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Investigating unmetabolized polycyclic aromatic hydrocarbons in adolescents’ urine as biomarkers of environmental exposure

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HIGHLIGHTS

• 12 of 16 parent PAHs were quantified in urine of Flemish adolescents.
• Benzo(a)pyrene showed a positive association with DNA damage.
• Parent PAH congeners do not correlate with 1-hydroxypyrene.
• Parent PAHs are useful as exposure biomarkers for biomonitoring studies.

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are of interest to human biomonitoring studies due to their carcinogenic potential. Traditionally metabolites of these compounds, like 1-hydroxypyrene, are monitored in urine, but recent methods allow the determination of the parent compounds in urine, which give additional information regarding sources and toxicity of PAHs. In order to assess the feasibility of incorporating these methods in a human biomonitoring study, the 16 USEPA parent PAHs were determined in 20 urine samples. These samples were obtained from 10 boys and 10 girls aged 14—16 years, participating in the third Flemish Environment and Health Study (Flanders, Belgium).

Of these 16 parent PAHs, nine could be determined in more than 95% of the samples and three (including benzo(a)pyrene) in more than 50%. Several correlations were found between different PAHs, but not between pyrene and its metabolite 1-hydroxypyrene. Diagnostic PAH ratios in urine and air samples pointed towards combustion sources and are in line with the ratios in environmental samples. Benzo(a)pyrene, naphthalene and fluorene have the highest carcinogenic potential in our cohort, when using toxic equivalency factors. Some associations between PAH congeners and determinants of exposure were found, while fluorene and acenaphthylene were positively associated with thyroid hormone levels and benzo(a)pyrene showed a positive correlation with DNA damage by comet assay. These results confirm that parent PAHs in urine are useful as biomarkers of exposure in biomonitoring studies.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a family of toxic...
compounds that are mainly of anthropogenic origin. As their name implies, PAHs are a family of compounds consisting of at least 2 aromatic carbon rings, without any incorporated heteroatoms. They are predominantly produced as a side product during incomplete combustion and are present in, amongst others, exhaust from fossil fuel engines, in smoke and soot from furnaces and cigarettes, and in charcoal grilled food. They are also present in asphalt, crude oil, coal and tar, and there is some deliberate production for applications such as medicine and research (ATSDR, 1995).

Although PAHs have common sources and similar properties, their environmental and toxicokinetic behaviour largely depends on their volatility and water solubility, which decreases with increasing molecular weight (Kamal et al., 2015; Li et al., 2010; Nagpal, 1993). After production, atmospheric PAHs will be distributed between the gaseous and particulate phases, where NAP is found in the gaseous phase, three-ring PAHs partitioned between gas and particulate phases, and heavier PAHs fully absorbed on the particulate phase (Kamal et al., 2015). Deposition, dry or wet, can then lead to uptake in the human food chain, both directly or via soil and surface water. Food can also be contaminated during preparation phases, either at home or by industrial preparation (EFSA, 2008). In a review Phillips (1999) concluded that ingestion after deposition on consumable plants is likely the most important exposure pathway of humans to PAHs, except for smokers and individuals that are occupationally exposed. However, inhalation and dermal exposure to PAHs are not necessarily negligible, especially when occupationally exposed (Kim et al., 2013). PAH have lipophilic properties, the logarithm of their octanol-water partition coefficient being between 3 and 8 (Nagpal, 1993). For this reason, PAH can easily cross cell membranes through passive diffusion both on the skin and inside the body. Parent PAH congeners do not directly induce DNA damage, but exert their effects mainly through metabolism and aryl hydrocarbon receptor (AhR) activation. PAHs will be metabolized by the human body into metabolites like diol-epoxides, radical cations and reactive redox active oquinones, which can all react with DNA to form DNA adducts, and can also alter proteins and lipids. On the other hand, several parent PAHs can associate with the AhR transcription factor that is present in all tissues, inducing AhR activity, which alters expression of many genes, indirectly causing changes in hormonal pathways, tumorigenesis, inflammation, cell proliferation and loss of cell adhesion (Moorthy et al., 2015; Murphy et al., 2007). The carcinogenic effects of PAHs are expected to be stronger when they have four or more rings, of which several are classified as proven, possible or probable carcinogens, but naphthalene is a notable exception that is classified by the IARC as a possible carcinogen, even though it is the lightest PAH (IARC, 2012a).

Over the last decades atmospheric emissions of PAHs have globally decreased, and will probably continue to do so (Shen et al., 2013). However, this trend is not followed in Belgium, where the levels are still high (Gao et al., 2013). Moreover, the concentrations of PAHs tend to be the highest in the most populated areas. Monitoring of atmospheric concentrations and of internal exposure of the general populations, as well as identification of important sources and potential health effects are still highly relevant in areas such as Flanders (Northern part of Belgium). Human biomonitoring is relevant since it integrates exposure by various routes.

