Internal exposure to pollutants and sexual maturation in Flemish adolescents

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Flanders is densely populated with much industry and intensive farming. Sexual maturation of adolescents (aged 14–15 years) was studied in relation to internal exposure to pollutants. Serum levels of pollutants and sex hormones were measured in 1679 participants selected as a random sample of the adolescents residing in the study areas. Data on sexual development were obtained from the medical school examination files. Self-assessment questionnaires provided information on health, use of medication and lifestyle factors. In boys, serum levels of hexachlorobenzene (HCB), p,p'-DDE and polychlorinated biphenyls (sum of marker PCB138, 153 and 180) were significantly and positively associated with pubertal staging (pubic hair and genital development). Higher levels of serum HCB and blood lead were associated with, respectively, a lower and a higher risk of gynecomastia. In girls, significant and negative associations were detected between blood lead and pubic hair development; higher exposure to PCBs was significantly associated with a delay in timing of menarche. Environmental exposures to pollutants at levels actually present in the Flemish population are associated with measurable effects on pubertal development. However, further understanding of toxic mode of action and sensitive windows of exposure is needed to explain the current findings.
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Introduction

Flanders is one of the most populated areas in Europe, with a dense network of traffic roads, industrial activities and intensive farming close to habitation. The Flemish Centre for Environment and Health performed a five-year (2001–2006) biomonitoring program on neonates, adolescents and adults (aged 50–65 years). The aim of the project was to measure internal exposure to pollutants in areas differing in pollution pressure and to assess whether place of residence or observed differences in internal concentrations of pollutants were associated with biological effects. The project was financed, steered and commissioned by the Flemish Government (Department of Environment, Flemish Agency of Care and Health, Department of Science). All public information on the project can be found on the website http://www.milieu-en-gezondheid.be/English/index.html.

Schroijen et al. (2008) reported on the internal exposure of 14- to 15-year-old adolescents to pollutants measured in blood, serum or urine, and Dhooge et al. (2010) reported on the hormone levels and body size of these adolescents in relation to internal exposure to environmental pollutants.

In this study, we report on sexual maturation of 1679 adolescents aged between 14 and 15 years in relation with internal exposure to environmental pollutants. Many of the pollutants that were studied are known to have endocrine-disrupting properties. It is, however, unknown whether they can be associated with measurable effects on pubertal development at levels actually present in the Flemish

population. Polychlorinated biphenyls (PCBs) were reported to have estrogenic, anti-estrogenic and anti-androgenic activities. Some PCBs exert dioxin-like activities mediated through the aryl hydrocarbon receptor, and possibly through interactions with other nuclear receptors. The three most abundant PCBs — PCB138, 153 and 180 — have pleiotropic effects on the estrogen and androgen receptor (Hansen, 1998; Bonefeld-Jorgensen et al., 2001). p,p'-DDE is known to have anti-androgenic properties. In pubertal male rats, p,p'-DDE inhibits androgen binding to the androgen receptor, androgen-included transcriptional activity and androgen action (Kelce et al., 1995). Hexachlorobenzene (HCB) was reported to significantly decrease uterine nuclear estrogen receptor levels, which may alter ovarian function and menstrual cycle characteristics (Foster et al., 1995; Alvarez et al., 2000). Cadmium was observed to mimic the effect of estrogen by activating estrogen receptor-α and to block binding to the androgen receptor (Stoica et al., 2000; Martin et al., 2002). Lead was reported to have xenoestrogenic activities mediated through estrogen receptor-α (Martin et al., 2003) and to affect pubertal development in girls (Selevan et al., 2003; Wu et al., 2003); polycyclic aromatic hydrocarbons display AhR-mediated as well as estrogen receptor-mediated activity (Hilscherová et al., 2000).

The aim of this study was to investigate dose–response relationships between levels of internal exposure to PCBs, chlorinated pesticides or heavy metals on the one hand and pubertal development on the other hand in a group of 14- to 15-year-old Flemish adolescents. We wanted to test whether gradients in exposure to compounds with endocrine-disrupting properties are associated with variance in sexual maturation.

Materials and methods

Selection and Recruitment of Participants
A stratified clustered multistage design was used to select 1600 participants as a random sample of the adolescents residing in the study areas, comprising 22% of the Flemish territory, 20% of the Flemish population and 20% of the Flemish municipalities as described in detail by Schroijen et al. (2008). The study areas were chosen to represent different types of environmental pressure occurring in Flanders. Sampling took place in three steps: first by study area, second by entities for access to participants (i.e., the schools) and third by selection of the participants in accordance with the inclusion criteria. Inclusion criteria were being born in 1988 or 1989, studying in the third year of secondary education, living for at least 5 years in the same study area and giving informed consent (both adolescent and parents). The adolescents were enrolled through 42 schools located in the selected regions, and were sampled between

October 2003 and July 2004. For the areas around waste incinerators, it was not possible to enroll adolescents through schools, because each separate area around a particular incinerator was small and comprised only a few streets. Therefore, adolescents living near an incinerator received a home addressed letter for participation.

