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**Biomarkers of human exposure to personal care products: results from the
Flemish Environment and Health Study (FLEHS 2007-2011)**

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Abstract

Personal care products (PCPs), such as soaps, perfumes, cosmetics, lotions, etc., contain a variety of chemicals that have been described as potentially hormone disrupting chemicals. Therefore, it is important to assess the internal exposure of these chemicals in humans. Within the 2nd Flemish Environment and Health Study (FLEHS II, 2007-2011), the human exposure to three classes of pollutants that are present in a wide variety of PCPs - i.e. polycyclic musks (galaxolide, HHCB and tonalide, AHTN in blood), parabens (urinary *para*-hydroxy benzoic acid, HBA) and triclosan (urinary TCS) - was assessed in 210 Flemish adolescents (14-15 years) and in 204 adults (20-40 years) randomly selected from the general population according to a stratified two stage clustered study design. The aim of this study was to define average levels of exposure in the general Flemish population and to identify determinants of exposure. Average levels (GM (95% CI)) in the Flemish adolescents were 0.717 (0.682 – 0.753) µg/L for blood HHCB; 0.118 (0.108 – 0.128) µg/L for blood AHTN; 1022 (723 – 1436) µg/L for urinary HBA and 2.19 (1.64 – 2.92) µg/L for urinary TCS. In the adults, levels of HBA were on average 634 (471 – 970) µg/L. Inter-individual variability was small for HHCB and AHTN, intermediate for HBA, and large for TCS. All biomarkers were positively associated with the use of PCPs. Additionally, levels of HHCB and AHTN increased with higher educational level of the adolescents. Both in adults and adolescents, urinary HBA levels were negatively correlated with BMI.

We define here Flemish exposure values for biomarkers of PCPs, which can serve as baseline exposure levels to identify exposure trends in future biomonitoring campaigns.

Keywords: human biomonitoring, parabens, musks, triclosan, personal care products

Introduction

Human biomonitoring (HBM) is a technique to evaluate human exposure to environmental chemicals. Biomarkers of exposure assess the internal dose of a xenobiotic compound and are therefore more likely to be directly associated with possible adverse health effects than external exposure monitoring (Den Hond et al., 2009; Lauwereys and Hoet, 2001). Between 2007 and 2011, an extensive HBM study (FLEHS II) was carried out in Flanders, the Dutch-speaking part of Belgium, for a large number of environmental pollutants in three age groups of the Flemish population, i.e. newborns, adolescents of 14-15 year old and adults between the age of 20 and 40 years (Schoeters et al., 2011; Schoeters et al., 2012). The aim of the FLEHS II study was firstly to assess the range of exposure, average levels as well as extremes. Secondly, the FLEHS study aimed at identifying the major determinants of exposure, both personal characteristics like age, gender or body composition and life style factors such as food consumption, smoking, traffic exposure, etc. Both historical pollutants such as heavy metals or persistent organochlorinated pollutants (POPs) and new emerging chemicals, such as phthalates, bisphenol A, perfluorinated compounds, etc. were studied. Here, we describe the Flemish results of three classes of chemicals that are present in personal care products (PCPs), namely musks, parabens and triclosan (TCS).

Synthetic musks are used as fragrances in detergents, fabric softener, household cleaning products, air fresheners, cosmetics, soaps, shampoos and perfumes (Balk and Ford, 1999; European Chemicals Bureau, 2005; Kafferlein et al., 1998). Musk compounds can be subdivided in two categories: polycyclic musks and nitromusks. The two largest volume products of the polycyclic musks are 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta- γ -2-benzo-pyran (HHCB) or galaxolide and 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetra-hydronaphtalene (AHTN) or tonalide (Balk & Ford, 1999). The two nitromusks investigated in this study are musk xylene (MX) and musk ketone (MK). The substances of both groups are stable against biological and chemical degradation and have a

high affinity for lipids (Balk & Ford, 1999). Human exposure mainly occurs via the use of body care and perfumed household products that could lead to ingestion, inhalation or skin contact, as well as through consumption of contaminated food or water (Reiner et al., 2007). Although musks have been found in human milk and human fat samples (Reiner et al., 2007; Lignell et al., 2008), data on the distribution within body compartments and elimination kinetics are sparse. For elimination, a half life of several months is suggested. Blood levels of nitromusks and polycyclic musks reflect aggregated exposure (Hutter et al., 2010). Musks are suspected estrogens (Bitsch et al., 2002; Schreurs et al., 2002; van der Burg et al., 2008) which have been identified as strong inducers of phase I enzymes and as cogenotoxicants (Mersch-Sundermann et al., 1996). The IARC (International Agency for Cancer Research (IARC), 1996) classified the nitromusks in group 2B, as possibly carcinogenic to humans.

Parabens are used as antimicrobial conservatives in food, drugs and PCPs (EFSA, 2004). Exposure to parabens occurs via ingestion, inhalation and predominantly via skin contact and their metabolism may differ depending upon the type of exposure (Calafat et al., 2010). In 2002 in the United States, an estimate of total paraben exposure was 77.5 mg/day (50 mg via cosmetics, 25 mg via pharmaceutical products and 2.5 mg via food) (EFSA, 2004). In the human body, parabens can be hydrolyzed to *p*-hydroxybenzoic acid (HBA), which is further conjugated before urinary excretion (Kiwada et al., 1980; Kiwada et al., 1981). Urinary HBA is a nonspecific metabolite of all parabens, and its conjugates in urine reflect short-term total exposure to parabens (Crow et al., 2008). *In vitro* experiments demonstrated that most parabens possess mild estrogenic activities (Miller et al., 2001; Routledge et al., 1998; Vinggaard et al., 2000), though *in vivo* only an uterotrophic effect could be demonstrated in mice for butylparaben and isobutyl paraben (Darbre et al., 2002; EFSA, 2004). The common metabolite of parabens, HBA, does not display estrogenic activity. Butylparaben, and to a lesser degree propylparaben, have a negative influence on

sperm quality in juvenile male rats (EFSA, 2004). Furthermore, parabens were found in breast tumors in humans (Darbre, 2004; Darbre et al., 2004).

