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Abstract: Human biomonitoring provides an integrated measure for individual exposure to environmental pollutants. Better insight in inter-individual variability of biomarkers of exposure may help in the interpretation of biomonitoring studies.
The aim was to study the impact of outliers, determine the optimal unit for fat-soluble biomarkers in serum and quantify the major determinants for biomarkers of exposure to polychlorinated aromatic hydrocarbons (PCAHs) in three age groups.
Data were obtained from the Flemish Environment and Health Study (2002-06). Marker PCBs (sum of 138, 153, 180), hexachlorobenzene (HCB) and p,p'-DDE were measured in cord blood samples of 1196 newborns, in serum samples of 1679 adolescents (14-15 years) and 1583 adults (50-65 years).

Exclusion of influential outliers in multiple linear regression models lead to models that are better applicable to the general population. In terms of adjusted R-square, the regression model with the pollutant expressed in volume-based units and blood fat as a separate independent variable was superior compared to models with other units. We found highly consistent relationships between the serum concentration of PCAHs and blood fat, age, changes in body weight, animal fat in the diet, local vegetable consumption (HCB and p,p'-DDE only) and being breastfed as a baby (in adolescents only). The impact of sex and BMI differed by age. For biomarkers of persistent pollutants that reflect long-term exposure, the relation between the covariates and the biomarkers can be well quantified.

Cover Letter

Mol, August 19, 2008

Environmental Research
To the Editor

Dear Editor,

Please find enclosed a manuscript entitled 'Determinants of polychlorinated aromatic hydrocarbons in serum in three age classes – methodological implications for biomonitoring' by E. Den Hond, E. Govarts, L. Bruckers and G. Schoeters which we would like to submit for publication in Environmental Research.

The manuscript presents results of the Flemish Environment and Health Study, a large biomonitoring trial (over 4500 participants) that was conducted in three age classes. The analysis of the data has allowed us to deduct general guidelines for biomonitoring and advices for the design and analysis of future biomonitoring trials.

We hope that the manuscript can be considered for publication in Environmental Research.

Yours sincerely,

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DETERMINANTS OF POLYCHLORINATED AROMATIC HYDROCARBONS IN SERUM IN THREE AGE CLASSES - METHODOLOGICAL IMPLICATIONS FOR BIOMONITORING.

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ABSTRACT

Human biomonitoring provides an integrated measure for individual exposure to environmental pollutants. Better insight in inter-individual variability of biomarkers of exposure may help in the interpretation of biomonitoring studies.

The aim was to study the impact of outliers, determine the optimal unit for fat-soluble biomarkers in serum and quantify the major determinants for biomarkers of exposure to polychlorinated aromatic hydrocarbons (PCAHs) in three age groups.

Data were obtained from the Flemish Environment and Health Study (2002-06). Marker PCBs (sum of 138, 153, 180), hexachlorobenzene (HCB) and p,p'-DDE were measured in cord blood samples of 1196 newborns, in serum samples of 1679 adolescents (14-15 years) and 1583 adults (50-65 years).

Exclusion of influential outliers in multiple linear regression models lead to models that are better applicable to the general population. In terms of adjusted R-square, the regression model with the pollutant expressed in volume-based units and blood fat as a separate independent variable was superior compared to models with other units. We found highly consistent relationships between the serum concentration of PCAHs and blood fat, age, changes in body weight, animal fat in the diet, local vegetable consumption (HCB and p,p'-DDE only) and being breastfed as a baby (in adolescents only). The impact of sex and BMI differed by age.

For biomarkers of persistent pollutants that reflect long-term exposure, the relation between the covariates and the biomarkers can be well quantified.

KEY WORDS

biomonitoring – biomarkers of exposure – polychlorinated aromatic hydrocarbons (PCAH) – pesticides – polychlorinated biphenyls (PCBs)

FUNDING SOURCES

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The database was obtained with permission from the Flemish Environment and Health Study Group. The Flemish Environment and Health Study was commissioned, financed and steered by the Flemish Community (department of Science, department of Public Health and department of Environment), without any responsibility for the scientific content.

APPROVAL

The study was approved by the medical ethical committee of the University of Antwerp (July 4, 2002).

INTRODUCTION

Biologic monitoring, i.e. biomonitoring, is used to assess human exposures to environmental and workplace chemicals. Biomarkers of exposure take into account inter-individual differences in absorption, distribution, biotransformation, and excretion of a substance which may be associated with differences in age, sex, height, weight, physiologic and nutritional status, duration of exposure, etc. Hence, biomarkers of exposure assess the internal dose of a xenobiotic compound and are therefore likely to be directly associated with possible adverse health effects (Lauwerys and Hoet 2001).

