

Renal function, cytogenetic measurements, and sexual development in adolescents in relation to environmental pollutants: a feasibility study of biomarkers

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Summary

Background Human exposure to chemicals is normally monitored by measurement of environmental pollutants in external media. We investigated whether biomarkers in adolescents can show exposure to, and health effects of, common environmental pollutants.

Methods We recruited 200 17-year-old adolescents (120 girls) from a rural control area and from two suburbs polluted by a lead smelter and two waste incinerators. We measured biomarkers of exposure and of effect in blood and urine samples, and obtained questionnaire data. School doctors measured testicular volume and staged sexual maturation.

Findings Internal exposure was mostly within current standards. Concentrations of lead and cadmium in blood, PCBs (polychlorinated biphenyls) and dioxin-like compounds in serum samples, and metabolites of VOCs (volatile organic compounds) in urine were higher in one or both suburbs than in the control area. Children who lived near the waste incinerators matured sexually at an older age than others, and testicular volume was smaller in boys from the suburbs than in controls. Biomarkers of glomerular or tubular renal dysfunction in individuals were positively correlated with blood lead. Biomarkers of DNA damage were positively correlated with urinary metabolites of PAHs (polycyclic aromatic hydrocarbons) and VOCs.

Interpretation Biomarkers can be used to detect environmental exposure to pollutants and measure their biological effects before overt disease develops. Our findings suggest that current environmental standards are insufficient to avoid measurable biological effects.

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Introduction

People worldwide are exposed to many environmental pollutants, which are usually monitored by measurements in air, food, water, soil, or dust. Extrapolation from these data to assess the total internal exposure of human beings or to the possible health effects is uncertain.¹ People are exposed via different routes. Variability between individuals in absorption, distribution, metabolism, and excretion of xenobiotics is huge. Several chemicals can act on the same target organs. Diseases caused by chronic exposure to low concentrations of pollutants might become clinically evident only after a long period of time. Concentrations of pollutants or their metabolites in blood, urine, or tissues show current or lifetime exposure via all routes. Biomarkers of exposure are more directly associated with biomarkers of effects than are measurements of pollutants in external media, and provide better estimates of health risk before onset of disease.²

Exposure to chlorinated pesticides has been compared between women aged 50–65 years in rural areas and in suburbs: serum-sample concentrations of pentachlorophenol, lindane, and active p,p'-DDT (dichlorodiphenyl-trichloroethane) and its inactive metabolite p,p'-DDE were significantly higher in rural areas than in suburbs (100 women per area), but the opposite was noted for hexachlorobenzene (Department of Welfare, Health and Equal Opportunities, Ministry of the Flemish Community, Brussels, 2000).

We have therefore investigated whether biomarkers can reveal exposure and early health effects in relation to four main classes of environmental pollutants: heavy metals, polychlorinated biphenyls (PCBs), volatile organic compounds (VOCs), and polycyclic aromatic hydrocarbons (PAHs). We chose 17-year-old adolescents as our target population, because in a society with a life expectancy of more than 74 years, biomarkers in young people show recent exposure, even for cumulative toxins such as heavy metals,³ polychlorinated biphenyls,^{4,5} or dioxins.⁴ Moreover, in Belgium, school attendance is compulsory until age 18 years and school doctors routinely examine adolescents. Hence, our study benefited from professional expertise and infrastructure.

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Methods

Geographical areas

The suburbs Hoboken and Wilrijk are 11–13 km south-east of the chemical industry in the seaport of Antwerp.⁶ We selected them for our study area because they included a large non-ferrous smelter,^{7,8} two waste incinerators,⁹ a crematory,⁹ a printing works, and other various industries. Both suburbs are crossed by motorways that carry over 80 000 vehicles per day.⁶ In 1998, the mean air concentrations of benzene, toluene, and ethylbenzene were 3.2, 13.0, and 3.6 $\mu\text{g}/\text{m}^3$, respectively (Vlaamse Milieumaatschappij; Erembodegem, Belgium). The waste incinerators (in Wilrijk) started working in 1971 and 1980. In 1997, they had annual turnovers of 23 000 and 110 000 tonnes,⁹ and were shut down because dioxin emissions exceeded recommendations (>2.0 vs <0.1 ng toxicity equivalents/ m^3).⁹ Dioxin concentrations in topsoil samples from 15 sites in a radius of 0.5–3.0 km around the incinerators, ranged from 3.9 to 27.2 ng toxicity equivalents per kg dry weight.⁹ Deposition of dioxins was also higher than acceptable in Hoboken (≥ 27 vs ≤ 6.8 pg toxicity equivalents/ m^2).¹⁰ Additionally, Hoboken has been polluted by lead since the end of the 19th century.^{7,8} In 1997, the lead concentrations in airborne particles ranged from 0.08 to 1.35 $\mu\text{g}/\text{m}^3$, and deposition from 3.3 to 7.2 mg/m^2 (Vlaamse Milieumaatschappij, 1997).

Our control area was the town of Peer and its surroundings. This rural area lies 15–25 km east of the nearest non-ferrous smelters and chemical plants, is not crossed by motorways,⁶ and has no large industrial settlements.

Participants

Eligible participants were adolescents (in 1999) who were life-long residents of the control area or the two suburbs. Our study protocol required 100 participants from the two suburbs combined, and 100 controls. In Peer (control area) and in Hoboken (study area), adolescents were enrolled from a large grammar school. Our fieldwork coincided with the school holidays in Wilrijk (study area); we enrolled adolescents from a local examination centre and recruited from only the area (Neerlandwijk) surrounding the main waste incinerator. Most pupils in Peer were girls. We therefore stratified recruitment by sex with the aim of enrolling at least 40% boys from all areas.