Of all pupils who received an invitation, 28.4% did not respond, because they did not fulfill the inclusion criteria or because they were not interested. Among the pupils who did respond, 14.7% refused to participate. Among the pupils who wanted to participate, 2.3% were excluded by the researchers because they did not reside in the area since 5 years, and 1.9% because of incomplete questionnaires or insufficient blood or urine samples. The recruitment resulted in a total of 1679 adolescents. All participants signed an informed consent form and had the right to withdraw from the study at any time. The study design was approved by the medical-ethical committee of the University of Antwerp on 4 July 2002.

Anthropometric Data and Collection of Blood and Urine
Height and body weight of the adolescents were measured by a study nurse. Body mass index (BMI) was calculated as the weight/height^2 (kg/m^2).

Each participant donated a urine sample of about 200 ml and a blood sample of 40 ml for subsequent analysis. Serum samples were prepared by immediate centrifugation of the coagulated blood. Urine, whole blood and serum samples were fractionated immediately and stored at −20°C until analysis.

Chemical Analysis of Biomarkers of Exposure
The laboratories involved in the analyses of biomarkers applied standard agreed quality control/quality assurance procedures. All analytical methods are described in detail by Schroijen et al. (2008).

In short, lead and cadmium concentrations in whole blood were determined after an acid digestion pretreatment destroying the organic matrix and a ten times dilution, followed by high-resolution inductively coupled plasma–mass spectrometry detection (ICP-MS) (Schroijen et al., 2008). The limits of detection (LOD) for cadmium and lead in the whole blood were 0.09 and 2.0 μg/l, respectively. Isotope Cd114 was used to quantify the amount of cadmium in urine using ICP-MS. Urine samples were diluted in nitric acid (0.7%). Rhodium was used as an internal standard. The LOD for urinary cadmium equaled 0.002 μg/l. Urinary cadmium levels were expressed in μg/g creatinine. Urinary creatinine was measured by a certified clinical laboratory using the Jaffé method on Modular Roche and Roche reagentia. The coefficient of variation on daily internal quality control ranged between 2.25% (Lyphochek Bio-Rad of 270 mg creatinine per dl) and 2.80% (Lyphochek Bio-Rad of 85 mg/dl).

HCB, p,p'-DDE and PCB congeners 138, 153 and 180 were measured in serum using gas chromatography-electron capture detection (Covaci and Schepens, 2001). The LOD of all chlorinated compounds in serum was $0.02\,\mu g/l$. Blood fat was calculated on the basis of serum cholesterol and serum triglycerides (Covaci et al., 2006). Levels of chlorinated compounds were expressed in ng/g lipid.

The measurement of urinary 1-hydroxypyrene (1-OHP) — a metabolite of pyrene — and urinary t,t'-muconic acid (t,t'-MA) — a metabolite of benzene — was performed using high-performance liquid chromatography (Angerer and Schaller, 1997). The LOD was $0.030\,\mu g/l$ and $0.0086\,mg/l$, respectively. Levels of 1-OHP and t,t'-MA were expressed in $\mu g/g$ creatinine and mg/g creatinine, respectively.

For all calculations, values below the LOD were set on half the LOD.

Measurement of Hormone Levels

Sex hormone levels were measured in boys. Commercial immunoassays were used to determine serum levels of total testosterone (T) (Medgenix, Fleurus, Belgium), luteinizing hormone (LH), sex hormone binding globulin (SHBG; Orion Diagnostica, Espoo, Finland) and total 17β-estradiol (E2) (Clinical Assay; DiaSorin s.r.l., Saluggia, Italy; adapted protocol with use of double amount of serum). The free fractions of testosterone (fT) and estradiol (fE2) were calculated from SHBG and T, respectively, E2 in serum, assuming a fixed albumin concentration using a validated equation (Szulc et al., 2004). The intra- and inter-assay coefficients of variation for all assays were less than 12%. For each individual, the aromatase index — the ratio of T on E2 — was calculated as pmol/pmol.

Questionnaires

Information on lifestyle, health status, personal factors, food intake (Bilau et al., 2008) and risk perception (Keune et al., 2008) was obtained by self-assessment questionnaires filled out by the adolescents and by their parents. The parents provided information on their education, health status, housing, residence history, family composition, social and financial situation, density of nearby traffic and in-house use of pesticides. The adolescents gave information on health status, use of medication, exposure to traffic, in-house exposures to pollutants and chemicals, sports, hobbies, contact with pets, smoking behavior and consumption of alcohol and drugs. Smokers were defined as smoking at least daily.

Adolescents also completed two food frequency questionnaires to assess the daily consumption of fruit and vegetables on the one hand, and fat-containing food items on the other hand during the last year. On the basis of these questionnaires, a number of parameters were calculated, including consumption (g/day) of fresh fruit and vegetables, cereals, fish, dairy products, meat and daily intake of animal fat (Bilau et al., 2008).