Human biomonitoring of PAH exposure is usually done by measuring PAH metabolites in urine. Typically, 1-hydroxypyrene (1-OHPYR) is measured, but naphthalene (NAP), phenanthrene (PHE) and fluorene (FLU) metabolite measurements have also been reported in the literature (Li et al., 2008). On the other hand, parent PAHs in urine are rarely measured, possibly because measurement of the metabolites was already a somewhat established method before viable techniques for parent PAHs became available (Waidyanatha et al., 2003). Investigating them alongside metabolized PAHs could have certain benefits. Often the concentration of one or a small amount of metabolites is extrapolated to the total PAH exposure. This ignores the fact that not all sources produce the same mixture of PAHs and the differences in environmental behaviour, so by only measuring one metabolite important information might be missed (Rossella et al., 2009). Additionally, parent PAHs are expected to be less sensitive to variability of urine concentration (creatinine content) than their metabolized counterparts, because they are eliminated from the kidney by passive rather than active diffusion. As such, no corrections for urinary dilution using either urine creatinine or specific gravity (SG) would be necessary (Boeniger et al., 1993; Campo et al., 2007; Waidyanatha et al., 2001). However, some correlation between creatinine and PHE or NAP levels was observed in the past, although it was much weaker than that between creatinine and PHE or NAP metabolites (Sobus, 2008). Initially, analysis of urinary parent PAHs was conducted in occupationally exposed individuals (Campo et al., 2007, 2006; Rossella et al., 2008), but recently an Italian study focused on cohorts with differing environmental exposures in which 10 PAH congeners were determined (Ranzi et al., 2013).

In Flanders, the Flemish Environment and Health Study (FLEHS) comprises human biomonitoring studies since 1999 and 1-OHPYR has been traditionally used to monitor for PAH exposure. In the present study, urine samples of a small subpopulation of the reference adolescent population of the 2012–2015 cycle of FLEHS (FLEHS III) were analysed for parent PAHs (the 16 US-EPA priority PAHs) to assess if they could be detected and monitored within this population. These PAHs are, by total number of rings:

- 2 rings: naphthalene(NAP), acenaphthylene(ACY), acenaphthene(ACE)
- 3 rings: anthracene(ANT), phenanthrene(PHE), fluorene(FLU)
- 4 rings: chrysene(CHR), fluoranthene(FLT), pyrene(PYR), Benz(a)anthracene(BAA)
- 5 rings: benzo(a)pyrene(BAP), benzo(p)fluoranthenes(BPF), benzo(k)fluoranthenes(BKF)
- 6 rings: benzo(ghi)perylene(BghiP), indeno (1,2,3-cd)pyrene(IP)

We calculated diagnostic PAH ratios in urine to distinguish between possible sources and compared them with those observed in the atmosphere in Flanders. We tried to determine the relative toxicological contribution of PAHs, and tested if levels of PAH congeners are correlated with each other, with 1-OHPYR and with t.t.-Muconic acid (TTMA). TTMA in urine is a benzene metabolite and proxy for benzene exposure. Since benzene shares some important sources with PAHs, like motor exhaust and cigarette smoking, its correlations with PAHs in urine were tested.

We also studied associations of parental PAH levels in urine with possible determinants of exposure and with biomarkers of health effects. Although the sample size in this study is quite small for such analyses, this was an explorative test to see if results in accordance with the literature could be generated, indicating the use of parental PAH in this kind of analysis. Due to the carcinogenicity of PAH and their effect on the thyroid hormonal pathways through AhR activation (Murphy et al., 2007), associations of PAH exposure with markers of DNA damage and thyroid hormones were examined.
2. Methods

2.1. Study design adolescent reference biomonitoring campaign FLEHS III

The human biomonitoring component of FLEHS III includes measurement of more than 20 biomarkers of exposure and 30 biomarkers of effect. Major objectives relevant to this article are:

- establishing or updating reference values for internal pollutant levels for Flanders
- the comparison of regional internal pollutant levels with health based guidelines and reference values from other international studies
- obtaining more detailed knowledge on potential sources of emerging pollutants

Specific benefits of including an adolescent cohort are that long-term exposure markers measured in adolescents better reflect the local situation compared to adults and that adolescents have no occupational exposure.

2.2. Study population and sample collection

Our study was conducted on a subgroup of the FLEHS III adolescent cohort. This cohort was recruited between March and December 2013 and consisted of 208 adolescents residing in Flanders (Belgium). The population was stratified by province, where the amount of samples per province was proportional to the amount of 14–15 year old adolescents living in each province on 01/01/2012. Recruitment was then done by contacting adolescents studying at specific schools. For this, 2 schools were selected randomly per province, one in an urban area (>600 inhabitants/km²) and another in a rural area (<600 inhabitants/km²). Afterwards, with approval of the school board, letters were sent to all pupils of the desired age group, and they received a presentation to inform them about the FLEHS study. Those adolescent consenting to participate and fulfilling the inclusion criteria were invited on one of the research days organised in their school.