Between 2002 and 2006, a large biomonitoring campaign was conducted in Flanders, the Dutch-speaking part of Belgium. As biomarkers of exposure to polychlorinated aromatic hydrocarbons (PCAHs), we studied the serum concentration of polychlorinated biphenyls (sum of marker PCB 138, 153 and 180) and serum concentrations of the organochlorine pesticides hexachlorobenzene (HCB) and p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE) in more than 4400 participants of the general population including newborns (n=1196), 14- to 15-year old teenagers (n=1679) and adults between 50 and 65 years (n=1583).

The aims of this study were to use the data of this large cohort to investigate in detail the impact of outliers on summary outcomes and regression models, to compare the use of fat-adjusted *versus* volume-based units, and to determine the major factors of inter-individual variability of serum PCBs, HCB en p,p'-DDE in the three age groups.

MATERIALS AND METHODS

Environment and Health Study

Between 2002 and 2006 we have introduced in Flanders a biomonitoring network which includes measurement of biomarkers of exposure in combination with biomarkers of effect and individual health data. Flanders is an industrialized region in the North of Belgium with 6 million inhabitants, it is densely populated and has a dense traffic network. Participants were systematically recruited in eight geographical areas covering 22% of the surface area of Flanders and 20% of the population. Two areas were urbanized (Antwerp city and Ghent city), four areas were characterized by industrial settings (Ghent and Antwerp harbor, non-ferro industry, chemical industry and areas around waste incinerators), one area had intensive fruit cultivation (fruit growing area) and one area was less densely populated and had no registered emissions (rural area). Three age groups were involved in the human biomonitoring study: newborns and their mothers, 14-15 year old adolescents and adults between 50 and 65. In total, about 4500 participants were systematically recruited. The recruitment campaign was scheduled over three years (2002-2004). Inclusion criteria for participation in the campaign were residing at least five years in the area, giving written informed consent and being able to fill in an extensive Dutch questionnaire. Participants were enrolled at random within primary sampling units. By stratified sampling by study area, we selected 26 maternities, 42 secondary schools and 46 communities as primary sampling units for the newborn, adolescent and adult study, respectively. In the newborn study, 97% of the eligible mothers agreed to deliver cord blood and answer the questionnaire. In the adolescent and adult study, invitation letters were sent via the schools and by regular post. 71.6% of the adolescents and 47.5% of the adults replied to the invitation and respectively 85.7% and 75.3% of

1 those who answered, agreed to participate. The study was approved by the medical
2 ethical committee of the University of Antwerp.
3
4 *Blood collection*
5 In newborns, minimal 30 mL cord blood was collected by leaving the umbilical cord
6 blood running off in 50 mL polypropylene tubes filled with 0.5 mL Na_2EDTA as
7 anticoagulant. Cord blood was aliquoted and plasma was separated by centrifugation
8 within 24h in the maternity laboratories. The aliquoted samples were kept in the
9 refrigerator and transported to the analytical laboratory within one week. After
10 transport in cool boxes, the samples were stored at -20℃ until analysis.
11 Both in the adolescents and the adults, 40 mL of non-fasting peripheral blood was
12 sampled. Na_2EDTA (10% v/v) was added to whole blood while serum was prepared
13 by immediate centrifugation of the coagulated blood. Samples were kept in cool
14 boxes and transported to the central laboratory within 24h where they were stored at
15 -20℃ until analysis.
16
17 *Measurements in cord blood*
18 Marker PCB 138, 153, 180 and the chlorinated pesticides HCB (hexachlorobenzene)
19 and p,p'-DDE (p,p'-dichlorodiphenyldichloroethylene) were analyzed by gas
20 chromatography equipped with an electron capture detector using the method of
21 Gomara et al. (Gomara et al. 2002). In all three biomonitoring campaigns, chemical
22 analyses were performed by two labs. Both laboratories participated in the AMAP
23 proficiency testing scheme (Institut National de Santé Publique, Quebec, Canada).
24 The measurement of uncertainty was estimated from the results of AMAP samples,
25 and ranged between 21% and 34% for all the compounds except for HCB (64%). The

limit of detection for all chlorinated compounds in plasma was 0.02 µg/L. The plasma total lipid concentration was determined gravimetrically. In case no value could be obtained gravimetrically, total lipid concentration was calculated on the basis of routinely measured triglycerides and total cholesterol by the following formula: total lipids = 1.33 x (triglycerides + cholesterol) + 50.5 mg/dL (Covaci et al. 2006).

Questionnaire data

All participants completed an extensive questionnaire (self-administered), assessing information on lifestyle, dietary intake, use of tobacco and alcohol, residence history, health, education, hobbies and occupation (if applicable). Body mass index (BMI) of the mothers was calculated, based on self-reported height and weight before pregnancy. For adolescents and adults, height and body weight were measured according to a standardized protocol (WHO 1995). Dietary intake was assessed via a semi-quantitative food frequency questionnaire (FFQ) as described in detail by Bilau et al. (Bilau et al. 2008). For each individual, dietary intake of fat from different sources was estimated in grams per day, for different sources of fat i.e. beef, pork, sheep, horse, chicken, turkey, cereals, yoghurt, milk, eggs, cheese, cooking and frying fats, seafood (shrimps and mussels) and fish (lean, fatty, smoked and canned). Participants were asked whether they regularly use local food products (meat or vegetables); these answers were used as binary variables in the analysis.