Data on Sexual Development

Data on growth and sexual development for 767 boys and 636 girls were obtained from the 25 centers for school health examination. In Flanders, school doctors routinely examine growth and pubertal development when pupils are attending the third year of secondary school (aged 14–15 years). The informed consent for the study — which was signed by the adolescent and by the parents — included the permission to apply for these data and use them as health outcome parameters in the biomonitoring study.

The human biomonitoring campaign and the school health examinations were performed within the same school year (running from September 2003 to June 2004). The median time period between the blood collection for measurement of biomarkers and the measurement of the sexual development equaled 35 days (interquartile range: −56 to 141 days). In boys, gynecomasty was reported. Sexual development was routinely measured through the international score of Marshall and Tanner (1968, 1969), on a scale from 1 (start of puberty) to 5 (adult stage). In boys, genital (stages G1–G5) and pubic hair (P1–P5) development was assessed; in girls, breast (B1–B5) and pubic hair (P1–P5) development were scored. Shortly before the biomonitoring study was conducted, all school doctors had received a re-training for the assessment of pubertal development since new Flemish growth curves were developed in the period 2000–2004 (Roelants et al., 2009). For boys, as their sexual development is slower and as stages 2 and 3 can be distinguished more reliably than stages 3 and 4, reaching stage 3 was used as a benchmark for male sexual development. For girls, reaching stage 4 was used as cutoff value.

In girls, information on age at menarche and regular/irregular menses was obtained through self-assessed questionnaires. Age at menarche was studied as a binary outcome; that is, the median age at menarche was used as a cutoff to classify individual below or above the median.

Statistical Analysis

Database management and statistical analyses, for male and female adolescents separately, were performed with SAS for Windows, version 9.1.3 and Statistica, version 7.1. Data that were not normally distributed were subjected to Neperian logarithmic transformation for use as dependent variables in the statistical analyses. Medians with 10th and 90th percentile or geometric means with 95% confidence interval (95% CI) are reported.

In boys, average hormone levels were calculated by pubertal stage (genital stage or pubic hair stage) and geometric means of hormone levels were compared between the five pubertal stages by analysis of variance.

In all regression analyses, data were adjusted for some prespecified, literature-based confounders and for covariates showing a significant association with effect parameters in single-regression analyses. Both in boys and girls, age, BMI

and smoking were selected as confounders for all outcome parameters; in girls, use of oral contraception was also considered as a confounder for pubertal development and age at menarche. In addition, parameters related to food intake and other lifestyle parameters were included as covariates in multiple regression or logistic regression models if they showed a significant association ($P < 0.1$) with the dependent variable under study in simple linear or logistic regression.

Separately for boys and girls, dose–response relationships were calculated between biomarkers of exposure and effect, using multiple logistic regression, allowing for the confounders and significant covariates. Odds ratios with 95% CI were calculated from the logistic regression coefficients for a twofold increase in the biomarker of exposure. Multiple linear regression models were used to study the relation between exposure and age at menarche. From the regression coefficients, the days of delay in age at menarche were calculated for a doubling of the exposure.

To study the association of biological effect parameters and exposure makers after adjustment for other concurrent markers of internal exposures, we performed a series of forward stepwise logistic regressions or multiple regressions with the biological effect parameter as dependent variable and predetermined confounders, significant covariates and all exposure markers as independent variables in the model. In stepwise logistic regressions, the P-value was set at 0.25 to enter the model and at 0.26 to stay in the model; in stepwise multiple regressions, the F-value was set at 1.2 to enter the model and at 1.1 to stay in the model.

Results

Characteristics of Participants, Nutritional and Other Lifestyle Factors
Of the boys ($n = 887$), 14.5% drank alcoholic beverages at least weekly, 8.2% smoked daily and 46.6% lived in a family in which at least one parent had received higher education. Respective data in girls ($n = 792$) were 13.8%, 7.7% and 53.1%. Of the girls, 9.3% used oral contraception. Other characteristics of participants are summarized in Table 1.

Internal Exposure to Pollutants
Data on internal exposure (median values and 10th and 90th percentiles) to pollutants for boys and girls separately are given in Table 2. Levels of lead and persistent chlorinated compounds were significantly higher in boys than in girls ($P < 0.001$).

Both in boys and in girls, significant ($P < 0.05$) and positive correlations were observed between the different biomarkers of exposure: the concentrations of the sum of PCB138, 153 and 180 correlated significantly with the concentration of HCB (Pearson correlation coefficient on ln-transformed data: $r = 0.45$ and 0.44, respectively, in boys

Table 1. Characteristics of the participants and food intake by sex.