Inclusion criteria were: 1) residing at least 10 years in Flanders; 2) giving written informed consent which was approved by the ethical committee of the University of Antwerp; 3) being able to fill in an extensive Dutch questionnaire; 4) being between 14 and 16 years old. All participants provided a questionnaire completed by both the adolescent and the parents. This included data on personal characteristics, asthma and allergy, food consumption, perception of environmental problems and recent exposure. Additionally, a questionnaire with possibly sensitive questions on smoking, drugs, etc. was answered by the adolescents during the research day.

A spot urine sample of at least 30 mL and a 35 mL blood sample were collected during the field work in heavy metal free polyethylene containers.

A subgroup of 20 Flemish adolescents (including ten boys and ten girls) was randomly selected for a feasibility study on the quantification of PAH congeners in urine.

2.3. Analysis of exposure and effect markers

A full overview of all analysed markers is described in the final report (Steunpunt Milieu en Gezondheid, 2014). Whole blood samples for the comet assay were kept at room temperature and transported to the analysing lab within 12 h, where analysis was started immediately. All other samples (blood and urine) were treated and fractionated at the local sampling centre and transported to the central lab (Vlaams Instituut voor Technologisch Onderzoek, VITO) where they were stored at −20 °C in a biobank within 12 h after sampling. Analyses (PAH measurements, creatinine, specific gravity, urinary 8-OH-deoxyguanosine, 1-OHPYR) were performed in batch when all samples were collected.

Parental PAHs were measured in the 20 selected adolescents’ urine samples and in 6 additional quality control (QC) samples, by the Laboratory of the Toxicology Department of Clinical Sciences and Community Health, University of Milan. The QC samples were collected from two smoking volunteers to test if there was an influence on the recovery of PAHs when different tubes (cryotube, 5 mL polyethylene tube) and different volumes (60% or 90% of total tube volume) were used as containers. PAHs were analysed as described previously (Campos et al., 2011, 2014), with modifications. Briefly, urine samples were thawed at room temperature and then centrifuged at 3000 rpm for 15 min. An aliquot of 1500 μL urine was transferred to a silanized glass vial, and 1 μL of internal standard solution containing 12 deuterated PAHs was added. Analyses were extracted from samples with a 100 μm polydimethylsiloxane SPME fiber (Supelco, Milan, Italy) for 60 min at 80 °C and then desorbed into the chromatograph injection port for 10 min at 270 °C. GC-MS-MS analysis was performed using an Agilent 7890A gas chromatograph equipped with an Agilent 7000 triple quadrupole mass spectrometer (both from Agilent Technologies, Cernusco sul Naviglio, Italy), with an inert electron impact source (70 eV) operating in multiple reaction monitoring (MRM) mode and an autoinjector (CTC CombiPal, Agilent). Analyte separation was performed on a VF-Xms capillary column (30 m, 0.25 mm inner diameter [i.d.], 0.10 μm thickness; Varian, Milan, Italy).

t₀′-Muconic acid (TTMA) and 1-OHPYR were measured in urine by VITO using methods developed by Angerer and Schaller (Angerer and Schaller, 1998). TTMA is separated by means of ion chromatography and eluted using 10% acetic acid. The eluent is further separated by HPLC. Standard addition calibration is used, a reference sample is also analysed each cycle. 1-OHPYR is measured by UPLC-MS/MS after overnight enzymatic incubation. A 12C6 1-OHPYR internal standard is added. Standard addition calibration and control samples are analysed each batch.

For assessing thyroid function free triiodothyronin (FT3), free thyroxin (FT4) and thyroid stimulating hormone (TSH) were measured by the Algemeen Medisch Laboratorium (AML) in serum using a commercial immuno-assay.

The extent of DNA damage was determined by comet assay on whole blood by VITO, using single-cell gel electrophoresis following a literature method (van Goethem et al., 1997). 8-OH-deoxyguanosine was analysed at VITO using enzyme-linked immunosorbent assay (ELISA).

2.4. Data treatment

LOQ was determined as described by Campos et al. (2011). For analytes with a signal present in the zero sample, the LOQ was the concentration corresponding to five times the standard deviation of the signal in the zero sample. For analytes without a signal in the zero sample, the LOQ was the lowest point in the calibration curve giving a signal with a signal-to-noise ratio greater than 5:1.