The ethics committee of the University of Leuven approved the study. We obtained informed written consent from the parents of participating adolescents.

Procedures

Four trained school doctors recorded medical history, stages of sexual maturation according to Marshall and Tanner,^{11,12} and in boys measured testicular volume with Prader's orchidometer.¹³ Two doctors examined the teenagers recruited in Peer and two others staged the pupils in Wilrijk and Hoboken.

Nurses used questionnaires to assess lifestyle, use of tobacco and alcohol, food intake, special dietary habits, intake of medicines, and social class of parents.¹⁴ We calculated the amount of animal fat per person from their intake of meat, fish, and dairy products in the year before study, by use of Dutch food composition tables.¹⁵ Regular alcohol intake was defined as a positive answer to the question "do you regularly consume alcohol?", and specification of

at least one type of drink containing alcohol in a subsequent question.

To validate our lifestyle questionnaire¹⁴ for teenage smoking habits, we measured participants' urinary concentration of cotinine.¹⁶ About 50 mL of blood and 200 mL of urine were taken from every participant in the morning. Girls were not examined when they were menstruating. Blood samples were spun immediately. Split samples of serum, plasma, whole blood, and urine were stored at 4°C or immediately deep frozen. All tests were done in specialised laboratories that met national and international quality-control standards. Blood samples for cytogenetic tests reached the laboratory within 6 h of withdrawal.

Exposure to heavy metals was estimated from concentrations of lead and cadmium in blood samples, and from urinary excretion of cadmium.¹⁷ We estimated exposure to benzene and toluene (VOCs) from concentrations of their urinary metabolites *t,t'*-muconic acid¹⁸ and orthocresol,¹⁹ respectively. PAH exposure was estimated by measurement of 1-hydroxypyrene²⁰ in urine. The dioxin congener 2,3,7,8-tetra-chlorodibenzo-p-dioxin (TCDD)^{21,22} is the reference compound for polychlorinated aromatic hydrocarbons (PAHs), which include dioxins, PCBs, and polychlorinated dibenzofurans. Concentrations are usually expressed in toxicity equivalents relative to toxicity of TCDD. We measured concentrations of congeners 138, 153, and 180 in serum samples as biomarkers of exposure to PCBs.²² Direct chemical measurement of serum-sample concentrations of dioxins would have required an additional 50 mL of blood. Therefore, we estimated exposure to biologically-active polychlorinated chemicals by the calux assay,²³ which measures in-vitro activation of the aryl hydrocarbon receptor of cultured H4IIE cells by dioxin-like compounds in 2.5 mL of serum.

Cystatin C in serum samples²⁴ and β_2 microglobulin in alkalinised urine samples²⁵ were measured to detect early glomerular and tubular renal dysfunction, respectively. DNA damage was assessed from whole-blood samples by comet assay:²⁶ 50 cells per person were processed and the median proportion of DNA in the tail area was calculated. Chromatid breaks, chromosome breaks, and chromosome aberrations (including gaps) were counted in 100–200 cultured lymphocytes from 100 randomly selected adolescents.²⁷ Urinary 8-hydroxy-deoxyguanosine^{28,29} was measured as a biomarker of the DNA repair response to oxidative stress. Urinary measurements were standardised to 1 mmol of creatinine.

Houses and potential sources of pollution were located by use of the global positioning system, GPS Pathfinder Pro XL (Trimble Navigation Europe; Hampshire, UK). Degrees longitude and latitude (ellipsoid WGS84) were converted into kilometres with the Lambert projection system of Belgium maps. We used SAS/GRAPH mapping software (Cary, NC, USA) and the database of Teleatlas (Gent, Belgium). To protect privacy, we calculated spatial summary statistics for small statistical units, as defined by the National Institute of Statistics (Brussels, Belgium). Mean and maximum daily temperatures, and atmospheric ozone concentrations were obtained from the Royal Meteorological Institute (Brussels, Belgium) and the Vlaamse Milieumaatschappij, respectively. We expressed concentrations of pollutants in molar units,

rather than SI, to allow comparison of the effects of a wide range of pollutants on a similar scale. Conversion factors: cadmium, $1\mu\text{g}=8.897\text{ nmol}$; lead, $1\mu\text{g}=4.826\text{ nmol}$; PCB congeners 138 and 153, $1\mu\text{g}=2.771\text{ nmol}$; PCB congener 180, $1\mu\text{g}=2.530\text{ nmol}$; t,t'-muconic acid, $1\text{mg}=7037\text{ nmol}$; orthocresol, $1\text{mg}=9246\text{ nmol}$; 1-hydroxypyrene, $1\mu\text{g}=481\text{ pmol}$. To standardise per mmol creatinine: creatinine, $1\text{g}=8.840\text{ mmol}$; cadmium, $1\mu\text{g/g}=1.006\text{ nmol/mmol}$; t,t'-muconic acid, $1\text{mg/g}=796\text{ nmol/mmol}$; orthocresol, $1\text{mg/g}=1046\text{ nmol/mmol}$; 1-hydroxypyrene, $1\mu\text{g/g}=518\text{ pmol/mmol}$.

Statistical analyses

Database management and statistical analyses were done with SAS software (version 6.12). Data that were not normally distributed were log-transformed and described by geometric mean and 95% CI, or by median and IQR.

In the first part of the statistical analysis, we compared unadjusted means and proportions across the three areas with analysis of variance and Fisher's exact test, respectively. We then traced confounders by linear regression for continuous variables or by logistic regression for categorical outcomes. We used stepwise-regression procedures in which we set $p=0.05$ for the independent variables to enter and to stay in the model. Potentially important covariates were forced into the models irrespective of statistical significance. With allowance for the covariates, we looked for differences across the three areas, by use of analysis of covariance for continuous outcomes and logistic regression for odds ratios. If we found significant geographical differences, we did multiple comparisons between individual areas with Bonferroni's correction of significance levels.