TCS is a broad spectrum antimicrobial agent commonly used in a wide-spread variety of antibacterial soaps, deodorants, toothpastes, cosmetics, fabrics, plastics, toys and other products (Glaser, 2004; Scientific Committee on Consumer Safety, 2010; United States Environmental Protection Agency (USEPA), 2008). A pharmacokinetic study (Sandborgh-Englund et al., 2006) showed that the major fraction of a single oral dose of 4 mg triclosan was excreted in urine within the first 24 h (calculated urinary excretion half-life of 11 h), and approached baseline levels within 8 days after exposure. These data suggest that the concentrations of TCS in urine can be used as biomarkers of exposure to TCS. Exposure to TCS occurs via inhalation, ingestion or skin contact. TCS has a lipophilic nature and is resistant to degradation. TCS has been found to be acutely toxic to some aquatic organisms, it may be a weak endocrine disrupter in fish and can modulate thyroid function in amphibians (Chu and Metcalfe, 2007; Ishibashi et al., 2004; Veldhoen et al., 2006). Furthermore, contact dermatitis, photoallergic contact dermatitis and skin irritation were reported after exposure to TCS (Durbize et al., 2003; Storer et al., 2004). A number of studies have raised concerns since TCS can induce TCS resistance in bacteria, or even may be associated with changes in antibiotic susceptibility of bacteria and hence may induce antibiotic resistance (Glaser, 2004; Heath et al., 2000). Up to date, no evidence was found concerning any carcinogenic, mutagenic or teratogenic effects (Bhargava and Leonard, 1996). In 2010, a biomonitoring equivalent (BE), based on NOAEL safety targets as proposed by the European Scientific Committee on Consumer Products, was derived for total urinary TCS and equals 2600 µg/L (or 3300 µg/g creatinine) (Krishnan et al., 2010).

The aim of the current study was to assess the range of internal levels of musks, parabens and TCS in a representative sample of adolescents and adults from the Flemish population and to identify the major determinants of exposure to these three PCP compounds.

Methods

Study population

Adolescents (14-15 years) and adults (20-40 years) were targeted in this study. Based on sample size calculations of the first Flemish Environment and Health Study (FLEHS I, 2001-2006), 200 participants per age group were defined as the required number. A stratified clustered multi-stage design was used to select participants as a random sample of the population residing in Flanders. Firstly, the number of participants in each of the five Flemish provinces was determined, proportional to the population density at June 1st, 2007. In a second step, primary sampling units (PSU) were selected. These PSU's were schools for the adolescents and provincial institutes for the adults. Within each province, one or two PSUs per age class were randomly selected taking into account that the selected PSUs should be located at least 20 km apart. In a third step, individuals were selected at random within the PSU. In order to account for seasonal variation, recruitment was spread over one year. Inclusion criteria were: 1) residing at least 10 years in Flanders; 2) giving written informed consent; and 3) being able to fill in an extensive Dutch questionnaire. This study was approved by the ethical committee of the University of Antwerp on May 18th, 2008 (UA A08 09).

Fieldwork

In both age groups, invitation letters, information brochures and informed consent forms were distributed. The adolescents were contacted via the schools (n = 10; 2 per

province); the adult participants were recruited through provincial institutes (n = 5; 1 per province). Information about socio-economic status, living conditions, life style, food intake, tobacco-smoke, diseases and medication was gathered through self-administered questionnaires that were filled out by the participants at home (and in case of the adolescents partly by the parents). First morning urine was collected at home on the day of the examination. The examination was performed at school or at local centers in the provincial administration and involved blood sampling, and standardized weight and height measurements. During the field examination, a quick check of the home questionnaires took place. Additionally, a short questionnaire on recent exposure during the last three days was filled out. All participants were included in the study between May 2008 and May 2009, thus accounting for seasonal variation.

Laboratory analysis

Standard quality assurance and quality control (QA/QC) and validation dossiers were fulfilled. All samples were handled, frozen and stored within one day. Biomarker values were reported if they were above a quantifiable level as determined in the laboratory.

Musks. Polycyclic musks (HHCB and AHTN) and nitromusks (MX and MK) were measured in whole blood samples by stir bar sorptive extraction (SBSE), followed by thermal desorption and gas chromatography – mass spectrometry (GC-MS). SBSE is a micro-extraction technique previously used for trace analysis of organic compounds in biofluids (Tienpont et al., 2002; Tienpont et al., 2003). The advantages of SBSE include the reduction of sample size, of solvent use, and of contamination (important in musk determination). For musk determination, 0.5 mL of blood was diluted with 4.5 mL of mineral water. The diluted samples were spiked with 500 pg of MX-d15 and 300 pg of AHTN-d3 (Dr Ehrenstorfer GmbH, Augsburg, Germany) as internal standard for quantification (isotope dilution