Statistical Analysis

Database management and statistical analyses were done with SAS software version 9.1 (SAS Institute Inc.).

Biomarkers below the detection limit (0.02 µg/L for all compounds) were first replaced by half the detection limit. Then, concentrations were expressed in molar units by using the following conversion factors: PCB congeners 138 and 153: 1 µg = 2.771 nmol; PCB congener 180: 1 µg = 2.530 nmol; HCB: 1 µg = 4.02 nmol; and p,p'-DDE: 1 µg = 3.14 nmol. Finally, marker PCBs in molar units were added.

Data that were not normally distributed, were ln-transformed (natural logarithm) and described by their geometric means and 95% confidence intervals (CIs).

Multiple linear regression models of serum PCBs in adolescents and adults. Multiple linear regression models were build to identify the major determinants of the biomarker levels. In first instance, this was done for serum PCBs in adolescents and adults. For both age groups, separate models were investigated for different units of PCBs, i.e. (A) PCB concentration expressed in nmol/L without any adjustment for blood fat (volume-based units); (B) PCB concentration expressed in nmol/L with blood fat (mg/dL) added as a separate explanatory variable in the model (volume-based units with adjustment for blood fat); and (C) PCB concentration expressed in pmol per gram blood fat (fat-based units). Possible covariates that determine inter-individual variation of the concentration of PCBs were listed based on a literature search. A multiple linear regression model was build including covariates significant at a 10% level in the univariate analyses. Important covariates are identified by stepwise regression procedures in which we set the p-value at 0.10 for the independent variables to enter and at 0.05 to stay in the model. The adjusted R-square (coefficient of determination, R^2_a) of the obtained multiple linear regression models was used to select the "best" model. This coefficient shows the proportion of variability in the biomarker values that is accounted for by this model, penalizing for the number of explanatory variables in the model.

Variance inflation factors (VIFs) were used to analyse the effects of multicollinearity. If the VIFs were larger than 10 multicollinearity was concluded.

The assumptions of normality, constancy of variance, independence (randomness) and linearity were checked with informal diagnostic plots and formal tests (White's General test for constancy of variance, Kolmogorov-Smirnov test for normality and the lack of fit test for linearity).

Impact of outliers. An observation is considered to be outlying with respect to its biomarker value when its value lies more than 1.5 times above or below the interquartile range (IQ = the 75^{th} percentile minus the 25^{th} percentile). The impact of outliers on the summary statistics was investigated. A case may also be outlying if it has dramatic effects on the multiple regression analysis. A case may be outlying with respect to its Y value (biomarker value), its X value (covariates in the model), or both. In a first step, we identified outlying cases based on the fitted model; outliers with respect to the response were assessed using the studentized deleted residuals and outliers with respect to the predictor variables were assessed using the leverages or the diagonal elements of the hat matrix. In a second step, specific methods (DFFITS and Cook's Distance) were used to detect if these outlying cases were influential, that is cases that heavily influenced the fitted model. Models were fitted with and without influential cases and it was studied whether exclusion of the influential cases significantly changed the regression parameters.

Quantification of impact of determinants. Quantitative relationships between the determinants and the biomarkers were calculated from the estimates of the multiple linear regression model, assuming that, when quantifying the relation of one covariate with the biomarker, all other covariates in the model are fixed at the population mean.

RESULTS

Descriptive statistics and mean exposure values for the sum of marker PCBs, HCB and p,p'-DDE in the three study populations are given in Table 1.

Choice of units: volume-based versus fat-based units

Both in adolescents and in adults, we build multiple linear regression models to identify the determinants for inter-individual variability of serum PCB levels. These regression models were constructed for different units of serum PCBs, i.e. serum PCBs in nmol/L without adjustment for blood fat (model A), serum PCBs in nmol/L with blood fat as explanatory variable in the model (model B) and serum PCBs expressed in pmol/g blood fat (model C). In both age groups, inclusion of blood fat in the analysis, either as an independent variable in the model (B) or as a correction factor in the formula (C), considerably increased the variability in serum PCB levels that is explained by the model (Table 2). In adolescents, very similar results were obtained with blood fat added as an independent variable in the model (R^2_a=0.43) and with fat-based units (R^2_a=0.44). In adults, inclusion of blood fat as a separate variable in the model resulted in a considerably higher adjusted R^2 (R^2_a=0.30) compared to a model with fat-based units (R^2_a=0.19). Thus, we conclude that the model with PCBs in volume-based units with blood fat as separate independent variable in the multiple regression model is superior to the other units (i.e. explaining more variability) and will therefore be used in all further analyses.