The internal exposure of the population, expressed per volume of urine, was described by medians and percentiles and the number of samples above limit of quantification (LOQ) was checked for each congener. Variability in the population for each congener was described using the coefficient of variation (CV). For all statistical treatments, samples below the LOQ were replaced by LOQ/2. Their relative carcinogenic strength was checked by comparing their toxic equivalents (TEQ). TEQ values are obtained by multiplying the concentrations of PAHs in urine with their corresponding
3. Results and discussion

3.1. Stability during sample storage

To test the stability during storage of the samples, tubes were filled for respectively 60% and 90% of the total volume and kept at −18 °C until analysis. Analysis of all samples was executed as described in Methods section 2.3. Using the replicates of two test samples from smokers, a lower recovery was noticed for naphthalene when sample tubes were only 60% filled (sample 1: 162 ng/L; sample 2: 54.6 ng/L) as opposed to 90% filled (sample 1: 192 ng/L; sample 2: 88.5 ng/L). For the other PAHs, there were no noticeable differences, and there was no difference between the tested counting tubes and cryotubes. This confirms the loss of naphthalene, but not of less volatile PAHs with more than two aromatic rings, can occur during collection/storage when headspace is present above urine in the storage vial, due to the partitioning of naphthalene between air and the urine. Thus, we suggest filling tubes to 90% of the tube volume for both short and long term storage.

3.2. PAH concentrations in urine and diagnostic ratios

The urinary PAHs measured in the study sample are presented in Table 1. 11 of the 14 PAH congeners studied (for PHE + ANT and BKF + BBF only the sum is available) were measured in more than 50% of the samples, and 8 in 95% or more. This is a much higher frequency compared to prevalences obtained from metabolite measurements. For example, large biomonitoring studies like GERes measured metabolites of PYR, PHE and NHANES of PYR, PHE, NAP, FLU, BAA and CHR (Li et al., 2008; Schulz et al., 2009). Only metabolites of NAP, FLU, PHE and PYR are generally detected in more than 50% of the samples, which means that for other PAHs no information on population exposure could be obtained.

To our knowledge, this is the first study to report detectable median concentrations of ACY, ACE, BAP, iP and BghiP in environmentally exposed subjects. BAA and DAHA were however not detectable in any sample, and BKF + BBF only in one, thus these are not included in further discussion. Variability within the population was the biggest for NAP and ACE. Those congeners that are often not detected might have an erroneously low CV because samples below LOQ were replaced with LOQ/2.

Reported PAH concentrations were not corrected for specific gravity as it is not expected to have an important influence on urinary concentrations of PAHs. According to Campo et al. (2007), the urinary concentration of un-metabolized PAHs is generally expected to be less dependent than metabolite concentration on potential variability of urine output. Unmetabolized volatile organic compounds are thought to be eliminated via the kidney by passive diffusion, which would cause them to be independent of creatinine level and thus urine flow rate, and reflective of blood concentrations (Boeniger et al., 1993; Waidyanatha et al., 2001).

Only Ranzi et al. (2013) and Campo et al. (2014) reported values of unmetabolized PAHs (respectively 10 and 11 congeners) in urine of an unexposed population. In our study, the 16 US-EPA congeners were measured and 11 PAHs (when PHE + ANT are considered together) could be quantified. In the studies of Ranzi et al. (2013) and Campo et al. (2014) only 5 and 6 congeners respectively could be quantified in at least 50% of samples, probably because of the higher LOQs in these studies. Higher sensitivity and specificity were achieved in this study thanks to the use of a GC-MS-MS method instead of GC-MS.

In all populations, NAP showed the highest concentration, followed by PHE (or PHE + ANT in our case), and then FLU. The values reported in Table 1 are however significantly lower than those measured in occupationally exposed individuals like construction workers, road pavers, asphalt workers or coke oven workers (Campo et al., 2014, 2007, 2006; Ranzi et al., 2013; Rossella et al., 2009).

Of all PAHs, BAP is of specific interest as it is classified by IARC as carcinogenic to humans, making it a concern for policy makers. BAP was quantified in 50% of the samples in this cohort. This is considerably more than in similar cohorts using BAP metabolites. During the 2006–2011 cycle of FLEHS, BAP-tetroles were measured in 10 pooled samples of environmentally exposed adolescents and adults (5 samples for each cohort), but almost all values were below the limit of detection (unpublished results). In a study on environmentally exposed cohorts separated according to recent smoking behaviour, less than 20% of the samples showed 3-hydroxybenzo(a)pyrene (3-OHBAP) above LOQ for all but the heaviest smokers (Zhang et al., 2015). In urine samples of both steel workers and controls, no 3-, 7- or 9-OHBAP was quantifiable at all (Onyemauwa et al., 2009), nor was any BAP metabolite quantifiable in urine of native Americans before and after fish smoking (Motorykin et al., 2015). To our knowledge, the method used in this study shows highest amount of quantifiable samples. This shows that the current method is suitable for routine analysis of non-occupationally exposed individuals.

Exposure to PAHs can occur through several sources. To investigate possible environmental sources, diagnostic ratios were toxicological equivalent factor (TEF) from the literature, using BAP as the reference (TEF = 1). TEF for NAP, ACE and ACY were obtained from Nisbet and LaGoy, 1992, the others from Hester, 1998.