quantification). Internal standard concentrations correspond to 1 µg/L blood and 0.6 µg/L blood respectively for MX-d15 and AHTN-d3. SBSE was performed with a 1 cm × 0.5 mm PDMS stir bar (Gerstel GmbH, Mullheim, Germany) at room temperature (20°C) during 1h in a closed 20 mL headspace vial. After extraction, Twisters were removed, rinsed with distilled water and dried with a tissue. Analyses were performed on an Agilent 7890 GC combined with an Agilent 5975 MSD (Agilent Technologies, Santa Clara, USA). The system was equipped with a multipurpose (MPS2) autosampler, a programmed temperature vaporizing (PTV) inlet (CIS4) and a thermal desorption system (TDU), mounted on the PTV inlet (all Gerstel GmbH). Thermal desorption of the stir bar (introduced in a TDU liner) was performed at 300°C in splitless mode. The desorbed compounds are cryo-trapped in the PTV at -100°C and injected in the GC-MS in splitless mode by programming the PTV at 600°C/min to 280°C. GC-MS separation was performed on a 30 m x 0.25 mm ID x 0.25 µm HP-5MS column (Agilent Technologies) and MS detection was done in selected ion monitoring (SIM) mode. Additional details on the musk analyses and method validation is included in the supporting material. The limit of detection (LOD) – corresponding to a signal/noise ratio of 3 – and the limit of quantification (LOQ) – corresponding to a signal/noise ratio of 6 – were calculated using a blood sample spiked with 50 pg of standards (corresponding to 0.1 µg/L). The LODs were 0.016 µg/L for HHCB, 0.014 µg/L for AHTN, 0.019 µg/L for MK and 0.038 µg/L for MX; the respective LOQs were 0.032 µg/L, 0.028 µg/L, 0.038 µg/L and 0.076 µg/L (see supporting material).

Para-hydroxy benzoic acid (HBA). The paraben metabolite HBA was analyzed in urine samples of adolescents and adults using liquid chromatography – tandem mass spectrometry (LC-MS/MS) without prior derivatisation. Quantification was done by isotope dilution standardization. After filtration (0.2 µm cellulose acetate syringe filter) of an aliquot of the urine sample, ring-¹³C₆-p.hydroxybenzoic acid (Cambridge Isotope laboratories,

Andover, USA) was added at a concentration level of 200 µg/L. From this solution, 10 µL was injected directly onto an Agilent 1200 LC combined with an Agilent 6410 triple quadrupole MS (Agilent Technologies). Separation was done on a 100 mm x 2.1 mm ID x 3.5 µm Zorbax SB C18 column (Agilent Technologies). The column was thermostated at 30°C. Mobile phase A was 20 mM ammonium formate (adjusted to pH 3 with formic acid), mobile phase B was methanol. A gradient from 2% B to 100% B at 10 min (2 min hold) was used at a flow rate of 0.2 mL/min. Electrospray ionization in negative ion detection mode was used. The MS was operated in MRM mode, monitoring the transition 137.2 > 93.1 for HBA and the transition 143.1 > 99.1 for the internal standard. The method was validated (see Supporting Information). LOD of 50 µg/L and LOQ of 100 µg/L were obtained for this method.

TCS. TCS was measured in 3 mL urine of adolescents with GC-MS in negative ionization mode after enzymatic hydrolysis, solid phase extraction on Oasis[®] HLB, derivatization with pentafluorobenzoylchloride and subsequent clean-up of the extract on acidified silica, as described by Geens et al. (2009). The method LOQ was described as three times the standard deviation (SD) of procedural blanks and was 0.1 µg/L.

Urinary creatinine and serum lipids. Urinary creatinine was measured spectrophotometrically by the Jaffé method. Serum triglycerides (TG) and serum cholesterol (CH) levels were measured individually by spectrophotometry on a Modular analyzer (Roche diagnostics, Belgium). Total lipid (TL) concentration (mg/dL) was calculated by the following formula: $TL = 1.33 \cdot (TG + CH) + 50.5$ (Covaci et al., 2006)

Statistical analysis

Database management and statistical analyses were done with SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA).

Based on self reported questionnaire data, a score for PCP use was defined, both for adolescents and adults. The following PCPs were included in the score: day- or night crème, lip balm, body lotion, hand crème, shampoo, hair care products (hair balm or conditioner), make-up, bath- or shower products, deodorant, perfume and eau de toilette. Based on the frequency of use the following scores were given per product: 0 for ‘never’; 1 for ‘less than once a month’; 2 for ‘several times a month’; 3 for ‘once a week’; 4 for ‘several times a week’ and 5 for ‘daily’. Subsequently, the scores of all products were summed up. PCP score was either used as a continuous parameter or as a categorical parameter. In the latter case, a score below 19 was defined as low, between 19 and 26 as moderate and 27 or higher was defined as high.

Descriptive statistics of the biomarkers included geometric mean (with 95% confidence intervals (95% CI)), median (25th - 75th percentile) and 90th percentile. Values below the LOD were replaced by ½ LOD. The coefficient of variation (CV) was calculated as the ratio of the standard deviation to the mean (*100). For each of the biomarkers, the total number may be lower than 210 in adolescents or 204 in adults due to missing samples.