Impact of outliers

An observation may be outlying with respect to its biomarker values. For serum PCBs in nmol/L in adolescents, 17 cases were found to be beyond the fences. Dropping

these outlying cases from the study population resulted in a similar geometric mean and 95% confidence interval (mean: 0.779 nmol/L; 95% CI: 0.761-0.798 nmol/L) compared to the whole study population (mean: 0.781 nmol/L; 95% CI: 0.762-0.801 nmol/L). In adults, 37 cases were identified as outlying. Here also, the geometric mean and confidence interval without the outlying cases (mean: 5.500 nmol/L; 95% CI: 5.396-5.605 nmol/L) were comparable with those of the whole population (mean: 5.485 nmol/L; 95% CI: 5.370-5.603 nmol/L).

A case may also be an outlier if it has dramatic effects on the regression coefficients in the linear regression models. In adolescents, 57 cases were found to be outlying with respect to the biomarker values while 55 cases were outliers with respect to the covariates; respectively 40 and 13 of them were influential. Fitting the model with or without the 53 influential cases changed the adjusted R^2 of the model from 0.43 to 0.51. In adults, 67 cases were outliers with respect to the biomarker values and 50 cases with respect to the covariates. Respectively 54 and 14 of them were found to be influential. Exclusion of the 68 influential cases resulted in an adjusted R^2 of 0.38 compared with an adjusted R^2 of 0.30 with influential cases. Thus, identification and exclusion of influential cases in multiple regression models will lead to better prediction models, with an higher proportion of the variability explained. Also, exclusion of influential outliers sometimes changed the regression coefficients (but not the sign) of the covariates and had an influence on the covariates that were retained as significant ($p<0.05$) in the model. When we exclude influential cases, these models will be better applicable to the general population, and are therefore preferential for studies in which we want to extrapolate general guidelines for biomonitoring.

Determinants of serum PCBs, HCB and p,p'-DDE.

For all age groups separately, we constructed multiple linear regression models with PCAHs expressed in nmol/L and without influential outliers in order to identify the factors that determine inter-individual variability in the serum levels of each of the three PCAHs. For serum PCBs, the model explained 39.1%, 50.8% and 38.0% of the variability in respectively newborns, adolescents and adults (Figure 1). For the chlorinated pesticides, the total variability explained was lower than in PCBs and amounted in the three age groups to respectively 21.5%, 26.0% and 43.0% for HCB, and to 22.1%, 27.9% and 26.0% for p,p'-DDE. The factors that contributed most to the variability were blood fat, age, sex, body-mass index, change in body weight, area of residence and local meat consumption (Figure 1).

Quantification of impact of determinants.

Starting from the multiple linear regression models, we used the estimates of the regression coefficients to quantify the relationship between the biomarkers of exposure and significant determinants at the 5% level (Table 3).

We found a highly consistent relationship between changes in the blood fat content and variations in the serum concentration of the three fat-soluble pollutants. In peripheral serum (adolescents and adults), an increase of the blood fat content with 100 mg/dL was associated with a 11 to 14% increase ($p<0.001$) in the concentration of PCBs, HCB or p,p'-DDE. In cord plasma, the variation among the three markers was higher with increases between 11 and 23% ($p<0.001$) for an augmentation of the blood fat content with 100 mg/dL.

Both in mothers and adults, there was a strong association between the age of the respondent and the serum/plasma concentration of all three persistent compounds.

Increases in the serum levels of the three PCAHs were highly consistent in 50 to 65 year old adults (+11 to 14% per 5 year increase of age, $p<0.001$) and more diverse in 18 to 40 year old mothers (+18 to 34% per 5 year increase of age, $p<0.001$). As the age range in adolescents was only 2.7 years, it was not surprising to find a weak relationship between the PCAHs concentration and age; we only found a significant ($p<0.01$) association between serum HCB and the adolescents' age. On the other hand, the serum PCB concentration at the age of 14 to 16 years was significantly ($p<0.01$) influenced by the age of the mother at the adolescent's birth. Also, birth weight of the adolescent was associated with HCB levels and the event of being breastfed as a baby had a major impact on the serum concentration of all three PCAHs of the teenager. Compared to adolescents who only received formula, those who were breastfed had 7%, 26% and 32% higher serum concentrations of respectively HCB, PCBs and p,p'-DDE (all $p<0.001$).

With respect to sex, we found different effects in the various age groups. In adults, serum levels of p,p'-DDE and HCB were respectively 28 and 40% ($p<0.001$) higher in women compared to men, while serum PCBs did not differ significantly between men and women. In adolescents, we found 20 to 40% ($p<0.001$) higher serum levels in boys compared to girls for all three PCAHs. Also for body-mass index, the results varied by age. In adults, serum levels of p,p'-DDE and HCB (but not PCB) were significantly and positively related to BMI, while in adolescents, the serum levels of all three PCAHs decreased significantly when BMI was rising. In the mothers of the newborns, we found contradicting results, i.e. BMI was negatively associated with serum PCBs and positively with serum HCB. For different age groups and PCAHs, however, we found very consistent relationships between changes in body weight

and serum levels of persistent compounds: a weight gain of 10 kg was associated with a decrease in the serum levels of PCBs, HCB or p,p'-DDE up to 24%.