Pearson correlation coefficients between PAH congeners and between those congeners and TTMA and 1-OHPYR were calculated. Significant (p < 0.05) associations with determinants of exposure were determined by univariate linear regression of several variables, including sex, age, BMI, school type, education of parents, socioeconomic status, birth country parents/grandparents, smoking, alcohol consumption, food consumption, breastfeeding after birth, home use of fireplace or heater and weather variables. A description of the study population based on these variables is available as Supplementary Information Table 1.

Relationships between exposure markers (PAHs in urine, and the sum of their TEQs) and the effect markers of thyroid function and DNA damage (COMET test and 8-OH-deoxyguanosine) were examined. As the distributions of all exposure markers were significantly skewed to the right according to a Shapiro-Wilk test, they were Ln-transformed for further statistics. The sum of the PAHs’ TEQ-values was not significantly skewed, so it was not transformed. All effect markers, except % DNA damage by comet assay, were tested after Ln-transformation.

For these exposure-response relationships, relevant confounders were selected based on a literature study and internal discussion within FLEHS. For thyroid hormones, these were sex, age and BMI (Hollowell et al., 2002; Nyrnes et al., 2006; Reinehr et al., 2008). For markers of DNA damage, confounders were sex, age and smoking status (Hoffmann et al., 2005; Möller et al., 2000; Pilger and Rüdiger, 2006). Covariates were selected after univariate regression of PAHs with lifestyle factors from questionnaire data of 408 adolescents (208 from the reference population + 200 from a hotspot biomonitoring) at a significance level of p < 0.2. For our subgroup of 20 participants, these confounders and covariates were introduced into a multivariate regression model along with the exposure biomarker of interest. While confounders were forced into the model, covariates were only retained in the final model if they were significant at p < 0.05.

Microsoft Excel 2010 and Statsoft Statistica 12 were used to process the data for the study population. The whole dataset was made in Statistical Analysis Software.
calculated. Diagnostic ratios of atmospheric PAHs in Flanders are in good agreement with those in local river sediments, and indicate combustion sources rather than petrogenic ones (Charriau et al., 2009; Sanctorum et al., 2011). These atmospheric ratios display some differences with recent measurements in Flanders (Vlaamse Milieumaatschappij, 2015), but the latter still point towards combustion as the more important source. In our study, the median of the individual ratios of FLT/(FLT + PYR) in urine is 0.58, which would indicate combustion of grass, wood and coal as important sources (Yunker et al., 2002). Similarly, the IP/(IP + BghiP) urine ratio is 0.31, indicating combustion is a more important source (Yunker et al., 2002).

It should be noted that not only atmospheric concentrations, but also differences in diet and food preparation methods might be responsible for the ratios that are eventually observed (Phillips, 1999). Moreover, even when the exposure route is the same, metabolism rate in the human body might be different for each congener (Campo et al., 2007).

### 3.3. Toxic equivalents

For all compounds that were quantifiable in at least half of the samples, a TEQ was calculated using the population median, as shown in Table 1. In order to compare the toxic contribution of these doses, toxic equivalency factors from the literature were used to express the doses in terms of their toxicity relative to BAP.

The toxic equivalent of BAP was the largest, followed by that of NAP and FLT. Expressed as a percentage of the total toxicity of the measured PAHs, the contributions of BAP, FLT and NAP are respectively 54, 20 and 10%, or in total 84%. This seems to confirm the notion that toxicity needs to be taken into account as well as internal concentration levels in order to determine priority toxic compounds.

However, care should be taken with interpreting results this way. The fraction of unmetabolized PAH congeners excreted in urine is likely to be different for each congener (Campo et al., 2007). In addition, the TEF relates the carcinogenicity to the total exposure, typically doses used in lab tests, and not the concentration of PAHs in urine. Hence, the relative toxicity as presented here is only indicative, and further research is needed to confirm how the urinary concentrations relate to the total toxic effect of the compounds that entered the body. With that information, the most important PAHs for carcinogenicity could be identified, which would allow guided policy action to reduce the relevant sources for these PAHs.

### 3.4. Correlations between PAHs’s, TTMA and 1-OHPYR

As can be seen from Table 2, most correlations are found between the 3 and 4-ring PAHs FLU, PHE + ANT, FLT and PYR. CHR, a 4 ring PAH, shows fewer and less strong correlations. Of the 2-ring PAHs, ACY shows significant correlations with some 3 and 4-ring PAHs, while NAP and ACE have no correlation whatsoever. Likewise, the 5 and 6-ring PAHs show no correlations, not even with each other. The fact that most of the 5–6 ring PAHs have many samples below LOQ might contribute to this.