In the final part of our analysis, we calculated dose-effect relations in individuals between biomarkers of exposure and of effect; and dose-response relations between biomarkers of exposure and odds ratio for a disorder, by use of multiple-linear regression and multiple-logistic regression, respectively. Effects sizes and odds ratios with 95% CI were calculated from linear

and logistic regression coefficients for a two-fold increase in the biomarker of exposure.

Results

Participants

524 adolescents, born in 1980–83 were eligible. 169 children were excluded: seven because they had not lived all their lives in the study areas, and 162 because the sex quota by area had already been filled. Of 355 invited youngsters, 207 (58%) volunteered to take part. We did not examine seven adolescents: three had recently moved out of the study area, two were unavailable because of illness, and two were away travelling.

The 200 adolescents included 120 girls (60%), none of whom were pregnant. Mean age was slightly but significantly higher in Wilrijk, because these adolescents were examined after the end of the school year. Sex distribution and demographic characteristics did not differ between areas (table 1). In Hoboken, the sample included six descendants of non-European immigrants (one boy and five girls). Exclusion of these children did not alter our results. None of the participants had a part-time job in industry or was grossly obese ($\text{BMI} > 30\text{ kg/m}^2$).

Background characteristics of the 155 non-participants were similar to participants with respect to: mean age (17.4 *vs* 17.3 years, respectively, $p=0.67$), sex distribution (105 [68%] *vs* 120 [60%] girls, respectively, $p=0.13$), and parental social class (low, medium, and high: 44 [28%], 99 [64%], and 12 [8%] *vs* 47 [24%], 129 [65%], and 24 [12%], respectively, $p=0.30$). Of the non-participants, 97 lived in Peer, 41 in Wilrijk, and 17 in Hoboken. In the suburbs, non-participants and participants lived at similar distances from the lead smelter (1896 *vs* 1993 m, $p=0.61$) and the largest waste incinerator (1297 *vs* 1376 m, $p=0.71$).

Proportions of current smokers were similar in control and polluted areas (table 1). Geometric mean concentration of urinary cotinine was higher in 50 smokers than in 150 non-smokers, of whom 81 (54%) were passive smokers (309.2 *vs* 22.7 nmol/mmol creatinine, $p=0.0001$). Pearson's correlation coefficient

Characteristics	Peer (control group) n=100	Wilrijk (study group) n=42	p* between Wilrijk and Peer	Hoboken (study group) n=58	p* between Hoboken and Peer	p* between Wilrijk and Hoboken	p between all 3 areas
Demographics							
Mean (SD) age (years)	17.2 (0.8)	17.8 (0.8)	<0.0001	17.2 (0.8)	0.91	<0.001	<0.001
Mean (SD) height (cm)							
Girls	166 (6)	165 (8)	0.62	165 (6)	0.53	0.99	0.51
Boys	179 (6)	180 (6)	0.66	177 (8)	0.23	0.15	0.31
Mean (SD) body weight (kg)							
Girls	57.7 (8.1)	57.9 (11.9)	0.93	58.7 (9.8)	0.62	0.76	0.62
Boys	66.2 (10.3)	71.4 (15.2)	0.10	66.8 (10.4)	0.86	0.22	0.63
Mean (SD) BMI (kg/m ²)							
Girls	21.0 (2.5)	21.3 (3)	0.72	21.6 (3.1)	0.37	0.72	0.36
Boys	20.5 (2.4)	21.9 (3)	0.07	21.2 (2.7)	0.37	0.44	0.24
Sociodemographics							
Girls	60 (60%)	21 (50%)	0.47	39 (67%)	0.62	0.14	0.22
Girls on oral contraceptives	21 (35%)	11 (52%)	0.27	17 (44%)	0.68	0.88	0.35
Smokers	23 (23%)	14 (33%)	0.39	13 (22%)	0.93	0.35	0.37
Consume alcohol	50 (50%)	22 (52%)	0.01	15 (26%)	0.005	0.80	0.005
Take vocational education	36 (36%)	16 (38%)	0.62	26 (45%)	0.95	0.57	0.51
Social class of parents							
Workers	31 (31%)	5 (12%)		11 (19%)			
Middle class	60 (60%)	29 (69%)		40 (69%)			
Educated professionals	9 (9%)	8 (19%)	0.05	7 (12%)	0.43	0.80	0.08
Serum-sample lipids							
Mean (SD) total cholesterol (mmol/L)	4.21 (0.74)	4.63 (0.86)	0.003	4.30 (0.73)	0.51	0.03	0.01
Mean (SD) triglycerides (mmol/L)	1.07 (0.46)	1.26 (0.50)	0.03	1.06 (0.50)	0.90	0.04	0.07
Mean (SD) total fat (g/L)	5.19 (1.14)	5.36 (1.24)	0.43	4.80 (1.10)	0.04	0.02	0.01

*Bonferroni's method.