In the mothers of the newborns, no associations were found between the food intake pattern of the last year before pregnancy and serum levels of PCAHs in cord blood. In adolescents, a positive association was found between serum PCBs, HCB or p,p'-DDE on the one hand and different sources of animal fat (dairy fat, fish fat and added fats) on the other hand. The serum level of p,p'-DDE was positively associated with fresh vegetable consumption in adolescents. In adults between 50 and 65 years old, a positive association was detected between animal fat intake (eel, mussels, chicken) and serum PCB levels. Contradictory, both total fat and milk fat were associated negatively with serum levels of HCB or p,p'-DDE in adults.

Over and beyond food intake we studied the effect of locally grown food products on serum levels of PCAHs. In mothers of newborns, consumption of local dairy products was associated with higher serum PCB levels. In adolescents, consumption of local meat was related to higher levels of all three PCAHs. In all age groups, local vegetable consumption and/or being owner of a vegetable garden was associated with increased serum levels of the persistent pesticides.

Finally, a number of variables show small effects in some age groups and for some exposure markers. Smoking was identified as confounder in adults for PCBs and for HCB, but the results were inconsistent and the impact was small. In adolescents, a higher educational level of the respondent itself was significantly associated with higher serum levels of HCB; the educational level of the family (highest of mother or father) was significantly and positively related to serum PCBs of the adolescent. In adults and mothers, significantly higher values of HCB and p,p'-DDE were found in the winter season.

DISCUSSION

In this study, we determined the major covariates of the serum levels of marker PCBs, HCB and p,p'-DDE in three different age groups. We found consistent relationships between the serum concentration of the three fat-soluble pollutants and blood fat, age, changes in body weight and sources of animal fat in the diet. The serum levels of the two persistent pesticides were positively associated with local vegetable consumption. In 14- to 15-year old adolescents, being breastfed as a baby was an important predictor of serum levels of PCAHs during puberty. The impact of sex and body-mass index differed by age. We also showed that exclusion of influential outliers in multiple linear regression models resulted in multiple regression models that are better applicable to the general population. The regression model with the pollutant expressed in volume-based units and blood fat as a separate independent variable was superior compared to models with other units in terms of adjusted R-square.

The levels reported in this study are in line with former measurements of serum levels of PCBs, HCB and p,p'-DDE in Flanders. In 1999, 200 17-18 year old adolescents and 200 adult women between the age of 50 and 65 were studied in a pilot trial for biomonitoring. The geometric mean of the summed PCBs in the youngsters was 293 pmol/g fat (Staessen et al. 2001), which is considerably higher compared to the current study (i.e. 178 pmol/g fat). Yet, this is in line with the expected, since the teenagers in the pilot trial were on average 3 years older and the levels of PCBs in the environment are declining over time. Studies in breast milk have shown that levels of PCBs have decreased by 80-90% over a period of 12 years (Van Leeuwen and Malisch 2002; Schade and Heinzow 1998). In the pilot

project, median levels in 200 women equaled 1054 pmol/g fat for PCBs, 442 pmol/g fat for HCB and 2736 pmol/g fat for p,p'-DDE (Koppen et al. 2002). These levels are also higher than the average values measured in the current study, that is 919, 273 and 2341 pmol/g fat, respectively. Possible explanations for the lower values in the current trial are the decreasing levels in the environment and the fact that in the pilot project only women were studied, which may result in higher values for this age class.

Up to now, there is no general agreement in literature on the use of units for biomarkers assessing exposure to persistent pollutants. In some studies, concentrations (mol/L) are reported, while others use fat-based units (mol/g blood fat). In this study, we could explain more of the observed variability (highest adjusted R^2) if PCAHs were expressed as volume-based units and blood fat was included as an explanatory variable in the model. Results were only presented for PCBs, but similar conclusions can be drawn for the chlorinated pesticides, HCB and p,p'-DDE (data not shown). For all three components, the relation between the serum concentration and the blood fat content was highly consistent: an increase of the blood fat content by 100 mg/dL was associated with a 10 to 14% higher concentration of PCAHs in peripheral serum (both in adolescents and in adults), and a 11 to 23% higher concentration in cord blood. Therefore, for multiple regression analysis of PCBs or chlorinated pesticides, we recommend to use the values expressed as volume-based units (nmol/L) with blood fat added as a separate independent variable in the model. This approach allows the serum concentrations of persistent pollutants to be appropriately adjusted for blood fat, and the statistical significance of other variables in the model to be independent of effects of blood fat

concentrations. These recommendations are in line with guidelines for the use of creatinine in measurements of urinary biomarkers by Barr et al. (Barr et al. 2005). Their advice for biologic monitoring measurements in spot urine is to use the urinary analyte concentration and add creatinine as a separate value in the multiple regression model. Urinary creatinine concentrations are used to adjust for dilution of spot urine samples, and thus are comparable to the use of blood fat as dilution marker for non-fasting serum or plasma.