Starting from the biomarkers expressed in µg/L, univariate linear regression models were constructed with all possible determinants of exposure, i.e. age, gender, BMI, educational level, smoking, urbanization, PCP use (score), serum lipid levels (for musks only) and urinary creatinine levels (for HBA and TCS only). Multiple linear regression models were built between the biomarker of exposure (in µg/L) and variables showing a linear relationship with a p-value ≤0.25 in the univariate models. In the univariate models, all continuous variables (e.g. age, blood fat, urinary creatinine, etc.) were tested as categorical classes and were only considered further if linearity was demonstrated; in the multivariate models, continuous variables were used on the original scale. Serum lipid levels and the use of PCPs were forced in the models for musks, independently of the significance level in the univariate

models. In case of HBA and TCS, the same was done for urinary creatinine levels and the use of PCPs. From the multivariate models, the effect sizes for significant determinants were calculated. For the PCPs score, the effect size was calculated for a change of 8 units in the PCP score, thus reflecting the change from the upper limit of the lowest category to the lower limit of the highest category. In a second model, the separate personal care products from the questionnaire data were included in a stepwise multiple regression model, together with the variables that showed a p-value ≤ 0.25 in univariate models. This model allows identifying the most prominent determinant of the biomarker. However, since the order of introducing the products to the model may influence the strength of the relation, one should be aware that different models with slightly different results may be built.

Results

Characteristics study population

Basic characteristics for both age groups (210 adolescents and 204 adults) are presented in Table 1. The mean age of the adolescents was 14.8 (SD 0.5) years; adults were on average 33.9 years (SD 4.6). Both in the adolescent and in the adult group, there was a selection bias towards higher educated participants.

Based on self-reported questionnaire data, a score for PCP use was calculated. In the adolescents, the use of PCPs was significantly ($p < 0.001$) higher in girls (57% high; 26% moderate; 17% low) compared to boys (3% high; 25% moderate; 72% low). No significant differences were seen according to age ($p = 0.74$) possibly due to the narrow age range in the study population. High PCP use occurred more in adolescents with a higher educational level: respectively 21%, 22% and 32% of the pupils following vocational, technical or general education reported a high PCP use, but these differences were not significant ($p = 0.45$). In adults, a significantly ($p < 0.001$) higher PCP use was observed in women (71% high, 22%

moderate, 7% low) compared to men (10% high, 34% moderate and 56% low). PCP use in adults was inversely related with educational level: participants with a primary or secondary education reported a significantly ($p=0.02$) higher use of care products (58% high, 29% moderate and 13% low) compared to subjects with a tertiary education (39% high, 28% moderate and 33% low). No relation was observed between PCP use and age class in adults.

Exposure to musks

In a first step, polycyclic musks and nitromusks were analysed in 20 randomly selected blood samples of adolescents. Since MK and MX were below the LOD ($0.019\text{ }\mu\text{g/L}$ for MK and $0.038\text{ }\mu\text{g/L}$ for MX) in all samples, they were not further analysed in the rest of the samples. HHCB and AHTN were measured in all blood samples.

Descriptive statistics of the polycyclic musks are given in Table 2. Within the group of adolescents, the ratio of the minimum/maximum value was approximately 15 for AHTN, compared to about 5 for HHCB. The coefficient of variation (CV) was similar for both components, i.e. 35.7 for AHTN and 43.9 for AHTN, thus showing limited inter-individual variability.

Univariate models were used to identify the major determinants of musk concentrations in blood (in $\mu\text{g/L}$). Concentrations of HHCB in blood were significantly ($p<0.001$) and positively associated with the self reported use of PCPs (Figure 1A). Higher HHCB levels were found in girls compared to boys (GM: 0.826 (95% CI: $0.775 - 0.880$) $\mu\text{g/L}$ versus 0.645 ($0.603 - 0.690$) $\mu\text{g/L}$; $p<0.001$). These differences are most probably in line with a higher PCP use in girls. Further, blood HHCB levels were significantly and positively associated with educational level: youngster with a vocational, technical or general education showed average levels of respectively 0.552 ($0.471 - 0.648$), 0.691 ($0.637 - 0.750$) and 0.779 ($0.729 - 0.833$) $\mu\text{g/L}$ ($p=0.001$). Yet, the self-reported use of PCPs did not differ significantly

by educational level. In univariate linear regression models, blood HHCB levels were not significantly ($p>0.05$) associated with blood lipid concentration, age, BMI, smoking or urbanization (urban/rural). In a next step of the analysis, multiple linear regression models were built for HHCB. The final model explained 19% of the variance (Table 3). Blood HHCB was significantly related to PCP use ($p=0.01$) and to educational level of the adolescent ($p<0.001$). After adjustment for blood lipids, age, gender, educational level and urbanization, a change of 8 units of the PCPs score - i.e. the difference between 'low' and 'high' users - was associated with an increase of 7% in blood HHCB (Table 3). If each of the type of PCPs is considered separately, a dose-specific increase of blood HHCB was found with higher use of day- or night crème, lip balm, body lotion, shampoo and make-up (all $p<0.001$). In a stepwise linear regression model, these different types of PCPs were considered simultaneously. After adjustment for age and educational level, day and night crèmes ($p=0.03$) and body lotions ($p=0.03$) showed a significant relationship with blood HHCB.