Table 1: Characteristics of participants

Biomarkers	Peer (control group) n=100	Wilrijk (study group) n=42	p* between Wilrijk and Peer	Hoboken (study group) n=58	p* between Hoboken and Peer	p* between Wilrijk and Hoboken	p between all 3 areas
Blood							
Lead in blood (nmol/L)†	72.0 (65.0–79.0)	87.0 (75.0–101)	0.04	132 (116–149)	<0.0001	<0.0001	<0.0001
Cadmium in blood (nmol/L)†	3.58 (3.19–4.03)	3.66 (3.06–4.39)	0.84	2.62 (2.24–3.05)	0.002	0.006	0.003
Marker PCBs in serum (nmol/L)‡	1.19 (1.10–1.28)	1.48 (1.31–1.67)	0.003	1.19 (1.07–1.32)	0.99	0.007	0.008
(pmol/g fat)‡	234 (217–253)	278 (246–314)	0.02	259 (234–287)	0.14	0.31	0.050
Dioxin-like compounds in serum§ (TEQ ng/L)‡	0.13 (0.11–0.14)	0.16 (0.13–0.20)	0.09	0.21 (0.17–0.25)	<0.0001	0.06	0.0002
(TEQ pg/g fat)‡	24.9 (21.4–29.0)	29.8 (23.4–38.0)	0.20	45.8 (37.5–56.0)	<0.0001	0.01	<0.0001
Urine (standardised to 1 mmol of creatinine)							
Cadmium (nmol)†	0.14 (0.13–0.15)	0.14 (0.12–0.16)	0.81	0.15 (0.13–0.17)	0.30	0.54	0.570
t,t'-muconic acid (nmol)	33.3 (28.3–39.2)	50.0 (37.6–66.0)	0.02	45.8 (35.0–60.0)	0.08	0.72	0.020
Orthocresol (nmol)	47.6 (39.5–57.5)	120.5 (87.1–167)	<0.0001	61.6 (45.1–84.1)	0.22	0.01	<0.0001
1-hydroxypyrene (pmol)	30.8 (25.1–37.8)	38.5 (26.9–55.2)	0.28	36.2 (25.7–51.1)	0.48	0.83	0.460

Data are geometric mean (95% CI). PCB=polychlorinated biphenyls. TEQ=toxicity equivalents. *Bonferroni's method. †Adjusted for sex and smoking. ‡Adjusted for sex, BMI, weeks of breastfeeding, parental social class, and dietary fat intake. Marker PCBs (sum of congeners 138, 153 and 180) were not measured in one resident of Wilrijk and two of Hoboken. Calux assay results were unavailable in one resident of Wilrijk. §Calux assay²³ measures biologically active dioxin-like compounds. ||Adjusted for sex, smoking, mean daily temperature, and mean atmospheric ozone concentration in the week before samples were obtained.

Table 2: Biomarkers of exposure

between urinary cotinine concentration and number of cigarettes smoked per day was 0.45 ($p=0.001$). Median daily tobacco consumption was 11 cigarettes (IQR 6–16) in 19 male smokers, and six cigarettes (4–9) in 31 smoking girls. Participants who smoked had higher blood concentrations of cadmium (geometric mean 8.65 *vs* 2.38 nmol/L) and lead (104 *vs* 85 nmol/L), and higher urinary concentrations (standardised to 1 mmol of creatinine) of t,t'-muconic acid (56.1 *vs* 35.5 nmol), orthocresol (84.9 *vs* 56.4 nmol), and 1-hydroxypyrene (59.1 *vs* 28.1 pmol). All other exposure and effect biomarkers, which included those for DNA damage, were similar in smokers and non-smokers.

Among 52 boys and 35 girls who drank alcohol, median intake per week was 11.4 g (IQR 4.3–24.7) and 4.3 g (1.1–7.1), respectively. Smoking and consumption of alcohol were significantly associated ($p=0.02$). In Hoboken, fewer participants reported regular alcohol intake than in the other areas (15/58 [26%] *vs* 72/142 [51%]).

Reported food intake was similar in all areas. Median servings per months were: 30 (IQR 20–30) for meat, three (1–8) for fish, and 30 (20–60) for dairy products. However, in the rural area ($n=100$), compared with the two suburbs ($n=100$), more adolescents consumed locally produced meat (33 *vs* 5%; $p=0.001$),

dairy products (47 *vs* 20%; $p=0.001$), and vegetables or fruit (39 *vs* 24%; $p=0.02$). 113 adolescents (57%) had been breastfed for a median of 9 weeks (IQR 6–13); their serum-sample PCBs (sum of congeners 138, 153, and 180) increased by 17% (95% CI 9–27%; $p<0.001$) per 10 weeks of breastfeeding. Adolescents who reported eating fish on more than 3 days per month (median) had a higher urinary concentration of 1-hydroxypyrene (44.0 *vs* 30.6 pmol per mmol creatinine; $p=0.02$) than those who did not.

Dietary fat intake was similar in all areas (63.3 g per day [IQR 49.3–75.2, $p=0.82$]). Serum-sample cholesterol was significantly higher in Wilrijk than in the other areas. Mean concentration of total fat in serum-samples was lowest in Hoboken (table 1).

Meteorological conditions

Adolescents from Peer were investigated from May 20, to June 3, and from Sept 16, to Oct 28, those from Wilrijk Aug 10–31, and those from Hoboken from Nov 9, to Dec 2, 1999. In the week before blood and urine samples were obtained, mean daily temperatures were 13.6 (3.4) °C in Peer, 16.8 (5.2) °C in Wilrijk, and 5.3 (4.5) °C in Hoboken; and mean ozone concentrations in air measured from 10:00 to 18:00 h were 58.1 (23.0) $\mu\text{g}/\text{m}^3$, 52.6 (13.5) $\mu\text{g}/\text{m}^3$, and