In most studies, the major determinant of biomarkers that reflect persistent pollutants is the age of the respondent (Schade and Heinzow 1998; Dallaire et al. 2002; Falk et al. 1999; Jonsson et al. 2005; Solomon and Weiss 2002; Sweeney et al. 2001). In the current study, we also found a highly consistent relationship between biomarkers of exposure and age in 50 to 65 year old adults (+11% for PCBs, +14% for HCB and + 14% for p,p'-DDE per 5 year increase of age), while the effects were less constant in 18 to 40-year old pregnant women (+34%, +18 and +24%, respectively). Possibly, other factors such as parity or previous breastfeeding, although not identified as significant determinant, may interact with age in young mothers. The age dependency can be explained by larger buildup of PCAHs in the body throughout life. Moreover, as environmental loads op PCAHs are decreasing over time, older people have been exposed to higher levels in the past.

In adolescents, serum PCAHs were negatively related to BMI, while a positive relationship with BMI was observed in adults. These findings are in accordance with earlier studies in youngsters (Nawrot et al. 2002; Ryan et al. 1994) and in adults (Schade and Heinzow 1998; Falk et al. 1999; Jonsson et al. 2005). In adults, we

expect a positive relationship in view of the higher absolute body burden in obese subjects and the assumption of a steady state between PCAH concentration in fat tissue and in serum. In adolescents, a transient dilution effect in adipose tissue during growth spurt may explain the negative relationship between serum PCAH levels and BMI. As body fat content in teenager girls is about double that of boys in adolescence, the same mechanism may explain the lower serum values in girls compared to boys. Also the negative relationship between changes in body weight and serum levels of PCAHs is in line with this mechanism. Weight loss and lipolysis may lead to an up-concentration of PCAHs in the lipid tissue and subsequently, if we accept the assumption of steady state between fat tissue and serum, to higher serum values of PCAHs.

Nutrition is a major source of exposure to PCAHs. In mothers of newborns, we found no significant associations between serum levels of PCAHs and food consumption in the year before the pregnancy. Although this best represents the ‘usual’ food intake, the relationship between serum levels and food consumption is probably is probably troubled by the long time gap, by changing food habits during pregnancy and possibly also by a changing metabolism during pregnancy. In adolescents, the serum levels of PCAHs at the age of 14 to 16 years were associated with the use of breastfeeding as baby. This observation has been reported several times in the last decade and in different Western countries (Schade and Heinzow 1998; Falk et al. 1999; Jonsson et al. 2005; Solomon and Weiss 2002; Nawrot et al. 2002; Glynn et al. 2007; Grimvall et al. 1997). Still, according to WHO guidelines, mother’s milk remains the best nutrition for a baby (WHO 2001). With some exceptions, we can state that both in adolescents and in adults, serum levels of PCAHs were positively related to

animal food intake (meat, chicken or fish). This is in accordance with literature (Falk et al. 1999; Glynn et al. 2007; Glynn et al. 2003; He et al. 2001) and with the fact that animal fats are the major source of fat-soluble bio-accumulating pollutants. The serum levels of the chlorinated pesticides (but not PCBs) were positively associated with local vegetable consumption and/or with being owner of a vegetable garden. This indicates that there may be local contamination, either as a result of former use of DDT and HCB in the area, or even due to current illegal or ignorant use of these products in Flanders. As a result of the current biomonitoring study, the government has launched a campaign to collect old pesticides through the local community authorities.

Finally, a number of variables that are associated with the serum levels of PCAHs, are probably no determining factor in itself but rather a dummy for other underlying factors that determine exposure to or metabolism of the persistent compounds. Smoking was identified as confounder in adults, but the results were inconsistent and the impact was small. Possibly, there is co-linearity between smoking and other life style factors such as nutrition, or social class. In adolescents, a higher educational level of the respondent itself was significantly associated with higher serum levels of HCB; the educational level of mother or father was significantly and positively related to serum PCBs. It is likely that educational level is associated with nutritional habits (e.g. fish or milk consumption), living conditions, or other life style factors that may influence exposure to persistent compounds. The serum concentrations of HCB and p,p'-DDE differed by season. In adults and mothers, significantly higher values were found in the winter season. We can hypothesize that this may be due to nutritional

factors (e.g. higher animal fat consumption in winter) or to differences in body composition (e.g. weight gain in winter).