	Boys ($n = 887$)	Girls ($n = 792$)
Age (years)	14.8 (14.3–15.7)	14.9 (14.3–15.6)
Height (cm)	171 (159–180)	164 (157–172)
BMI (kg/m^2)	19.8 (17.3–23.9)	20.4 (17.3–24.8)
Food and nutrient intake (g/day)		
Fresh fruit and vegetables	238 (76–549)	270 (89–591)
Cereals	168 (76–313)	138 (60–242)
Fish	14.6 (2.5–40.6)	14.1 (1.4–35.6)
Dairy products	262 (67–593)	185 (57–464)
Meat	111 (44–196)	90 (31–171)
Animal fat	30.3 (16.6–58.6)	23.6 (12.6–46.0)

Data are medians (10th–90th percentile).

Table 2. Internal exposure to pollutants in boys and girls.

	Boys ($n = 887$)	Girls ($n = 792$)	P-value
Blood cadmium (µg/l)	0.39 (0.05–1.27)	0.39 (0.05–1.24)	0.51
Blood lead (µg/l)	25.0 (12.0–51.2)	18.1 (8.8–38.1)	<0.001
HCB in serum (ng/g fat)	22.8 (15.1–34.5)	18.3 (12.3–6.5)	<0.001
p,p'-DDE in serum (ng/g fat)	104 (47–404)	84 (39–247)	<0.001
Sum of marker PCBs in serum (ng/g fat)	79.8 (42.7–141.3)	53.1 (30.3–98.5)	<0.001

HCB, hexachlorobenzene; PCB, polychlorinated biphenyl.
Data are medians (10th–90th percentile).

and in girls) and p,p'-DDE ($r = 0.19$ and 0.26). The serum levels of HCB were significantly and positively correlated with levels of p,p'-DDE ($r = 0.19$ and 0.19). Blood levels of cadmium were significantly correlated with blood lead levels ($r = 0.28$ and 0.29). The high correlation coefficients show that humans are exposed to these compounds by common sources. PCBs, HCB and p,p'-DDE are persistent lipophilic compounds that accumulate in the food chain. The main source for humans is contaminated food. Exposure to lead and cadmium mainly occurs through inhalation, and to a lesser extent through drinking water and soil contamination.

Sexual Development and Its Relation to Hormone Levels
Table 3 shows the number of boys that reached the different stages of genital development (G1–G5) and of pubic hair development (P1–P5), and the hormone levels in blood by pubertal stage. For T, fT, E2, fE2, the aromatase index and LH, a gradual rise of hormone levels was observed between stages 1 and 4, whereas all hormone concentrations reached a plateau between stages 4 and 5. For SHBG, an opposite trend was detected; that is, decreasing levels between stages 1 and 4 and a leveling off between stages 4 and 5. These trends were observed both for genital stages and for pubic hair stages. Our observations are perfectly in line with the changing hormone levels in boys that are reported during

Table 3. Hormone levels in relation to pubertal stage in male adolescents.

	Stage of genital development					ANOVA	Stage of pubic hair development					ANOVA
	G1	G2	G3	G4	G5	*P*-value	P1	P2	P3	P4	P5	*P*-value
Number of boys	8	61	214	282	202		17	77	209	260	206	
Total testosterone (T) (ng/dl)	65	156	327	442	449	<0.001	121	176	352	443	438	<0.001
Free testosterone (fT) (ng/dl)	0.93	2.23	6.30	9.63	10.08	<0.001	1.57	2.47	6.84	9.81	9.82	<0.001
Total estradiol (E2) (pg/ml)	9.3	10.0	13.0	16.4	17.3	<0.001	9.1	10.2	13.7	16.4	18.9	<0.001
Free estradiol (fE2) (pg/ml)	0.131	0.135	0.221	0.305	0.323	<0.001	0.121	0.140	0.235	0.306	0.347	<0.001
Aromatase index	7.3	14.4	24.3	26.9	26.0	<0.001	12.3	16.0	25.2	27.2	25.1	<0.001
SHBG (nmol/l)	63.2	68.7	42.6	31.2	30.2	<0.001	70.6	66.7	39.7	29.7	31.3	<0.001
LH (IU/ml)	1.55	1.79	2.66	3.27	3.43	<0.001	1.48	1.85	2.66	3.44	3.49	<0.001

Pubertal stages are defined according to Marshall and Tanner (1968). Hormone levels are geometric means.
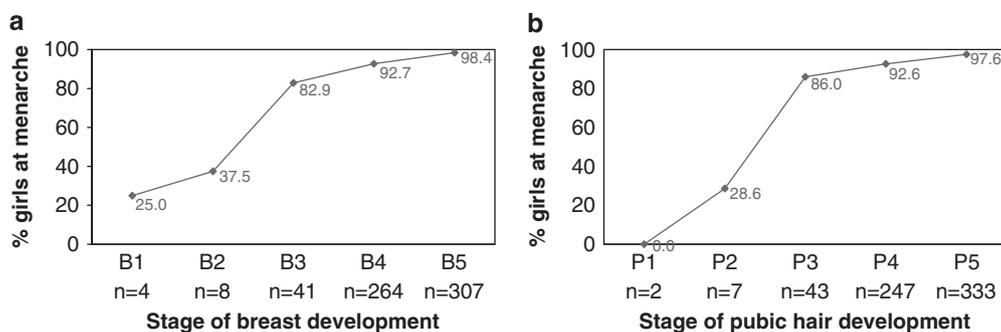