Characteristics	Peer (control group) n=100	Wilrijk (study group) n=42	p* between Wilrijk and Peer	Hoboken (study group) n=58	p* between Hoboken and Peer	p* between Wilrijk and Hoboken	p between all 3 areas
Renal function†							
Cystatin-C in serum (mg/L)	0.65 (0.08)	0.63 (0.08)	0.13	0.71 (0.08)	<0.0001	<0.0001	<0.0001
β_2 microglobulin in urine ($\mu\text{g}/\text{mmol}$ creatinine)	5.22 (4.59–5.94)	5.30 (4.34–6.48)	0.90	9.09 (7.67–10.8)	<0.0001	<0.0001	<0.0001
Cytogenetic‡							
8-hydroxy-deoxyguanosine ($\mu\text{g}/\text{mmol}$ creatinine)	0.44 (0.40–0.48)	0.57 (0.49–0.66)	0.004	0.49 (0.42–0.56)	0.31	0.19	0.01
Comet assay (percentage DNA in the tail)	1.02 (0.44)	1.70 (0.49)	<0.0001	1.01 (0.42)	0.98	<0.0001	<0.0001
Chromatid breaks§	31 (62%)	19 (68%)	0.30	12 (55%)	0.81	0.22	0.28
Chromosome breaks§	23 (46%)	12 (43%)	0.17	11 (50%)	0.08	0.21	0.17
Chromosome aberrations§	36 (72%)	20 (71%)	0.61	18 (82%)	0.74	0.58	0.62
Sexual development							
Left plus right testicular volume (mL)	47.3 (6.50)	42.8 (6.70)	0.02	42.1 (6.30)	0.004	0.72	0.005
Boys with genital stage G3–G4¶	3 (8%)	8 (38%)	0.003	0	0.96	0.001	0.003
Girls with breast stage B3–B4¶	6 (10%)	7 (33%)	0.03	8 (21%)	0.10	0.08	0.04
Adolescents with stages G3–G4 or B3–B4¶	9 (9%)	15 (36%)	<0.0001	8 (14%)	0.27	0.01	0.0002

Data are mean (SD), geometric mean (95% CI), or number of participants (% of those examined). *Bonferroni's method. †Adjusted for sex and smoking; β_2 microglobulin was also adjusted for initial urinary pH. ‡Adjusted for sex, smoking, mean atmospheric ozone concentration, and mean daily temperature in the week before blood samples were obtained. §Lymphocytes of 50, 28, and 22 randomly selected adolescents from Peer, Wilrijk, and Hoboken, respectively, were cultured; number of participants who had one or more lymphocytes with a specified chromosomal abnormality are shown. ||Adjusted for age (no data from one boy of Peer). ¶Two boys from Peer were not staged. Adjusted for age, BMI, parental social class, and use of oral contraceptives (girls).

Table 3: Biomarkers of effect

Biomarkers of effect	Related biomarker of exposure	Effect type	Effect size* (95% CI)	p
Renal effects				
Cystatin-C in serum	Lead in blood	% increase	3.6 (1.5 to 5.7)	<0.0001
β_2 microglobulin in urine	Lead in blood	% increase	16.0 (2.7 to 31)	0.02
Cytogenetic effects				
8-hydroxy-deoxyguanosine in urine	Orthocresol in urine	% increase	6.8 (2.3 to 11.5)	0.003
Comet assay (percentage DNA in the tail)	t,t'-muconic acid in urine	% increase	4.3 (-0.70 to 9.3)	0.09
	Orthocresol in urine	% increase	5.3 (1.1 to 9.5)	0.01
	1-hydroxypyrene in urine	% increase	7.0 (3.1 to 10.9)	0.0005
Chromatid breaks	t,t'-muconic acid in urine	Odds ratio	1.74 (1.13 to 2.66)	0.01
	1-hydroxypyrene in urine	Odds ratio	1.58 (1.10 to 2.26)	0.01
Chromosome aberrations	1-hydroxypyrene in urine	Odds ratio	1.56 (1.07 to 2.27)	0.02
Effects on sexual development				
Genital stage G3-G4 in boys	Sum of marker PCBs in serum	Odds ratio	3.80 (0.94 to 8.00)	0.06
Breast stage B3-B4 in girls	Dioxin-like compounds in serum†	Odds ratio	2.26 (1.15 to 4.46)	0.02

For number of participants and factors for which the relations were adjusted, see table 3. *Effect sizes were calculated for a two-fold increase in the biomarker of exposure.

†Calux assay.²³

Table 4: Dose-effect relations

15.1 (3.5) $\mu\text{g}/\text{m}^3$, respectively ($p < 0.001$ compared with Hoboken).

Orthocresol and 1-hydroxypyrene concentrations in urine-samples and comet assay results were significantly ($p < 0.0001$) correlated with mean temperature and atmospheric ozone concentration. In single regression analysis, r for mean temperature and atmospheric ozone concentration were, respectively, 0.56 and 0.40 for orthocresol, 0.29 and 0.31 for 1-hydroxypyrene, and 0.53 and 0.45 for the comet assay.

Regional differences in biomarkers of exposure

Table 2 shows concentrations of biomarkers of exposure adjusted for various factors. Before and after these adjustments, blood lead concentration was higher in Hoboken than in the control area and in Wilrijk, whereas the opposite was noted for blood cadmium concentrations (table 2). Urinary cadmium concentrations were similar in all areas. Marker PCBs in serum samples were significantly higher in Wilrijk than

in Peer. Exposure to dioxin-like compounds was highest in Hoboken. Urinary concentration of t,t'-muconic acid was significantly increased in Wilrijk compared with the control area. Urinary concentration of orthocresol was significantly higher in Wilrijk than Peer and Hoboken (table 2).

Regional differences in biomarkers of effect

Table 3 shows biomarkers of effects adjusted for various factors. Before and after these adjustments, cystatin-C in serum samples and urinary β_2 microglobulin were significantly higher in Hoboken than the other areas (table 3). Urinary concentrations of 8-hydroxy-deoxyguanosine and comet assay results were higher in Wilrijk than Peer. Among 100 randomly selected adolescents, median percentage of cultured lymphocytes with chromatid breaks, chromosome breaks, or chromosome aberrations was 1 (IQR 0-1), 0 (0-1), and 1 (0-2), respectively. The number of adolescents who had one or more cultured lymphocytes with these cytogenetic characteristics was similar in all areas (table 3).