A major strength of this study was the availability of a large data set (more than 4400 individuals) for three different biomarkers of the same chemical class in different age classes (from newborn to adults). The chemical measurements, the collection of the questionnaire data, the calculation of secondary variables and the statistical analyses were performed in a consistent and uniform method for the three biomarkers and the three age groups. This has resulted in high quality data and thus allows to generalize the findings and deduct a number of recommendations for biomonitoring studies on how to handle outliers, use of units and selection of major confounders. The data show that methodological aspects and host factors are important to take into account in biomonitoring studies that perform between-group comparisons or describe relations between internal PCAH exposure and health effects.

Although the results are based on a large data set, the conclusions are still limited to the three biomarkers at exposure levels that were assessed in this study and to the age ranges of the subgroups that were included in the study. To further improve our insight in the determinants of variability in these and other biomarkers and the methodological aspects of biomonitoring studies, we need well-designed studies with large and well-documented gradients in environmental, biological and life style factors. A European biomonitoring project, enabling comparison of biomarker data and information on covariates of citizens of different countries could meet these requirements.

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ABBREVIATIONS

95% CI: 95% confidence interval

AMAP: arctic monitoring and assessment programme

BMI: body-mass index

FFQ: food frequency questionnaire

HCB: hexachlorobenzene

IQ: interquartile range

ln: natural logarithm

Na_2-EDTA: sodium ethylenediaminetetraacetic acid

ns: not significant

PCAHs: polychlorinated aromatic hydrocarbons

PCBs: polychlorinated biphenyls

p,p'-DDE: p,p'-dichlorodiphenyldichloroethylene

R^2_a: adjusted coefficient of determination

VIF: variance inflation factor

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Table 1. Descriptive statistics in three age groups.

		Mothers & newborns	**Adolescents**	**Adults**
N		1169	1679	1583
Sex	female / male	1196 / 0	792 / 887	808 / 775
Age (years)	mean ± SD	29.6 ± 4.3	14.9 ± 0.5	57.6 ± 4.1
	range	18.1 - 44.0	13.8 -16.5	49.8 - 65.3
BMI	mean ± SD	23.3 ± 4.2	20.6 ± 3.1	26.9 ± 4.2
	range	14.0 – 44.6	13.7 – 36.6	15.1 – 48.1
Smoking	% non-smokers	64.2%	86.4%	44.6%
	% former smokers	19.6%	-	37.2%
	% current smokers	16.2%	13.6%	18.3%
Blood fat in mg/dL	mean (95% CI)	209 (205-213)	444 (440-448)	612 (604-620)
Serum PCBs in nmol/L	geometric mean (95% CI)	0.333 (0.318-0.349)	0.781 (0.762-0.801)	5.485 (5.370-5.603)
Serum PCBs in pmol/g fat	geometric mean (95% CI)	226 (215-236)	178 (174-183)	919 (901-937)
Serum HCB in nmol/L	geometric mean (95% CI)	0.189 (0.180-0.198)	0.364 (0.358-0.370)	1.370 (1.329-1.412)
Serum HCB in pmol/g fat	geometric mean (95% CI)	101 (95-106)	87.9 (86.4-89.4)	273 (264-281)
Serum p,p'-DDE in nmol/L	geometric mean (95% CI)	0.993 (0.929-1.056)	1.462 (1.404-1.522)	8.885 (8.441-9.352)
Serum p,p'-DDE in pmol/g fat	geometric mean (95% CI)	515 (481-548)	529 (486-572)	2341 (2202-2479)

PCBs: sum of marker PCB 138, 153 and 180; HCB: hexachlorobenzene; p,p'-DDE: p,p'-dichlorodiphenyldichloroethane (metabolite of DDT).

Table 2. Adjusted R-square of multiple linear regression models with different units of serum PCBs as dependent variable.

	Adolescents	**Adults**
Adjusted R^2 of multiple linear regression models*		
(A) volume-based units of serum PCBs	$R^2_a = 0.3860$	$R^2_a = 0.1602$
(B) volume-based units of serum PCBs with adjustment for blood fat	$R^2_a = 0.4281$	$R^2_a = 0.2990$
(C) fat-based units of serum PCBs	$R^2_a = 0.4443$	$R^2_a = 0.1862$

* the multiple regression model was adjusted for the following covariates: *in adolescents*: BMI, sex, educational level of the family, breastfeeding, smoking during pregnancy of the mother, age mother at childbirth, study area, local meat consumption, eel fat consumption, animal fat consumption and milk fat consumption (only for A); *in adults*: age, smoking, change in body weight, BMI (only for C), study area, local meat consumption (only for B and C), eel fat consumption, mussel fat consumption, chicken fat consumption, vegetable fat consumption (only for C), consumption of liver fat (only for A) and alcohol use (only for C).

Table 3. Quantification of determinants of serum PCBs, hexachlorobenzene (HCB) and p,p'-DDE.