The lack of correlations between PAH congeners and NAP is not surprising, since it has been shown to behave differently from other PAH owing to its chemical properties (lowest molecular weight and boiling point, highest solubility in water). Its higher volatility could also result in higher loss during sample collection (Campo et al., 2007).

Based on correlations between air and urine samples, Li et al. (2010) suggested inhalation might be a larger contributor to internal exposure for NAP than for other PAHs, where ingestion may play a bigger role (Phillips, 1999). This is possibly linked to the fact that NAP is almost entirely in the gaseous phase in the atmosphere, while structures with more than 3 rings are only found to rapid metabolisation into 1-OHPYR (Campo et al., 2010; 0.2% on average (range 0.05–1.78%). This average value is in agreement to 0.2% previously reported (Campo et al., 2010). However, variation is large because the PYR concentrations show very small variations between individuals compared to the 1-OHPYR concentrations (CV: 1-OHPYR: 97%; PYR: 15%), possibly due to the variation in the polymorphism of metabolic enzymes of genetic or environmental origin. This lack of correlations suggests that extrapolation of 1-OH-PYR measurements to indicate exposure to all PAH’s has its limits, while the measurement of parental PAH’s clearly offers more information.

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Unit</th>
<th>LOQ</th>
<th>%&lt; LOQ</th>
<th>Median</th>
<th>P95</th>
<th>CV (%)</th>
<th>Median Cao</th>
<th>TEF</th>
<th>TEQ</th>
<th>% TEQ</th>
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<tr>
<td>NAP</td>
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<td>15.0</td>
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<td>41</td>
<td>&lt;LOQ</td>
<td>0.001</td>
<td>0.0015</td>
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<td>100.0</td>
<td>1.4</td>
<td>29</td>
<td>65</td>
<td>&lt;LOQ</td>
<td>0.001</td>
<td>0.0014</td>
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<td>100.0</td>
<td>10.2</td>
<td>20.9</td>
<td>41</td>
<td>20.6 + 13</td>
<td>0.0005</td>
<td>0.0051</td>
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<tr>
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<td>100.0</td>
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<td>1.58</td>
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<td>3</td>
<td>0.05</td>
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<td>0.57</td>
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<td>0.15</td>
</tr>
<tr>
<td>CHR</td>
<td>ng/L</td>
<td>0.2</td>
<td>55.0</td>
<td>0.16</td>
<td>0.24</td>
<td>42</td>
<td>0.7</td>
<td>0.03</td>
<td>0.0048</td>
<td>1.23</td>
</tr>
<tr>
<td>BAP</td>
<td>ng/L</td>
<td>0.3</td>
<td>50.0</td>
<td>0.21</td>
<td>0.44</td>
<td>45</td>
<td>&lt;LOQ</td>
<td>1</td>
<td>0.2089</td>
<td>54.02</td>
</tr>
<tr>
<td>IP</td>
<td>ng/L</td>
<td>0.3</td>
<td>65.0</td>
<td>0.29</td>
<td>0.37</td>
<td>35</td>
<td>&lt;LOQ</td>
<td>0.1</td>
<td>0.0285</td>
<td>7.37</td>
</tr>
<tr>
<td>BghiP</td>
<td>ng/L</td>
<td>0.5</td>
<td>95.0</td>
<td>0.67</td>
<td>0.80</td>
<td>19</td>
<td>&lt;LOQ</td>
<td>0.02</td>
<td>0.0134</td>
<td>3.46</td>
</tr>
<tr>
<td>BAA</td>
<td>ng/L</td>
<td>0.3</td>
<td>5</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>NC</td>
<td>2.3</td>
<td>&lt;LOQ</td>
<td>0.005</td>
<td>NC</td>
</tr>
<tr>
<td>BKF + BBF</td>
<td>ng/L</td>
<td>0.5</td>
<td>5</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>NC</td>
<td>0.5</td>
<td>1.1</td>
<td>0.05</td>
<td>NC</td>
</tr>
<tr>
<td>DAHA</td>
<td>ng/L</td>
<td>0.5</td>
<td>0</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>NC</td>
<td>&lt;LOQ</td>
<td>1.1</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>1-OHPYR</td>
<td>ng/L</td>
<td>5</td>
<td>75</td>
<td>180</td>
<td>998</td>
<td>97</td>
<td>Not applicable</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TTMA</td>
<td>µg/L</td>
<td>3</td>
<td>100.0</td>
<td>76</td>
<td>390</td>
<td>131</td>
<td>Not applicable</td>
<td></td>
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</tr>
</tbody>
</table>

*Due to median below LOQ.*

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**a** (Campo et al., 2014) control group of 40 adult men from Silesia, south Poland.

**b** (Ranzi et al., 2013) control group of 103 men and women living and working outside of the waste incinerator influence area in Modena, Italy.