For blood AHTN, univariate regression analyses showed no significant ($p=0.12$) relationship between blood AHTN concentrations and self-reported use of PCPs, although there was a trend for increasing blood levels with a higher consumption pattern (Figure 1B). Mean levels of blood AHTN were higher in girls compared to boys (GM: 0.129 (95% CI: 0.116 – 0.144) $\mu\text{g/L}$ *versus* 0.110 (0.097 – 0.124) $\mu\text{g/L}$); this difference was borderline significant ($p=0.047$). A highly significant ($p<0.001$) association was found between blood AHTN concentrations and educational level of the adolescents: levels increased from vocational (0.079 (0.059 – 0.106) $\mu\text{g/L}$) over technical (0.102 (0.087 – 0.122) $\mu\text{g/L}$) to general educational level (0.142 (0.133 – 0.152) $\mu\text{g/L}$). Blood AHTN levels were not significantly associated with blood lipid concentration, age, BMI, smoking or urbanization. In multiple regression models, blood AHTN was significantly associated with PCP use ($p=0.04$), blood lipid concentrations ($p=0.04$) and educational level ($p<0.001$) (Table 3). An 8-unit

increase of the PCP score was associated with a rise in blood AHTN concentration of 8%, after adjustment for blood lipids, age, gender, and educational level. With respect to specific PCPs, only the use of body lotion was significantly ($p=0.02$) related to higher blood AHTN concentrations.

Exposure to parabens

HBA, a major metabolite of parabens, was measured in urine of adolescents and adults. HBA reflects recent exposure to the sum of parabens. Descriptive statistics, both in $\mu\text{g/L}$ and in $\mu\text{g/g}$ creatinine are given in Table 2. The ratio between the minimum/maximum value (in $\mu\text{g/L}$) was 16 in adolescents and 22 in adults; the CV equaled 59.0 in adolescents and 79.6 in adults, thus pointing to a moderate inter-individual variability.

In univariate models, urinary HBA concentrations in adolescents correlated significantly positive with urinary creatinine ($p<0.001$). Also, higher values were observed in boys compared to girls (GM: 1098 (95%CI: 1007 – 1196) $\mu\text{g/L}$ versus 926 (825 – 1038) $\mu\text{g/L}$; $p=0.02$). In univariate models, no significant associations were found with age, BMI, educational level, smoking, urbanization or the use of PCPs (see Figure 1C). In multiple regression models, urinary HBA was significantly associated with creatinine, age, gender and BMI; the model explained 39% of the variance (Table 4). The quantitative relationships between urinary HBA and the determinants were as follows: for an increase of creatinine with 50 mg/dL, HBA rose by 25% ($p<0.001$); for each year of age, HBA increased with 15% ($p=0.008$); in boys compared to girls, HBA levels were 19% higher ($p=0.02$); for an increase of the body-mass index by 5 kg/m^2 , HBA levels decreased by 10% ($p=0.03$). After adjustment for creatinine, age, gender, BMI and urbanization, HBA levels rose with 6% for an increase in PCP score with 8 units, but this results was borderline non-significant ($p=0.057$). None of the separate PCPs from the questionnaire were associated significantly with HBA concentrations.

In adults, a strong and positive association was found between HBA concentrations and urinary creatinine levels ($p < 0.001$). In univariate models, urinary HBA concentrations were significantly ($p = 0.005$) and negatively associated with BMI, i.e. average levels equaled 760 (95% CI: 686 – 843) $\mu\text{g/L}$ in subjects with underweight or normal weight ($n = 136$); 585 (510 – 672) $\mu\text{g/L}$ in overweight ($n = 43$) and 537 (426 – 677) $\mu\text{g/L}$ in obese ($n = 18$). In univariate models, urinary HBA concentrations were not associated with age, gender, educational level, smoking, urbanization or PCP use (Figure 1D). In multivariate models, after adjustment for age, gender, educational level and urbanization, urinary HBA in adults was significantly associated with creatinine (+24% for a creatinine increase of 50 mg/dL ; $p < 0.001$), with BMI (+9% for a BMI increase of 5 kg/m^2 ; $p = 0.05$) and with use of PCPs (+10% for an 8-unit increase of PCPs score; $p = 0.04$) (Table 4). In total, 21% of the variance of urinary HBA was explained by these variables. When the separate PCPs were studied in univariate models, the use of day and night crème ($p = 0.02$), body lotion ($p = 0.01$), hand cream ($p = 0.001$), hair products ($p = 0.04$), make-up ($p = 0.04$) and deodorant ($p = 0.01$) were significantly associated with HBA in urine. In a stepwise multiple regression model, after adjustment for creatinine and BMI, only the use of body lotion remained in the model as significant determinant of HBA concentrations in urine.

Exposure to triclosan

TCS concentrations were measured in urine of adolescents, and represent recent exposure to TCS. Descriptive statistics is given in Table 2. The ratio between the minimum (0.10 $\mu\text{g/L}$) and maximum (706 $\mu\text{g/L}$) value amounted to more than 7000; the CV was 307, thus illustrating a very large inter-individual variability.

In univariate models, urinary TCS concentrations augmented with increasing use of PCPs ($p = 0.003$; Figure 1E). Also, levels were higher in girls compared to boys (GM: 3.50

(95% CI: 2.14 – 5.72) $\mu\text{g/L}$ *versus* 1.55 (1.11 – 2.18) $\mu\text{g/L}$, respectively; $p=0.007$), possibly due to the higher use of PCPs in girls. Urinary TCS levels were not significantly associated with age, BMI, educational level, smoking or urbanization. In multiple regression models, only the use of PCPs was retained as a significant determinant of urinary TCS levels. The model explained 8% of the variance (Table 4). After adjustment for creatinine and gender, an 8-unit increase of the PCPs score was associated with a 45% increase of TCS in urine. If the individual PCPs were considered, urinary TCS increased with a more frequent use of day- or night crème ($p=0.01$), lip balm ($p=0.02$), shampoo ($p<0.0001$), hair care products ($p=0.01$), make-up ($p=0.01$), bath- or shower product ($p=0.04$) and perfume ($p=0.01$). In a stepwise multiple regression model, day and night crème ($p=0.005$) and hair care products ($p=0.07$) were retained in the model as strongest predictors.