Measurements of sexual development and testicular volume, done by the two school doctors in Peer, did not differ significantly (p values ranged from 0.21 to 0.85). In a separate validation study of the school doctors who had examined the teenagers in Peer and Wilrijk, each examined on the same day ten boys and 12 girls in random order. Mean (SD) age of the teenagers was 16.6 (0.6) years. With the physician who had worked in Wilrijk as a reference, κ coefficients³⁰ for staging sexual maturity were 0.64 (95% CI 0.27-1.00, $p=0.009$) and 0.58 (0.23-0.94, $p=0.01$), and mean differences in estimated testicular volume were -3.0 (4.8) mL ($p=0.08$) and 0 (3.3) mL ($p>0.99$), respectively. κ coefficients between 0.40 and 0.75 represent good agreement beyond chance.³⁰ Although

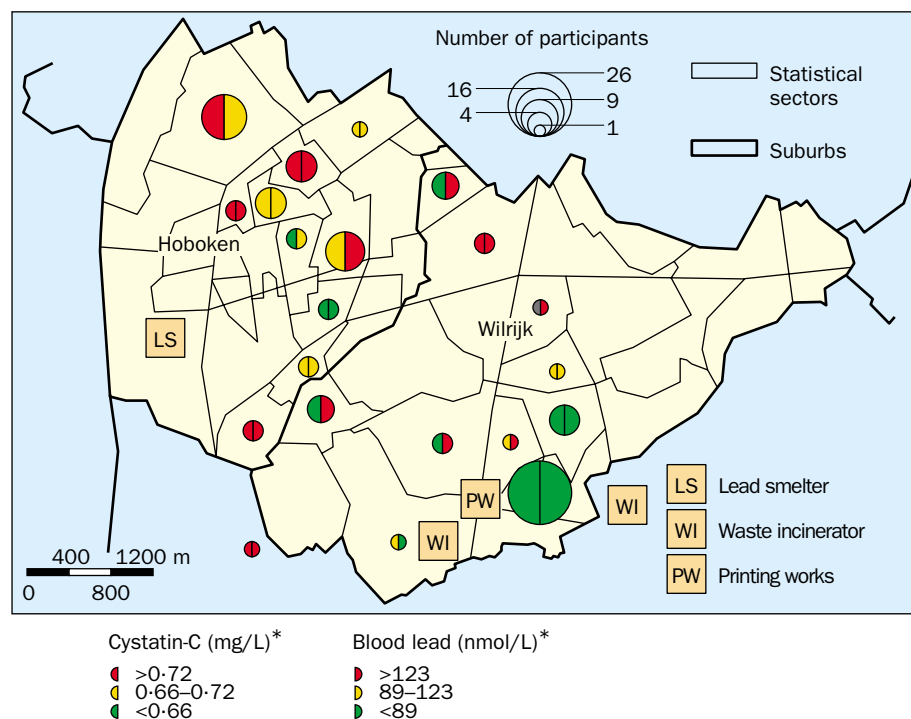


Figure 1: Location of study-group participants and heavy industry, and concentrations of lead in blood and cystatin-C in serum in Hoboken and Wilrijk

*Shades from green to red represent increasing levels of biomarkers. The right half of each circle represents the biomarker of exposure and the left half the biomarker of effect.