**c** Values reported as < LOQ are lower than the LOQ in the respective study.

**d** Not calculated (due to median below LOQ).
No significant correlations with TTMA were found. In the literature, the associations between BTEX (benzene, toluene, ethylbenzene, xylene) and PAHs in the atmosphere have been tested, although there was not always a significant correlation (Miller et al., 2010). Not all sources will lead to internal exposure to both PAH and BTEX. Ranzi et al. (2013) found a significant effect of the proximity to a solid waste incinerator for unmetabolized urinary PAHs, but not for urinary BTEX. However, it should be noted that TTMA is a not a specific biomarker of benzene for low exposures such as those investigated in this study.

To our knowledge this is the first attempt to find correlations between PAHs in urine for an environmentally exposed population, but this has previously been done for occupationally exposed populations (Campo et al., 2007, 2006; Rossella et al., 2009). While individual correlations differ between these studies, more significant correlations were found between PAHs in occupationally exposed populations, especially for NAP and ACE. These populations that are professionally exposed to high atmospheric concentrations of PAH also show significantly higher PAH levels than ours (Campo et al., 2014). These higher levels could lead to new or strengthened correlations which were not present or weaker in less exposed individuals. Even in such populations, NAP correlations are reported to be weaker than those of other PAHs (Campo et al., 2007). No correlations between 5 and 6 ring congeners themselves or with lighter PAHs could be found, neither in our study nor in the literature.

### 3.5. Determinants of exposure

Table 3 shows the most significant associations between PAHs and determinants of exposure (on level of p < 0.05; sex, age, BMI, school type, socio-economic status, birth country parents/grandparents, passive smoking, recent consumption of ready-to-eat meals, weather variables and season). A complete table of relationships between determinants of exposure and PAH concentrations in urine is shown as Supplementary Information Table 1. Since there is no publication that statistically assessed determinants of PAH exposure using parent PAH's in urine, studies on PAH metabolites are used for comparison, if available. The results presented in this section are not conclusive evidence of associations between the determinants and the PAH congener, nor representative of the FLEHS III population of which this samples is a subgroup. Many associations have been tested. In order to avoid giving too much weight to false positives, only those with p < 0.01, or with p < 0.05 where most other PAH show the same trend, will be further discussed. For many of these associations some support can be found in the literature, and as such, our results suggest it might be useful to perform these analyses using parental PAH on larger cohorts.

Older adolescents had significantly higher levels of ACY, FLU and PHE + ANT. No literature was found confirming or contradicting these results for unmetabolized PAHs. Results of a metabolite study show lower internal exposure in an adolescent age cohort of 12–19 than both a child cohort aged 6–11 and an adult cohort (Li et al., 2008). This suggests a minimal concentration at some point during adolescence but the age analysis made by Li et al. (2008) is not detailed enough to allow comparison with our cohort.

### 3.6. Exposure-response associations

Significant exposure-response relationships between PAH congeners and thyroid hormone concentrations or percentage DNA damage are presented in Table 4. For FT3 and FT4, no previously reported significant associations
DNA damage compared to unexposed controls (Einaudi et al., 2014). Accordingly, BAP-exposed mice showed increased DNA-damage (Frenzilli et al., 2000; Hanelt et al., 1997; Speit, 1996; LaGoy, 1992). However, no associations with 8-OH-deoxyguanosine were found. Comet tests following exposure of rats and mice after oral administration of PAHs showed that many PAH metabolites activate thyroid receptor beta and are associated with transthyretin, a thyroid hormone transporting protein. Other in vitro tests by Song et al. (2012) showed effects of PAHs on the activity of an enzyme catalysing thyroid hormone formation. So although the results cannot be directly compared to the reference: *p < 0.01, **p < 0.005.

Table 3

<table>
<thead>
<tr>
<th>Determinant</th>
<th>NAP</th>
<th>ACY</th>
<th>ACE</th>
<th>FLU</th>
<th>PHE + ANT</th>
<th>FLT</th>
<th>PYR</th>
<th>CHR</th>
<th>BAP</th>
<th>IP</th>
<th>BghiP</th>
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</thead>
<tbody>
<tr>
<td>Sex: boys (reference girls)</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>School type: arts/technical (reference: general education)</td>
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<td>Socioeconomic status</td>
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<tr>
<td>Birth country parents/grandparents: Belgium (reference: not Belgium)</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Passive smoking</td>
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<td>Ready-to-eat meal past 3 days</td>
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<tr>
<td>Ozone 2 days before sampling</td>
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<td>Ozone 7 days before sampling</td>
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<tr>
<td>Temperature 7 days before sampling: 5–10 °C (reference &lt;5 °C)</td>
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<td>Solar radiation 2 days before sampling</td>
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<td>UV radiation 2 days before sampling</td>
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<tr>
<td>Temperature 7 days before sampling: 5–10 °C (reference &lt;5 °C)</td>
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<td>10–20 °C</td>
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<tr>
<td>Solar radiation 2 days before sampling</td>
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<tr>
<td>UV radiation 2 days before sampling</td>
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</tr>
<tr>
<td>Season: autumn/winter (reference: spring)</td>
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<td></td>
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</tr>
</tbody>
</table>