		Mothers & newborns cord plasma			**Adolescents** peripheral serum			**Adults** peripheral serum		
		PCBs	**HCB**	**p,p'-DDE**	**PCBs**	**HCB**	**p,p'-DDE**	**PCBs**	**HCB**	**p,p'-DDE**
N		1011	1003	1094	1387	1390	1493	1438	1506	1468
% change in biomarker concentration for a given change of the covariate										
covariates related to blood sampling										
blood fat	+ 100 mg/dL	+23%***	+11%***	+20%***	+14%***	+11%***	+11%***	+11%***	+10%***	+12%***
season	spring *vs*. winter	ns	-20%***	-23%***	ns	ns	ns	ns	-17%***	-14%**
	summer *vs*. winter	ns	-18%**	-18%**	ns	ns	ns	-	-	-
	autumn *vs*. winter	ns	-19%**	ns	ns	ns	ns	ns	ns	ns
biological covariates										
age	+5 years	+34%***	+18%***	+24%***	ns	+5%**[a]	ns	+11%***	+14%***	+14%***
sex	male *vs*. female	-	-	-	+40%***	+20%***	+26%***	ns	-40%***	-28%***
BMI	+5 kg/m²	-15%***	+6%*	ns	-40%***	-14%***	-24%***	ns	+24%***	+22%***
weight change	+10 kg	ns	ns	ns	ns	-7%***[b]	ns	-14%***	-19%***	-24%***
birth weight	+1 kg	-	-	-	ns	+3%*	ns	-	-	-
age of mother at birth	+5 years	-	-	-	+3%**	ns	ns	-	-	-
socio-economic covariates										
highest educational level of the family (mother or father)	primary *vs.* higher	ns	ns	ns	-19%*	ns	ns	ns	ns	ns
	low secondary *vs.* higher				-10%***					
	high secondary *vs.* higher				-4%*					
secondary school level of the adolescent	technical *vs.* general	-	-	-	ns	-4%*	ns	-	-	-
	vocational *vs.* general					-7%**				

-: not applicable (information not available or not relevant); ns: not significant. * p < 0.05; ** p < 0.01; *** p < 0.001. § added fats = spreading and cooking fats
[a] for increase of age with 1 year; [b] for weight change of 5 kg
Quantitative relationships between a covariate and a biomarker were calculated from the estimates of the multiple linear regression model, assuming that, all other covariates in the model are fixed at the population mean.

Table 3, continued. Quantification of determinants of serum PCBs, hexachlorobenzene (HCB) and p,p'-DDE.

		Mothers & newborns			Adolescents			Adults		
		cord plasma			peripheral serum			peripheral serum		
		PCBs	**HCB**	**p,p'-DDE**	**PCBs**	**HCB**	**p,p'-DDE**	**PCBs**	**HCB**	**p,p'-DDE**
N		1011	1003	1094	1387	1390	1493	1438	1506	1468
% change in biomarker concentration for a given change of the covariate										
life style										
smoking	former *vs*. non-smoker	ns	ns	ns	-	-	-	ns	-6%*	ns
	current *vs.* non-smoker	ns	ns	ns	ns	ns	ns	+10%***	ns	ns
breastfed as baby	yes *vs*. no	-	-	-	+26%***	+7%***	+32%***	-	-	-
food intake										
total fat	+10 g/day	ns	ns	ns	ns	ns	ns	ns	ns	-10%**
milk fat	+5 g/day	ns	ns	ns	+1.3%**	+4%**	ns	ns	-7%**	-14%**
fish fat (all fish)	+5 g/day	ns	ns	ns	ns	+6%*	ns	ns	ns	ns
eel fat	+0.1 g/day	ns	ns	ns	+1.0%*	ns	ns	+1.3%***	ns	ns
mussel fat	+0.1 g/day	ns	ns	ns	ns	ns	ns	+4%**	ns	ns
chicken fat	+0.1 g/day	ns	ns	ns	ns	ns	ns	+1%*	ns	ns
added fats§	+5 g/day	ns	ns	ns	ns	+1.3%**	ns	ns	ns	ns
fresh vegetables	+100 g/day	ns	ns	ns	ns	ns	+2%*	ns	ns	ns
local food consumption										
local meat	yes *vs*. no	ns	ns	ns	+16%***	+7%**	+19%***	+10%***	ns	ns
local dairy	yes *vs*. no	+11%*	ns	ns	ns	ns	ns	ns	ns	ns
local vegetables	yes *vs*. no	ns	+12%*	+18%**	ns	+6%**	+13%*	ns	ns	ns
vegetable garden	yes *vs*. no	ns	ns	ns	ns	ns	+14%***	ns	ns	+11%*

-: not applicable (information not available or not relevant); ns: not significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. § added fats = spreading and cooking fats

[a] for increase of age with 1 year; [b] for weight change of 5 kg

Quantitative relationships between a covariate and a biomarker were calculated from the estimates of the multiple linear regression model, assuming that, all other covariates in the model are fixed at the population mean.

Figure

Figure 1. Adjusted R-square representing the percent variability explained by the covariates in the multiple regression models for serum PCBs, hexachlorobenzene (HCB) and p,p'-DDE in three age classes.