None of the participants in the study exceeded the biomonitoring equivalent of 2600 $\mu\text{g/L}$ or 3300 $\mu\text{g/g}$ creatinine (Krishnan et al., 2010).

Discussion

In a random population sample of 14-15 year old adolescents ($n=210$) and 20-40 year old adults ($n=204$), exposure to musks, parabens and/or TCS was assessed by measuring HHCB and AHTN in blood, HBA and TCS in urine. Inter-individual variability was small for HHCB (ratio between minimum and maximum values: order of magnitude of 5), intermediate for AHTN and HBA (minimum-maximum ratio: 15-20) and large for TCS (minimum-maximum ratio > 7000). For all measured biomarkers, a positive relationship was found with self reported use of personal care products. In ‘high’ compared to ‘low’ PCP users, levels increased by 7% ($p=0.01$) for blood HHCB, 8% ($p=0.04$) for blood AHTN, 6% for urinary HBA in adolescents ($p=0.06$), 10% for urinary HBA in adults ($p=0.02$) and 45% for urinary

TCS. After adjustment for PCP use, levels of blood HHCB and AHTN in adolescents increased significantly with higher educational level of the adolescent. Both in adults and in adolescents, urinary HBA concentrations were negatively associated with BMI. In adolescents, HBA levels also significantly differed by gender (higher in boys) and were significantly and negatively associated with age.

Information on the levels of musk compounds in the human body is sparse. In the Flemish population, the nitromusks - MK and MX - were below the LOD in a subset of 20 samples and therefore were not measured further. Both polycyclic musks could be detected in more than 92% of the samples. Accordingly, Hu et al. (2010) reported detection frequencies of respectively 91 en 77% for HHCB and AHTN, while MX and MK were all below the limit of quantification. The shift in production and usage from nitromusks to polycyclic musks (Hutter et al., 2005; Rimkus, 1999) probably can explain the low versus high detection of both musk groups. Geometric means for HHCB and AHTN in the general Flemish adolescent population were 0.717 and 0.118 µg/L, respectively. These values are in agreement with measurements of musks in other international studies (Bauer et al., 2005; Hutter et al., 2005). In this study, higher blood levels of HHCB and AHTN were associated with higher use of PCPs. Differences between girls and boys disappeared after adjustment for PCP use. In accordance, Hutter et al. (2005) described a significant positive association between HHCB and the use of PCPs, and higher HHCB levels in women compared to men. For AHTN, no significant predictors could be detected (Hutter et al., 2005). Even after adjustment of PCP use in the Flemish adolescents, significantly higher values were observed for higher educated pupils. This may indicate that there are other attitudes or characteristics that determine exposure to HHCB/AHTN that vary between adolescents from different educational level which were not taken into account in this study. This may be caused by additional types of

PCPs that were not included in the questionnaire or other potential sources of HHCB and AHTN in the environment, e.g. fish consumption (Rudel et al., 2006).

In this study, exposure to parabens was measured with the major metabolite *para*-hydroxybenzoic acid (HBA), which was detected in all samples. The geometric mean was slightly higher in adolescents (779 µg/g creatinine) than in adults (633 µg/g creatinine). The Flemish values for adolescents and adults were in the same order of magnitude as reported in an American study in children and adults (Crow et al., 2008).

In humans, parabens can be hydrolyzed to *p*-hydroxybenzoic acid (HBA), which can be conjugated before urinary excretion but they can also be excreted as intact esters. The fraction of the parabens excreted in the urine as the parent paraben (free or conjugated) versus the fraction excreted as HBA is unknown (Ye et al., 2006). One of the weaknesses of this study is that urinary HBA is a nonspecific metabolite of parabens, and its conjugates in urine may have different bioactivities. Although it is not known which fraction is excreted as parent compounds, the urinary concentration of the separate parabens (methyl, ethyl, propyl, and butyl parabens) may be a valid human exposure biomarker and therefore a better alternative for future biomonitoring studies (Ye, 2006; Soni, 2005).

Both in adolescents and in adults, a negative association was found between HBA and BMI. Similarly, Smith et al. (2012) observed lower concentrations of methylparaben and propylparaben in obese participants (BMI >30 kg/m²) compared to participants with a normal BMI, and Meeker et al. (2011) reported an inverse association of methylparaben, propylparaben and butylparaben with BMI in adult men. Pharmacokinetic differences (e.g. dilution effect) may contribute to the variation in urinary HBA levels in relation to body composition. Another possible explanation is that individuals with a high BMI may apply less

PCPs in relation with their body mass or may have different exposure profiles, concerning PCPs, food or medication (Smith et al., 2012).

In the American human biomonitoring program (NHANES 2005-2006), females had higher levels of parabens in their urine compared to males, both within the adolescent and within the adult group (Calafat et al., 2010). In the Flemish adolescents, after adjustment for urinary creatinine, age, BMI, educational level, urbanization and PCP use, boys had 19% higher levels of HBA compared to girls ($p=0.02$). These higher levels may be explained by the fact that native parabens were measured in the American study and by other sources of parabens in the environment that were not taken into account in this study, e.g. the presence of parabens in food (as preservatives) or in medication. The fact that HBA levels in adolescents were less strongly related to PCP use compared to the other biomarkers (musks and TCS) indeed suggests that the sources of parabens are much more diverse and thus more difficult to capture by means of questionnaires.

