. At the same time, urine spots (or other matrix representing short-term exposure) could be collected for comparison of exposures.

Since, there are many factors that might influence the nail growth (age, gender, nutritional state, climate), its properties (water, lipid, thickness, keratin, etc.) and other factors that might affect the partitioning of chemicals between blood (or other tissue) and

nail (e.g. lipophilicity of the chemicals, pH of the solvent media, keratin-chemical interaction), we consider that the evaluation of the exposure based exclusively on nails is not easy and straightforward. More, it is difficult at this stage (and considering that a unique nail sample was collected per participant) to understand the importance of all influencing factors (personal/environmental), once the routines reported in the questionnaires (during the sampling) might have changed. Also, there is yet no knowledge in relation to how exactly express a specific exposure (intake) based on nails data, likewise it is done for other matrices (e.g. urine and serum). Longitudinal (and cross-sectional) studies could help to understand some of these factors and explain variations between nail measurements (per participant). In addition, pharmacokinetics (metabolism and excretion) of some of these PEs has not been studied, as well as the partitioning between blood-nail and the contribution from external sources to the overall exposure, which might be an important research gap to overcome in future studies.

A more detailed questionnaire could unveil the importance of personal care products/cosmetics in the DEP and DBP exposure and food predictors in DEHP exposure. For assessing the feasibility of this new approach using nails these limitations are acceptable. Still, we need to be aware that more investigation is needed in order to consider nails as an alternative matrix to urine in HBM.

4. Conclusions

For the first time, a feasibility study was conducted to assess the human exposure to PEs in twenty Belgian individuals via nails and urine. A new normalization creatinine-based approach for: 1) correcting the dilution of PE metabolites in urine; 2) create a better comparison between matrices in terms of expressed units (ng/mL vs. ng/g) was proposed.

The first insights showed that nails can better identify the (long-term) exposure to some PEs such as DEHP, DBP (DBnP and/or DIBP) and DEP than urine. Therefore nails can be considered a valuable non-invasive alternative for estimating human exposure to phthalate esters. The levels of each individual metabolite did not correlate between matrices for the presented conditions (i.e. one spot urine vs. one nail collection), demonstrating therefore that these matrices have a distinct accumulation and clearance of PEs. Also, based on the results there is an evidence that part of the analytes that are not excreted in urine, are being accumulated in nails, although there is no clear assurance whether the exposure have

changed or not (in six months). Levels in nails were in the same order of magnitude as those determined in other author's studies.

As suggested by our results, the predictors of PEs exposure in univariate models were not further associated with the same metabolite expressed by a multivariate analysis. Instead, when all predictors are analyzed together in a multiple regression only few (and different ones than those highlighted in the univariate models) are evidenced as statistically significant in increasing exposure to a certain PE, including the use of hand care products (i.e. for DEHP), the body weight (i.e. for DBP and DEP) and number of hours of sport activity (i.e. for DEP).

Still, for better understanding of the bioaccumulation effect of PEs in nails, it would be important to have data from a more exposed and significant population, as well as more nail and urine collections from same individual that can represent the exposure to PEs at a same time point.

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Figure and Tables Captions

Table 1. Concentrations of the PE metabolites in urine (ng/mL) and in nails (ng/g) from twenty individuals.

Table 2. Average levels (and 50th percentile) of PE metabolites in nails (ng/g) for twenty individuals distributed according to personal and environmental predictors.

Table 3. Multiple linear regression analysis of personal/environmental predictors in nails (normalized values).

Table 4. Pearson correlation coefficients between PE metabolites in nails and urine.

Figure 1. Boxplot distribution of the total levels of the PE metabolites in nails (ng/g) (Boxplots A and B) and in urine (ng/mL) (Boxplots C and D) for the total Belgium individuals and grouped by gender. The box hinges represent the 1st quartile (bottom) and the 3rd quartile (top). The whiskers (top and bottom) extend from the hinge to 1.5 times the inter-quartile range. The middle line represents the median. Data beyond the end of the whiskers are outliers and are plotted by the individual n°.

Figure 2. Linear correlation between total PE metabolites in urine and in nails (normalized values).