Figure 1. Percentage of girls that has passed menarche for each stage of breast development (a) or stage of pubic hair development (b). Pubertal stages are defined according to Marshall and Tanner (1969). Data on breast development were missing in 12 participants; data on pubic hair development were missing in 4 participants.
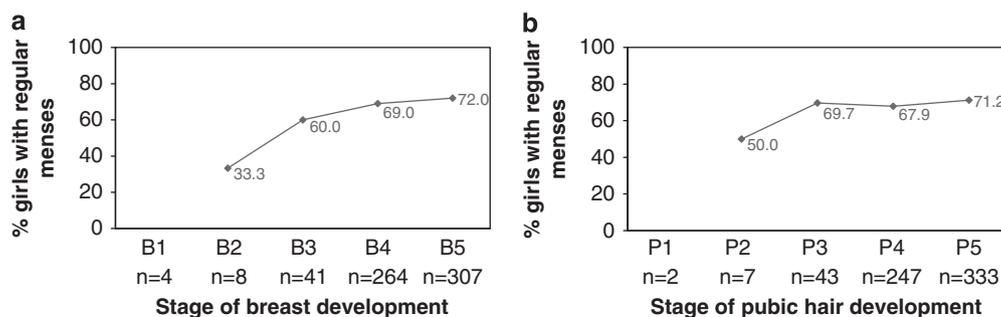

Figure 2. Percentage of girls having regular menses for each stage of breast development (a) or stage of pubic hair development (b). Pubertal stages are defined according to Marshall and Tanner (1969). Data on breast development were missing in 12 participants; data on pubic hair development were missing in 4 participants.

puberty (Kletter et al., 1993) and thus show the validity of assessment of the pubertal stages by the school doctors.

Proportions of girls by stage of breast development (B1–B5) and of pubic hair development (P1–P5) are given in Figure 1. As girls reach puberty earlier than boys, only few girls occur in the lower pubertal stages (B1 and P1). Pubertal stages recorded by the school physicians were compared with the data on menarche, reported by the adolescents in the questionnaires. Both for breast development (Figure 1a) and for pubic hair growth (Figure 1b), a clear positive relationship was observed between the pubertal stage and the proportion of the girls that had reached menarche. Similar positive associations were observed between sexual development and the proportion of girls having regular menses (Figure 2a and b). This, again, shows the validity of the data on pubertal development assessed by the school physicians.

Pubertal Stages in Boys in Relation to Internal Exposure to Pollutants

In boys, serum levels of HCB, p,p'-DDE and marker PCBs were significantly and positively associated with pubertal development (Table 4). A twofold increase in the serum concentration of HCB was significantly associated with higher odds ratios for the presence of genital stages G3-G4-G5 and pubic hair stages P3-P4-P5. These odds ratios were 3.01 and 3.92, respectively (Table 4). Also the internal dose of p,p'-DDE was associated with a higher chance of being in a higher stage of genital development of pubic hair development. A twofold increase of serum p,p'-DDE was associated with an odds ratio of, respectively, 1.52 and 1.50 (Table 4). Similarly, a twofold increase of the summed concentration of marker PCB138, 153 and 180 was associated with a higher odds ratio to have reached at least stage 3 of genital development (odds ratio = 2.98) or pubic hair development (odds ratio = 2.59) (Table 4).

No significant associations were observed between male sexual maturation and levels of blood lead, blood cadmium, urinary cadmium, urinary 1-OHP or urinary t,t'-MA.

As subjects are exposed to a mixture of pollutants and as strong correlations were observed between several biomarkers of exposure, the effect of multiple exposure was studied by forward stepwise logistic regressions starting with a model that included all confounders, all significant covariates and the different biomarkers of exposure. The results (Table 5) show that, after adjustment for the other pollutants, genital development was significantly associated with exposure to p,p'-DDE and PCBs. For pubic hair development in boys,

Table 4. Association between exposure and sexual maturation.

Exposure	Effect	Odds ratio for doubling of exposure (95% CI)	P-value
Boys			
HCB in serum	Reaching stage 3 for genital development[a]	3.01 (1.63–5.56)	<0.001
p,p'-DDE in serum	Reaching stage 3 for genital development[a]	1.52 (1.19–1.95)	<0.001
Sum of PCB138, 153 and 180 in serum	Reaching stage 3 for genital development[a]	2.98 (1.84–4.81)	<0.001
HCB in serum	Reaching stage 3 for pubic hair development[b]	3.92 (2.19–7.00)	<0.001
p,p'-DDE in serum	Reaching stage 3 for pubic hair development[b]	1.50 (1.19–1.89)	<0.001
Sum of PCB138, 153 and 180 in serum	Reaching stage 3 for pubic hair development[b]	2.59 (1.67–4.00)	<0.001
Blood lead	Gynecomastia[c]	1.84 (1.11–3.05)	0.018
HCB in serum	Gynecomastia[c]	0.38 (0.15–0.93)	0.035
Girls			
Blood lead	Reaching stage 4 for pubic hair development[d]	0.65 (0.45–0.93)	0.020
Sum of PCB138, 153 and 180 in serum	Menarche later than the median (12 years and 9 months)[e]	1.41 (1.07–1.86)	0.015