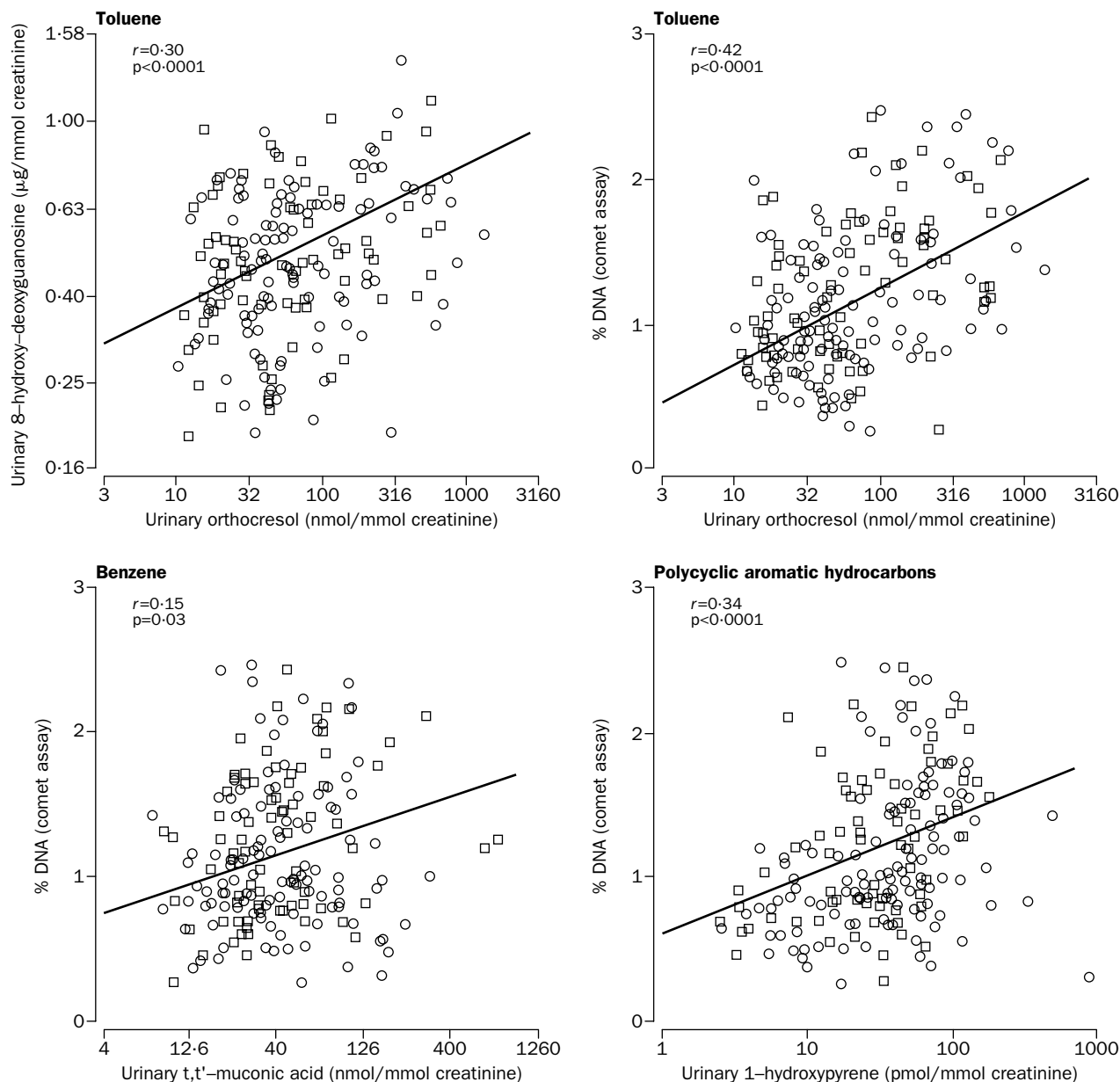


Figure 2: **Unadjusted dose-effect relations in 200 adolescents between two biomarkers of DNA damage,* and urinary biomarkers of exposure to benzene,†,‡ and polycyclic aromatic hydrocarbons§**

Circles and squares indicate girls and boys, respectively. *Urinary 8-hydroxy-deoxyguanosine and % DNA in the tail area in the comet assay. †,t,t'-muonic acid. ‡Orthocresol. §1-hydroxypyrene.

participants in Wilrijk were slightly older than those in Peer and Hoboken, more boys and girls had not reached the adult stage of genital or breast development and were rated G3-G4¹² or B3-B4,¹¹ respectively (table 3). Testicular volume was significantly lower in Hoboken and Wilrijk than in Peer (table 3).

Dose-effect and dose-response curves in individuals

Adjustments applied to calculate dose-effect and dose-response curves in individuals were the same as those used in table 3. Before and after adjustment, cystatin-C and β_2 microglobulin values rose with increasing blood concentration of lead (table 4) but not cadmium ($p>0.27$). Figure 1 shows the association between cystatin-C in serum-samples and the blood-sample lead concentration among adolescents living around the lead smelter in the two suburbs.

Before (figure 2) and after adjustment (table 4), concentrations of 8-hydroxy-deoxyguanosine were significantly correlated with those of orthocresol in urine. Comet assay results were also positively correlated with urinary concentrations of orthocresol and 1-hydroxypyrene. Relative risk of chromatid breaks (logistic regression) rose with higher urinary concentrations of t,t'-muonic acid or 1-hydroxypyrene. Probability of chromosome aberrations rose with increasing 1-hydroxypyrene concentration in urine (table 4). Odds of not having reached adult breast development in girls was positively correlated with estimated concentration of dioxin-like compounds in serum-samples. In boys, probability of less than adult genital development increased with higher serum-sample concentrations of marker PCBs (table 4).

Discussion

In adolescents, biomarkers were sensitive enough to detect significant geographical gradients in common environmental pollutants, in their metabolites, and in their biological effects. Across individual teenagers, dose-effect and dose-response curves were established, which were prespecified in our protocol on the basis of experimental data,^{21,22,31} hypotheses,³¹⁻³³ or observations mostly made at high levels of occupational^{1,18,20,29,34} or accidental³⁵ exposure to pollutants. We also showed spatial associations between biomarkers and probable sources of present or past pollution.

Our results are unlikely to be confounded by selection bias; participants and non-participants had similar sociodemographic characteristics such as sex, age, and parental social class. Self-selection of more exposed participants than less exposed participants did not occur in the polluted suburbs; participants and non-participants lived at similar distances from the lead smelter and the largest waste incinerator.

By contrast with traditional methods of environmental surveillance, biomonitoring does not require measurement of chemicals in external media. Nevertheless, we also assessed the effect of external factors such as atmospheric conditions and lifestyle on our results. Atmospheric ozone concentration and urinary concentration of metabolites of VOCs and PAHs varied seasonally. Diet affects non-occupational exposure to heavy metals,³ PCBs,^{4,5} dioxins⁴ and PAHs.³⁶ We confirmed the effect of breastfeeding on serum concentrations of PCBs⁵ and that of fish intake on urinary excretion of 1-hydroxypyrene.³⁶ Furthermore, we deliberately included smokers, because 10–20% of older teenagers smoke; cigarette smoke contains many xenobiotics and might increase the harmful effects of various environmental pollutants.^{5,36} Cigarette and alcohol consumption were significantly associated.¹⁴ Smokers compared with non-smokers had increased blood concentrations of cadmium and lead, and excreted greater quantities of metabolites of VOCs and PAHs in their urine. None of our adjustments for various factors removed the between-area difference in biomarkers, or the dose-effect or dose-response relations across individuals.