The abundant usage of TCS in consumer products was in this study reflected by a concentration well above the LOQ in all adolescents. The geometric mean for urinary TCS equaled $1.64 \mu\text{g/g creatinine}$ ($2.19 \mu\text{g/L}$), but the range of exposure was very wide (minimum-maximum range > 7000). Worldwide, TCS measurements in urine cover a wide range of concentrations with a difference of approximately three orders of magnitude for the average exposure. Biomonitoring studies in China (age 3-24 years) and Korea (age 18-69 years) showed similar urinary TCS levels, with geometric means of $3.4 \mu\text{g/g creatinine}$ ($3.8 \mu\text{g/L}$) and $1.58 \mu\text{g/g creatinine}$ ($1.68 \mu\text{g/L}$) respectively (Kim et al., 2011; Li et al., 2011). Geometric mean and median concentrations of the urinary TCS content of three studies conducted in the US were higher ($7.2 - 13.0 \mu\text{g/L}$) compared to our study (Calafat et al., 2008; Wolff et al., 2007; Ye et al., 2005).

In our study, TCS levels in urine were statistically significantly associated with the use of PCPs. Since the girls applied more PCPs, their urinary TCS concentration was higher than in male adolescents. Similarly, Li et al. (2011) found higher urinary TCS levels in females compared to males.

In 2010, biomonitoring equivalents (BE) were deduced for TCS. These health based guidance values are defined as the concentration or range of concentrations of a chemical or its metabolite(s) in a biological medium (blood, urine, or other medium) consistent with an existing health-based exposure guideline, and are derived by integrating available data on pharmacokinetics with existing chemical risk assessments (Krishnan et al., 2010). The estimated BE value for total urinary TCS, based on safety targets as proposed by the European Scientific Committee on Consumer Products, is 2.6 mg/L (or 3.3 mg/g creatinine) (Krishnan et al., 2010). This BE is based on no observed adverse effect levels in classical animal toxicological test after applying a safety factor of 100. This BE does not take into account possible low dose effects such as these observed for bisphenol A, effects that cannot be predicted by the outcomes observed at high doses (Vandenberg et al., 2012). The values of all the Flemish participants in our study were well below the BE for TCS, which is reassuring and might indicate that there is a considerable margin of safety for TCS in the Flemish population. Still, because of the possible existence of low dose effects, we cannot be certain that the observed internal exposure is entirely without significance in terms of biological or health effects.

In conclusion, the exposure values for HHCB, AHTN, HBA and TCS deduced in the second survey of the Flemish HBM campaign (2007-2011) provide a representative baseline exposure level that can be used for comparison with future biomonitoring campaigns to identify exposure trends. The high levels of detection of these biomarkers of PCPs should

505 encourage future research on the routes of exposure and toxicological properties of these
506 products.

507

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661 Table 1: Characteristics of the study population in the two age groups

Variable	Adolescents		Adults	
	Subgroup	N (%)	Subgroup	N (%)
Gender	<i>male</i>	121 (57.6%)	<i>male</i>	96 (47.1%)
	<i>female</i>	89 (42.4%)	<i>female</i>	108 (52.9%)
Age class (years)	≤ 14.5	67 (31.9%)	20-25	8 (3.9%)
	14.5-15.5	123 (58.6%)	25-30	44 (21.6%)
	> 15.5	20 (9.5%)	30-35	61 (29.9%)
			≥ 35	99 (48.5%)
BMI class (1)	<i>underweight</i>	15 (7.1%)	<i>underweight</i>	3 (1.5%)
	<i>normal weight</i>	169 (80.5%)	<i>normal weight</i>	137 (67.2%)
	<i>overweight</i>	26 (12.4%)	<i>overweight</i>	64 (31.4%)
Smoking habits	<i>non-smoker</i>	189 (91.3%)	<i>never smoked</i>	112 (55.7%)
	<i>less than daily</i>	9 (4.3%)	<i>ex-smoker</i>	42 (20.9%)
	<i>daily smoker</i>	9 (4.3%)	<i>less than daily</i>	20 (9.9%)
			<i>daily smoker</i>	27 (13.4%)
Educational level	<i>vocational</i>	20 (9.7%)	<i>primary level</i>	3 (1.5%)
	<i>technical</i>	86 (41.5%)	<i>secondary level</i>	42 (21.0%)
	<i>general</i>	101 (48.8%)	<i>tertiary level</i>	155 (77.5%)
Urbanisation (2)	<i>rural</i>	133 (63.3%)	<i>rural</i>	105 (51.5%)
	<i>urban</i>	77 (36.7%)	<i>urban</i>	99 (48.5%)
Season	<i>autumn</i>	47 (22.4%)	<i>autumn</i>	43 (21.1%)
	<i>winter</i>	62 (29.5%)	<i>winter</i>	99 (48.5%)
	<i>spring</i>	101 (48.1%)	<i>spring</i>	33 (16.2%)
	<i>summer</i>	0 (0.0%)	<i>summer</i>	29 (14.2%)
Use of personal care products (self reported) (3)	<i>low</i>	99 (49.0%)	<i>low</i>	59 (30.1%)
	<i>medium</i>	50 (24.8%)	<i>medium</i>	55 (28.1%)
	<i>high</i>	53 (26.2%)	<i>high</i>	82 (41.8%)

662 (1) For adolescents: cut-off values for BMI classes were based on Belgian growth curves,
663 specific for age and gender (<http://www.vub.ac.be/groeicurven/groeicurven.html>). For adults:
664 WHO guidelines were followed: underweight if BMI<18.5 kg/m²; normal weight if BMI:
665 18.5-25 kg/m²; overweight if BMI≥25 kg/m². (2) Rural: ≤600 inhabitants/km²; urban: >600
666 inhabitants/km². (3) Score based on use of day or night crème, lip balm, body lotion, hand

667 crème, shampoo, hair care products, make-up, bath- or shower products, deodorant, perfume
668 and eau de toilette (definition: see Methods).