Biomarkers of exposure were expressed in ng/g fat and ln transformed.
[a]Adjusted for age, BMI and smoking.
[b]Adjusted for age, BMI, smoking and consumption of meat.
[c]Adjusted for age, BMI, smoking and parental education.
[d]Adjusted for age, BMI, smoking and oral contraception use.
[e]Adjusted for age, BMI, smoking, parental education and alcohol consumption.

Table 5. Association between multiple exposure and sexual maturation in boys.

	Reaching G3 for genital development		Reaching P3 for pubic hair development		Gynecomastia	
	Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value	Odds ratio (95% CI)	P-value
HCB in serum	1.79 (0.85–3,77)	0.13	2.43 (1.24–4.76)	0.01	3.01 (1.63–5.56)	<0.001
p,p'-DDE in serum	1.32 (1.02–1.70)	0.035	1.25 (0.98–1.59)	0.068	Excluded	NA
Sum of PCB138, 153 and 180 in serum	2.25 (1.20–4.20)	0.011	1.60 (0.95–2.73)	0.080	Excluded	NA
Blood lead	No association	NA	No association	NA	1.84 (1.11–3.05)	0.018
Blood cadmium	No association	NA	No association	NA	No association	NA
Confounders, covariates in the model	Age, BMI		Age, BMI, meat consumption		BMI	

Odds ratios were computed by forward stepwise logistic regression starting with a model including all confounders, significant covariates and significant biomarkers of exposure. Odds ratios were calculated for a doubling of the exposure.
No association, variable showed no significant association in single regression and thus was not included in the stepwise regression; excluded, variable was not retained in the model resulting from stepwise regression; NA, not available.

only serum HCB remained significant in a multiple exposure model whereas the association with *p,p'*-DDE and marker PCBs became (borderline) insignificant (Table 5).

Gynecomastia in Boys in Relation to Internal Exposure to Pollutants

Boys who were described as having gynecomastia ($n = 31$) showed, after correction for age, BMI, smoking and parental education, higher blood lead levels (29.8 *vs* 23.9 μg/l; $P = 0.02$) and lower serum HCB levels (18.5 ng/g fat *vs* 22.8 ng/g fat, $P = 0.001$) compared with boys in whom no gynecomastia was reported. A doubling of blood lead levels was associated with significantly higher odds for having gynecomastia (odds ratio = 1.84). Higher serum levels of HCB, on the contrary, were associated with significantly lower odds for having gynecomastia (odds ratio was 0.38 for a doubling of serum HCB levels) (Table 4). When the effect of multiple exposure was studied by forward stepwise logistic regressions, blood lead and serum HCB remained significant in the multiple exposure model (Table 5). After adjustment for confounding factors, sexual maturation showed a positive association with gynecomastia, which was significant for sexual maturation in terms of having reached at least stage 3 of pubic hair development (data not shown).

Sexual Maturation in Girls in Relation to Internal Exposure to Pollutants

In girls, a twofold increase of blood lead was significantly associated with a 35% times lower odds of belonging to pubic hair stages P4-P5. The odds ratio equaled 0.65 (Table 4). After adjustment for age, BMI, smoking and contraception, girls in stages P4 or P5 had significantly lower blood lead levels (mean ± SE = 18.4 ± 1.06 μg/l) than girls in stages P1, P2 or P3 (22.5 ± 1.11 μg/l) ($P = 0.04$). However, blood lead concentrations were very similar in girls belonging to stages P4 or P5 (Figure 3). None of the other biomarkers of exposure were significantly associated with breast development or pubic hair development in girls.

The median age at menarche in girls equaled 12 years and 9 months. This value was used as cutoff to study the relationship with exposure markers. Age at menarche occurred later if exposure to PCBs was higher: a twofold increase of the summed concentration of marker PCB138, 153 and 180 was associated with a higher odds to reach menarche after the age of 12 years and 9 months. The odds ratio equaled 1.41 (95% CI: 1.07–1.86; $P = 0.01$). In multiple regression with parental education, smoking, use of alcohol and BMI as covariates, a doubling of internal exposure to marker PCB was associated with a delay of menarche of 80 days ($P < 0.01$).