Figure 3. Scatter Plot between the metabolites detected in both matrices (nails and urine) for: MEHP (**A**); 5-OH-MEHP (**B**); 5-oxo-MEHP (**C**); Σ (MnBP and MiBP) (**D**); MBzP (**E**); MEP (**F**).

Supporting Information

Table SI-1. MRM transitions, collision energy, cone voltage and retention times for the target metabolites and respective IS.

Figure SI-2. Scatter Plot between the metabolites detected in nails for: 5-OH-MEHP and MEHP (**A**); MBzP and MEHP (**B**); MEP and Σ (MnBP and MiBP) (**C**).

Figure SI-3. Scatter Plot between the metabolites detected in urine for: 5-oxo-MEHP and MEHP (**A**); 5-oxo-MEHP and 5-OH-MEHP (**B**); MBzP and Σ (MnBP and MiBP) (**C**); MEP and Σ (MnBP and MiBP) (**D**); MEP and MBzP (**E**).

References

1. Wittassek M, Koch HM, Angerer J, Brüning T (2011). Assessing exposure to phthalates - the human biomonitoring approach. *Mol Nutr Food Res* 55:7–31.
2. Koo HJ, Lee BM (2004). Estimated exposure to phthalates in cosmetics and risk assessment. *J Toxicol Environ Health A* 67: 1901-1914.
3. Hines EP, Calafat AM, Silva MJ, Mendola P, Fenton SE (2009). Concentrations of phthalate metabolites in milk, urine, saliva, and Serum of lactating North Carolina women. *Environ Health Perspect* 117:86–92.
4. Koch HM, Christensen KLY, Harth V, Lorber M, Brüning T (2012). Di-n-butyl phthalate (DnBP) and diisobutyl phthalate (DiBP) metabolism in a human volunteer after single oral doses. *Arch Toxicol* 86:1829–39.
5. Peck CC, Albro PW (1982). Toxic potential of the plasticizer Di(2-ethylhexyl) phthalate in the context of its disposition and metabolism in primates and man. *Environ Health Perspect* 45:11–7.
6. Wittassek M, Angerer J (2008). Phthalates: metabolism and exposure. *Int J Androl* 31:131–8.
7. Blount BC, Silva MJ, Caudill SP, Needham LL, Pirkle JL, Sampson EJ, Lucier GW, Jackson RJ, Brock JW (2000). Levels of Seven Urinary Phthalate Metabolites in a Human Reference Population 108:979-82.
8. Regulation (EC) N.º 1223/2009 on cosmetic products.
9. Directive 2005/84/EC relating to restrictions on the marketing and use of certain dangerous substances and preparations (phthalates in toys and childcare articles).
10. Ventrice P, Ventrice D, Russo E, De Sarro G (2013). Phthalates: European regulation, chemistry, pharmacokinetic and related toxicity. *Environ Toxicol Pharmacol* 36:88–96.
11. Braun JM, Just AC, Williams PL, Smith KW, Calafat AM, Hauser R (2013). Personal care product use and urinary phthalate metabolite and paraben concentrations during pregnancy among women from a fertility clinic. *J Expo Sci Environ Epidemiol* 24:459–66.
12. Barr DB, Silva MJ, Kato K, Reidy J.A., Malek NA, Hurtz D, Sadowski M, Needham L, Calafat AM (2003). Assessing Human Exposure to Phthalates Using Monoesters and Their Oxidized Metabolites as Biomarkers. *Environ Health Perspect* 111:1148–1151.
13. Romero-Franco M, Hernández-Ramírez RU, Calafat AM, Cebrián M.E., Needham L., Teitelbaum S, Wolff M.S., López-Carrillo L (2011). Personal care product use and urinary levels of phthalate metabolites in Mexican women. *Environ Int* 37:867–71.
14. Frederiksen H, Jørgensen N, Andersson A-M (2010). Correlations between phthalate metabolites in urine, serum, and seminal plasma from young Danish men determined by isotope dilution liquid chromatography tandem mass spectrometry. *J Anal Toxicol* 34:400-10.
15. Koch HM, Lorber M, Christensen KLY, Pálmke C, Koslitz S, Brüning T (2013). Identifying sources of phthalate exposure with human biomonitoring: Results of a 48h fasting study with urine collection and personal activity patterns. *Int J Hyg Environ Health* 216:672–681.
16. Preuss R, Koch HM, Angerer J (2005). Biological monitoring of the five major metabolites of di-(2-ethylhexyl)phthalate (DEHP) in human urine using column-switching liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 816:269–80.
17. Barr DB, Wilder LC, Caudill SP, Gonzalez AJ, Needham LL, Pirkle JL (2004). Urinary Creatinine Concentrations in the U.S. Population: Implications for Urinary Biologic Monitoring Measurements. *Environ Health Persp* 113: 192-200.