669 *Table 2: Descriptive statistics of the different personal care product biomarkers*

670

Biomarker	Age group	N	% >LOD/Q	Geometric mean (95% CI)	Median (P25 – P75)	P90	Min. – Max.
Polycyclic musks in blood							
Blood HHCB (galaxolide), µg/L	adolescents	204	100%	0.717 (0.682 – 0.753)	0.754 (0.546 – 0.905)	1.139	0.301 – 1.539
Blood AHTN (tonalide), µg/L	adolescents	204	92.2%	0.118 (0.108 – 0.128)	0.127 (0.102 – 0.166)	0.216	0.020 – 0.307
Paraben metabolite (HBA) in urine							
Urinary HBA, µg/L	adolescents	206	100%	1022 (953 – 1096)	1027 (723 – 1436)	1928	324 – 5149
Urinary HBA, µg/g creatinine	adolescents	206	100%	779 (735 – 826)	760 (586 – 994)	1443	300 – 2729
Urinary HBA, µg/L	adults	197	100%	696 (642 – 755)	634 (471 – 970)	1526	212 – 4712
Urinary HBA, µg/g creatinine	adults	197	100%	633 (581 – 686)	583 (408 – 893)	1297	137 – 3777
Triclosan in urine							
Urinary TCS, µg/L	adolescents	193	100%	2.19 (1.64 – 2.92)	1.30 (0.54 – 4.91)	63.65	0.10 – 706
Urinary TCS, µg/g creatinine	adolescents	193	100%	1.64 (1.22 – 2.19)	0.92 (0.37 – 3.69)	50.12	0.07 – 882

671 HHCB: 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta- γ -2-benzo-pyran; AHTN: 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetra-
672 hydronaphtalene; HBA: *para*-hydroxy benzoic acid; TCS: triclosan

673 *Table 3: Multiple regression models for blood HHCB and blood AHTN in adolescents*

674

675

		Blood HHCB (µg/L)		Blood AHTN (µg/L)	
N		198		198	
R²		0.19		0.15	
% change in blood concentration of the biomarker for a given change of the covariate (p-value)					
Blood lipids	+ 100 mg/dL	-2%	(p=0.63)	+11%	(p=0.04)
Age	+ 1 year	-0.04%	(p=0.94)	+1.4%	(p=0.13)
Gender	male vs. female	-11%	(p=0.08)	+10%	(p=0.36)
Educational level	general vs. vocational	+37%	(p<0.001)	+77%	(p<0.001)
	technical vs. vocational	+29%	(p=0.001)	+28%	(p=0.13)
Urbanisation	rural vs. urban	-4%	(p=0.45)	/	/
Score PCP use	+ 8 units	+7%	(p=0.014)	+8%	(p=0.04)

676 Multiple linear regression models were built between the biomarker of exposure (in µg/L) and variables showing a linear
 677 relationship with a p-value ≤0.25 in the univariate models. Blood lipids and PCP use were forced in the multiple models.

678

679 *Table 4: Multiple regression models for urinary paraben metabolite (in adolescents and adults) and for urinary TCS in adolescents*
680

		Urinary HBA in adolescents (µg/L)		Urinary HBA in adults (µg/L)		Urinary TCS in adolescents (µg/L)	
N		205		193		192	
R ²		0.39		0.29		0.08	
% change in urinary concentration of the biomarker for a given change of the covariate (p-value)							
Urinary creatinine	+ 50 mg/dL	+27%	(p<0.001)	+24%	(p<0.001)	+17%	(p=0.21)
Age	+ 1 year	-15%	(p=0.008)	-0.6%	(p=0.53)	/	
Gender	male vs. female	-19%	(p=0.02)	+8%	(p=0.48)	+25%	(p=0.43)
BMI	+5 kg/m ²	-10%	(p=0.03)	-9%	(p=0.050)	/	/
Educational level	tertiary vs. primary	/	/	-15%	(p=0.36)	/	/
	secondary vs. primary	/	/	-9%	(p=0.32)	/	/
Urbanisation	rural vs. urban	-5%	(p=0.41)	+9%	(p=0.25)	/	/
Score PCP use	+ 8 units	+6%	(p=0.057)	+10%	(p=0.038)	+45%	(p=0.015)

681 HBA: *para*-hydroxy benzoic acid, TCS: triclosan. Multiple linear regression models were built between the biomarker of exposure (in µg/L) and
682 variables showing a linear relationship with a p-value ≤0.25 in the univariate models. Urinary creatinine and PCP use were forced in the multiple
683 models
684

685 **Figure legend**

686 *Figure 1: Biomarkers of exposure to PCPs in relation with self-reported use of PCPs,*
687 *unadjusted data.*

688 (A) Blood HHCB (µg/L) in adolescents; (B) blood AHTN (µg/L) in adolescents; (C) urinary
689 *para*-hydroxy benzoic acid (HBA) (µg/L) in adolescents; (D) urinary *para*-hydroxy benzoic
690 acid (HBA) (µg/L) in adults; (E) urinary triclosan (µg/L) in adolescents

693

694 Figure 1

695

696