The effect of multiple exposure was studied by forward stepwise multiple regression starting with a model that included all confounders, all significant covariates and the different biomarkers of exposure. The sum of marker PCBs
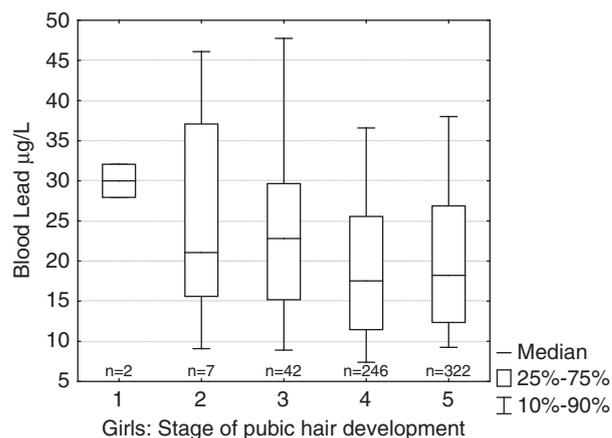

Figure 3. Blood lead levels in girls according to stage of pubic hair development. Pubertal stages are defined according to Marshall and Tanner (1969). Data on pubic hair development were missing in four participants.

was the only biomarker of exposure that remained significant in the multiple exposure model.

Discussion

The exposure levels reported in this study are in line with currently expected doses in Flanders. In 1999, a group of 17- to 18-year-old adolescents ($n = 200$) was studied in a pilot trial for human biomonitoring.

The geometric mean of the summed marker PCBs equaled 1.67 nmol/l or 377 pmol/g fat in the male adolescents and 1.02 nmol/l or 210 pmol/g fat in the female adolescents in the pilot trial (Staessen et al., 2001), which is considerably higher compared to this study (i.e., 0.92 nmol/l or 216 pmol/g fat in the boys and 0.65 nmol/l or 144 pmol/g fat in the girls). Possible explanations for the different exposure levels in the two studies are the differences in age (teenagers in the pilot trial were on average 3 years older) and the decreasing levels of persistent chlorinated compounds in the environment. Studies in breast milk have shown that level of PCBs in Western countries has decreased by 80–90% over a period of 10–12 years (Schade and Heinzow, 1998). Thus, the 30–40% decrease over a 5-year period observed in our studies is in accordance with the literature.

In spite of the fact that participants in the pilot study were 3 years older and smoked more frequently (25% in the pilot study (Staessen et al., 2001) *vs* 8% in this study), blood levels of heavy metals in both studies were very similar. Blood lead concentrations in the pilot trial equaled 93 nmol/l compared to 105 nmol/l in this study; blood cadmium levels were 3.32 and 3.21 nmol/l, respectively.

The median pubertal development of our 14- to 15-year-old participants was stage 4 for genital (G4) and pubic hair

development (P4) in boys and stage 5 for breast (B5) and pubic hair (P5) development in girls. These values are in accordance with the standard growth curves of the general Flemish population (Roelants et al., 2009); that is, for the age class 14–15 years, the median of the Flemish population lies between stage 4 and 5 for genital development (G4–G5) and pubic hair development (G4–G5) in boys and at stage 5 for breast (B5) and pubic hair (P5) development in girls.

We intentionally selected a population of 14- to 15-year-old teenagers, because they are at the end stage of sexual development, especially the girls. In view of the endocrine-disrupting properties of the chemicals that we study, we are especially interested in subjects at the extreme ends of the curve. By studying the subjects who are still at stage 1 or 2 at the age of 14–15 years, we focus on those with a slower than normal development.

The data on pubertal stage development are routinely collected in Flanders and probably in many other Western countries. Our study show that data recorded by school physicians and obtained from 25 different school health examination centers in Flanders correlated well with hormone levels in boys and with self-reported age at menarche in girls. This gave us confidence to use and integrate these health data with other monitoring data such as environmental health biomonitoring. Data on puberty development are not yet electronically recorded. Once this is possible they may be used for surveillance of puberty onset and puberty progression in function of age. These data can be further analyzed and interpreted in an environmental health and public health context.

In our study, PCBs, HCB and p,p'-DDE were positively associated with pubertal development in boys: an increase in the serum concentration of PCBs, HCB or p,p'-DDE was significantly associated with higher odds to have reached genital stages G3 or higher and pubic hair stages P3 or higher. In girls, a higher exposure to PCBs was associated with a delay in timing of menarche. These results are in contradiction with previous findings and with the stipulated working mechanism of the chlorinated compounds. In the Flemish pilot trial for human biomonitoring, exposure to PCBs was associated with a delay in pubertal development (both genital stage and pubic hair stage) in boys, whereas in girls, increased exposure to dioxin-like compounds was associated with a lower stage of breast development (Den Hond et al., 2002). The findings in the pilot trial were in accordance with the hypothesis of estrogenic properties of marker PCBs, especially PCB153 (Hansen, 1998) and of anti-estrogenic activities of dioxin-like compounds (Safe et al., 1991). In some other studies, similar results were reported. Higher serum concentrations of the potentially estrogenic PCB congeners 52, 70, 101 [+90] and 187 were associated with a significantly greater probability of having reached menarche (Denham et al., 2005), whereas in Yucheng boys accidentally exposed to high levels of PCBs

and polychlorinated dibenzofurans, shorter penile length suggesting pubertal delay was reported (Guo et al., 2004). Delayed initiation of breast development was found in girls with higher prenatal dioxin exposure (Leijs et al., 2008). In several other studies, no significant association was found between exposure to persistent chlorinated compounds and timing of puberty, in girls (Blanck et al., 2000; Gladen et al., 2000; Vasiliu et al., 2004) or in boys (Gladen et al., 2000; Mol et al., 2002).