18. Silva MJ, Reidy JA, Samandar E, Herbert AR, Needham LL, Calafat AM (2005). Detection of phthalate metabolites in human saliva. *Arch Toxicol* 79:647-652.
19. Hsu J-Y, Ho H-H, Liao P-C (2015). The potential use of diisononyl phthalate metabolites hair as biomarkers to assess long-term exposure demonstrated by a rat model. *Chemosphere* 118:219-228.
20. Chang YJ, Lin KL, Chang YZ (2013). Determination of Di-(2-ethylhexyl)phthalate (DEHP) metabolites in human hair using liquid chromatography-tandem mass spectrometry. *Clin Chim Acta.* 420:155-9.
21. Alves A, Vanermen G, Covaci A, Voorspoels S, (2015). Development of a New Ultrasound Extraction Coupled to Dispersive Liquid-Liquid microextraction (US-DLLME) for biomonitoring of phthalate metabolites in nails, *Submitted to Anal Bioanal Chem.*
22. Servaes K, Voorspoels S, Lievens J, Noten B, Allaerts K, Van De Weghe H, Vanermen G (2013). Direct analysis of phthalate ester biomarkers in urine without preconcentration: method validation and monitoring. *J Chromatogr A* 1294:25–32.
23. Sanbio B.V. website: <http://www.sanbio.nl/>.
24. Kato K, Silva MJ, Reidy JA, Hurtz D, Malek NA, Needham LL, Nakazawa H, Barr DB, Calafat AM (2003). Mono(2-Ethyl-5-Hydroxyhexyl) Phthalate and Mono-(2-Ethyl-5-Oxoheptyl) Phthalate as Biomarkers for Human Exposure Assessment to Di-(2-Ethylhexyl) Phthalate. *Environ Health Persp* 112:327-330.
25. WHO. 1996. Biological Monitoring of Chemical Exposure in the Workplace. Vol 1. Geneva:World Health Organization
26. Alves A, Koppen G, Vanermen G, Covaci A, Voorspoels S (2016). A novel fast method to measure phthalate metabolites in nails and its validation against urine. *Submitted to Sci Total Environ.*
27. Dewalque L, Pirard C, Charlier C (2014). Estimated daily intake and cumulative risk assessment of phthalate diesters in a Belgian general population *Toxicol. Lett.* 231:161-168.
28. Dewalque L, Pirard C, Charlier C (2014). Measurement of urinary biomarkers of parabens, benzophenone-3, and phthalates in a Belgian population. *Biomed Res. Int.* 649314: 1-13.
29. Geens T, Bruckers L, Covaci A, Schoeters G, Fierens T, Sioen I, Vanermen G, Baeyens W, Morrens B, Loots I, Nelen V, de Bellevaux BN, Van Larebeke N, Den Hond E (2014). Determinants of bisphenol A and phthalate metabolites in urine of Flemish adolescents. *Environ Res* 134:110-117.
30. Koch HM, Christensen KLY, Harth V, Lorber M, Brüning T (2012). Di-n-butyl phthalate (DnBP) and diisobutyl phthalate (DiBP) metabolism in a human volunteer after single oral doses. *Arch Toxicol* 86:1829-1839.
31. EPA website. Dibutyl phthalate. <https://www3.epa.gov/airtoxics/hlthef/di-n-but.html>
32. WHO, 1999. Concise International Chemical Assessment Document 17 for Butyl benzyl phthalate (BBzP).
33. Langer S, Bekö G, Weschler CJ, Callesen M, Toftum J, Clausen G (2014). Phthalate metabolites in urine samples from Danish children and correlations with phthalates in dust samples from their homes and daycare centers. *Int J Hyg Envir Heal* 217:78-87.
34. Bornehag C-G, Lundgren B, Weschler CJ, Sigsgaard T, Hagerhed-Engman L, Sundell J (2005). Phthalates in Indoor Dust and Their Association with Building Characteristics. *Environ Health Persp* 113:1399-1404.
35. Jen JF, Liu TC (2006). Determination of phthalate esters from food-contacted materials by on-line microdialysis and liquid chromatography. *J Chromatogr A* 1130:28-33.