Table 4

Significant relationships between urinary PAH concentrations and health effect markers.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Confounders</th>
<th>Covariates</th>
<th>PAH</th>
<th>Estimate (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT3</td>
<td>Sex, age, BMI</td>
<td>Season</td>
<td>FLU</td>
<td>1.061 (1.019–1.105)</td>
<td>0.0090</td>
</tr>
<tr>
<td>FT4</td>
<td>Sex, age, BMI</td>
<td>Season</td>
<td>ACY</td>
<td>1.037 (1.009–1.066)</td>
<td>0.0147</td>
</tr>
<tr>
<td>FT4</td>
<td>Sex, age, BMI</td>
<td>Season</td>
<td>FLU</td>
<td>1.055 (1.020–1.092)</td>
<td>0.0059</td>
</tr>
<tr>
<td>Comet assay</td>
<td>Sex, age, smoking</td>
<td>Education type</td>
<td>BAP</td>
<td>1.36 (0.541–2.180)</td>
<td>0.0050</td>
</tr>
</tbody>
</table>

* Estimates are the multiplicative factor by which the effect increases if exposure increases by inter-quartile range (IQR), except for %DNA migration in the comet assay which was not ln-transformed.

** Estimate of comet assay shows the additive difference in %DNA damage caused by increase by of the exposure marker by the IQR.

with parent PAHs in urine were found. Zhu et al. (2009) found positive correlations of metabolites of FLU with TSH in Chinese unexposed males, but none of the metabolites of NAP, FLU or PYR correlated with FT3 or FT4. In vitro tests by Bekki et al. (2009) showed that many PAH metabolites activate thyroid receptor beta and are associated with transthyretin, a thyroid hormone transporting protein. Other in vitro tests by Song et al. (2012) showed effects of PAHs on the activity of an enzyme catalysing thyroid hormone formation. So although the results cannot be directly compared, an association of PAHs with thyroid function is supported by the literature.

BAP shows a positive correlation with %DNA migration by comet assay. This is not surprising as BAP is considered a strong carcinogen and is expected to have the strongest carcinogenic potential of all detectable PAHs in our study (Hester, 1998; IARC, 2012b; Nisbet and LaGoy, 1992). However, no associations with 8-OH-deoxyguanosine were found. Comet tests following exposure of rats and in vitro human cell cultures to BAP previously showed increased DNA-damage (Frenzilli et al., 2000; Hanfelt et al., 1997; Speit, 1996; Uhl et al., 2000). Accordingly, BAP-exposed mice showed increased DNA damage compared to unexposed controls (Einaudi et al., 2014). A positive association between 1-OHPYR in urine and DNA damage was found previously in a FLESH biomonitoring study (Koppen and Verheyen, 2007). One limitation to our finding is that BAP was only detected in half of the samples, while measurements under the LOQ were replaced by LOQ/2. More research, including larger cohorts, is thus needed to confirm these findings.

3.7. Strengths and limitations

The most important limitation of this study is the small sample size, as it does not allow the obtained results to be used as reference values. Also, the statistical results presented above need to be carefully interpreted due to the limited sample size, and do not present conclusive evidence of any source of exposure, or exposure-response relationship. However, this research was done in the framework of a feasibility study of measuring parent PAHs in urine of adolescents in the Flemish population. As such, we have shown that several PAHs are clearly measurable using a state of the art method of analysis in adolescents that do not have any occupational exposure. This is at least partially due to high atmospheric emissions of PAHs in Flanders (Gao et al., 2013), which have not changed much since 1990. Furthermore, our dataset confirmed some determinants of exposure that are predicted by existing literature based on atmospheric measurements or PAH metabolites, and some plausible relationships between internal exposure and markers of health effects are observed.

4. Conclusions

NAP, ACY, ACE, FLU, PHE, + ANT, FLT, PYR and BghiP were detected by GC-MS-MS, after extraction from urine and pre-concentration on an SPME fibre, in at least 19 of the 20 adolescents; CHR, BAP and IP in at least half of the population, and BAA, BKF + BBF and DAHA were (almost) never detected. The analytical technique allowed thus determination of more PAH congeners compared to the traditional PAH-metabolite measurements, which

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**Table 4**

Significant relationships between urinary PAH concentrations and health effect markers.

- **Effect:** FT3, FT4, Comet assay
- **Confounders:** Sex, age, BMI
- **Covariates:** Season, Education type
- **PAHs:** FLU, ACY, BAP
- **Estimate (95% CI):** 1.061 (1.019–1.105), 1.037 (1.009–1.066), 1.36 (0.541–2.180)
- **p Values:** 0.0090, 0.0147, 0.0050