Overall, it is difficult to compare dose–response relationships from different studies. Participants are studied at different ages and stages of development, body fat stores are changing rapidly, serum levels of pollutants can vary considerably, the composition of the mixture of internal pollutants may fluctuate over time and this may lead to varying biological effects, background characteristics and confounding factors may differ and may be taken into account in different manners. Yet, it is unlikely that these methodological aspects can explain contradicting results such as opposite signs in dose–response relationships. One possible explanation for the conflicting results may be that serum PCBs, HCB and p,p'-DDE are not linked directly to the mode of action of the biological effect under consideration, but are surrogates for pollutants with a similar occurrence but a different biological action, for example, dioxins. Unfortunately, we did not measure dioxins or dioxin-like compounds (Calux assay) in this study. Also, we do not know the critical window of exposure for effects on sexual maturation. The triggers for puberty onset and development through puberty are not fully understood (Schoeters et al., 2008). We cannot exclude that the effects are "imprinted" at earlier time points of development; for example, during the perinatal period. In this study, we have only information of the internal doses of a selection of pollutants at the age of 14–15 years and not at other age windows that may be sensitive as well. Further on, a growing number of observations indicate that the biological and health effects of pollutants capable of binding to receptors show complex dose–response relationships. Even non-monotonic associations have been described (Bonefeld-Jorgensen et al., 2001; Endo et al., 2003; Gregoraszczuk et al., 2003; Eskenazi et al., 2005). The positive association found by Dhooge et al. (2010) between serum marker PCB levels and sex hormone levels for male adolescents in our study was much stronger at serum PCB concentrations below the median concentration of 0.92 nmol/l. As almost all male adolescents participating in the pilot biomonitoring study had marker PCB serum levels that were superior to 0.92 nmol/l, part of the contradiction between the earlier findings and ours might be due to this marked difference in range of internal exposures.

In boys, levels of HCB and lead were significantly associated with, respectively, a lower and higher risk of gynecomastia. As described in detail by Dhooge et al. (2010), a significant ($P<0.001$) and positive association was

observed between serum HCB concentration and the aromatase index (T/E_2) in this study. These results are in line with the hypothesis that HCB might protect against gynecomastia by its inhibiting effect on aromatase. Our findings are consistent with observations indicating that HCB lowered estradiol levels in female rats (Alvarez et al., 2000) and in monkeys (Foster et al., 1995). The positive relation between lead and the occurrence of gynecomastia may be explained by the xenoestrogenic properties of lead (Martin et al., 2003).

In girls, we found that higher blood lead levels were associated with a delay in pubic hair development; that is, girls with higher blood lead levels had a higher change of not having reached stage 4 or 5. As blood lead levels were similar in girls in stages P4 and P5, this finding could be due to chance. However, our observations are consistent with analyses of the data of the Third National Health and Nutrition Examination Survey showing that environmental lead exposure was related to retarded pubic hair development in girls (Selevan et al., 2003; Wu et al., 2003). Studies in rats have shown an inhibiting effect of lead on pubertal development in the female (Dearth et al., 2002; Iavicoli et al., 2006; Pine et al., 2006). Possible underlying mechanisms might include the inhibition by lead of the binding of estradiol to estrogen receptor-α, observed in human cells *in vitro* (Martin et al., 2003), and decreased serum concentrations of insulin-like growth factor-1, LH and estradiol, which in turn may delay the onset or progression of puberty.

Conclusion

In this study, we found significant associations between exposure to environmental pollutants and sexual maturation in 14- to 15-year-old Flemish adolescents. In boys, exposure to HCB, p,p'-DDE and the sum of marker PCB138, 153 and 180 were significantly and positively associated with pubertal development, both with genital and pubic hair development. Higher blood lead levels were significantly associated with a higher chance for gynecomastia, whereas a negative relationship was detected between HCB levels in serum and the occurrence of gynecomastia. In girls, higher blood lead levels were significantly associated with a delay in pubic hair growth and higher serum PCB levels were significantly associated with a delay in menarche.

The findings are partly in line with known modes of action of the pollutants and with previous findings in the literature. The effects of PCBs and chlorinated pesticides on pubertal staging in boys, however, are opposite to previous findings in Flanders. Further understanding of toxic mechanism and sensitive windows of exposure is needed to explain the current findings. Longitudinal studies with measurements of prenatal, perinatal and pubertal exposure to endocrine disrupters and with periodic follow-up of pubertal develop- ment could allow to achieve better insight in the associations between exposure and puberty.

Conflict of interest

The authors declare no conflict of interest.

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