36. Page DB, Lacroix GM (1995). The occurrence of phthalate ester and di-2-ethylhexyl adipate plasticizers in Canadian packaging and food sampled in 1985–1989: A survey. *Food Addit Contam* 12:129-151.
37. EU risk assessment report to Bis (2-ethylhexyl phthalate) (DEHP) (2008). <http://echa.europa.eu/documents/10162/e614617d-58e7-42d9-b7fb-d7bab8f26feb>
38. Cavallari JM, Simcox NJ, Wakai S, Lu C, Garza JL, Cherniack M (2015). Characterization of Urinary Phthalate Metabolites Among Custodians. *Ann Occup Hyg* 59:982-99.
39. Rudel RA, Gray JM, Engel CL, Rawsthorne TW, Dodson RE, Ackerman JM, Rizzo J, Nudelman JL, Brody JG (2011). Food packaging and bisphenol A and bis(2-ethylhexyl) phthalate exposure: findings from a dietary intervention. *Environ Health Perspect*; 119: 914–20.
40. Wormuth M, Scheringer M, Vollenweider M, Hungerbühler K (2006). What are the sources of exposure to eight frequently used phthalic acid esters in Europeans? *Risk Anal*, 26: 803–24.
41. Dirtu AC, Geens T, Dirinck E, Malarvannan G, Neels H, Van Gaal L, Jorens PG, Covaci A (2013). Phthalate metabolites in obese individuals undergoing weight loss: Urinary levels and estimation of the phthalates daily intake. *Environ Int* 59:344-53.
42. Hackney AC, Kraemer WJ, Hooper DR (2016). Hormonal changes associated with physical activity and exercise training. In Vaamonde D, du Plessis SS, Agarwal A (Eds), Springer New York, pp. 125-143.
43. Hubinger JC (2010). A survey of phthalate esters in consumer cosmetic products. *J Cosmet Sci* 61:457-465
44. Hauser R, Calafat AM (2005). Phthalates and Human health. *Occup Environ Med* 62:806–818.
45. Larsson K, Björklund KL, Palma B, Wennberg M, Kaj L, Lindh CH, Jönsson BAG, Berglund M. (2014). Exposure determinants of phthalates, parabens, bisphenol A and triclosan in Swedish mothers and their children. *Environ Int* 73, 323-333.
46. Frederiksen, H, Aksglaede, L, Sorensen, K, Skakkebaek, NE, Juul, A, Andersson AM (2011). Urinary excretion of phthalate metabolites in 129 healthy Danish children and adolescents: estimation of daily phthalate intake. *Environ. Res.* 111, 656–663.
47. Becker K, Göen T, Seiwert M, Conrad A, Pick-Fuss H, Müller J, Wittassek M, Schulz C, Kolossa-Gehring M (2009). GerES IV: phthalate metabolites and bisphenol A in urine of German children. *Int. J. Hyg. Environ. Health* 212, 685–692.
48. Huang P-C, Kuo P-L, Guo Y-L et al. (2007). Associations between urinary phthalate monoesters and thyroid hormones in pregnant women. *Hum Reprod* 22:2715-2722.
49. Fromme H, Gruber L, Schlummer M, Wolz G, Böhmer S, Angerer J, Mayer R, Liebl B, Bolte G. (2007). Intake of phthalates and di(2-ethylhexyl) adipate: results of the integrated exposure assessment survey based on duplicate diet samples and biomonitoring data. *Environ. Int.* 33, 1012–1020.