Lead and cadmium accumulate in the human body with age.^{1,37} Gastrointestinal absorption and inhalation of contaminated particulate, such as the cadmium-loaded particles in tobacco smoke, are the main routes of exposure. Blood lead concentration was highest in those who lived near the lead smelter.^{7,8} All teenagers but one who lived in Hoboken, had a blood lead concentration below 100 µg/L (483 nmol/L). Experts have proposed that in environmentally exposed adults, blood lead concentration should be lower than 250 µg/L (1208 nmol/L).¹

Cadmium is stored in the kidney from birth. Therefore, its urinary excretion shows life-time exposure.^{1,37} The young ages of our participants might be why concentrations of urinary cadmium did not differ between the groups. By contrast, blood cadmium concentration shows recent exposure.^{1,37} Cadmium in fertilisers,³⁸ unidentified point sources, or both, might have caused the blood cadmium concentrations to be higher in the rural control area than in Hoboken. Incineration of waste is an important source of cadmium emissions, because litter frequently includes different types of cadmium-containing products, such as plastics, batteries, or metal scrap.³⁸ Thus, the incinerators in Wilrijk probably raised the blood cadmium concentrations above those of Hoboken.

In adults³⁹ and children,⁴⁰ environmental lead exposure can affect glomerular³⁹ and tubular⁴⁰ renal function. Serum concentration of cystatin-C is a reliable index of glomerular function.²⁴ Unlike serum creatinine, this biomarker is independent of sex, age, height, and body composition.²⁴ In 184 children whose ages ranged from 0.2 to 18.0 years, and of whom 54% had renal impairment, serum cystatin-C averaged 1.75 mg/L.²⁴ We showed that at a young age glomerular function was independently and negatively correlated with blood lead concentration.

β₂ microglobulin is a circulating microprotein which can pass through the glomerular filter, but is then almost completely reabsorbed in the renal tubules.^{1,37} The independent and positive correlation between urinary β₂ microglobulin and blood lead concentration indicates slight some renal tubular dysfunction, and corroborates observations in 13-year-old Czech children living near a lead smelter.⁴⁰

VOCs^{1,41} and PAHs^{1,36} are common environmental pollutants. Benzene is a constituent of gasoline. Benzene^{1,41} and PAHs^{1,36} are formed by incomplete combustion of organic matter and fossil fuels (petroleum products, coal, and to a lesser extent wood). They are present in tobacco smoke and car exhaust fumes.^{1,36,41} VOCs also originate from organic solvents used in the chemical industry, printing works, or at home. Absorption of VOCs and PAHs occurs mainly through inhalation, and to a lesser extent, through skin contact.^{1,41} PAHs present in toast, barbecued food, or contaminated food are gastrointestinally absorbed.³⁶ Intakes of different food types did not differ between areas, which is probably why urinary concentration of 1-hydroxypyrene also did not vary.

Environmental exposure to toluene was highest in Wilrijk, and benzene exposure in both suburbs combined was higher than in Peer. Traffic or local effluents from point sources (eg, the printing works in Wilrijk) might have caused these findings. Across five studies in Europe, median urinary concentration of 1-hydroxypyrene, standardised to 1 mmol of creatinine, ranged from 80 to 270 pmol in non-smokers and from 170 to 510 pmol in smokers.⁴² Urinary excretions of *t,t'*-muconic acid, orthocresol, and 1-hydroxypyrene that we recorded were far below the reference values for the general population of 398 000 pmol, 314 000 pmol, and 1036 pmol per mmol creatinine, respectively.¹ VOCs and PAHs are potent carcinogens.^{1,36,41} 8-hydroxy-deoxyguanosine is formed in response to a specific form of DNA damage induced by reactive oxygen species⁴³ and is also mutagenic.⁴³ In workers occupationally exposed to asbestos, rubber, or azo-dye, urinary concentration of 8-hydroxy-deoxyguanosine was 30–80% higher than in controls.²⁹ In concordance with the biomarkers of exposure to VOCs, concentration of 8-hydroxy-deoxyguanosine in urine-samples and results of the comet assay²⁶ were highest in Wilrijk.

Furthermore, we also noted an independent and positive relation between urinary excretion of 8-hydroxy-deoxyguanosine and orthocresol. Comet assay²⁶ results were positively correlated with urinary concentration of orthocresol or 1-hydroxypyrene. Results of logistic regressions also showed an increased risk of chromatid breaks with high urinary concentrations of *t,t'*-muconic acid and 1-hydroxypyrene, and accorded with the greater risk of chromosome aberrations with high 1-hydroxypyrene concentration in urine. Thus, three independent

measurements of cytogenetic damage, two of which were unrelated to atmospheric conditions (8-hydroxydeoxyguanosine in urine and chromosome abnormalities in cultured lymphocytes), were positively correlated with urinary marker metabolites of VOCs or PAHs.

However, our cytogenetic findings must be interpreted carefully. None of the adolescents had abnormally raised numbers of cultured lymphocytes with chromatid breaks or chromosome abnormalities. The prognostic value of cytogenetic markers in adolescents is unknown. Nonetheless, in a pooled analysis of 3541 Nordic and Italian people (age >15 years), chromosome aberrations in peripheral lymphocytes were a biomarker of the cancer risk, reflecting either early biological effects of genotoxic carcinogens or individual cancer susceptibility.³⁴

Dioxins and PCBs are byproducts of many chemical and thermic reactions that contain organic substances and chlorine. They contaminate emissions of waste incinerators and smelt furnaces. PCBs were first produced commercially in the 1920s, although it was not until the 1950s that industrial applications of PCBs increased substantially.⁵ They were used as hydraulic or transformer fluids, as plasticisers in paint, and in carbonless copying paper.⁵ PCBs have entered the environment and contaminated the food chain, most notably fish.^{4,5} PCAHs are common in the environment, although usually present in very small amounts. However, they are lipophilic substances and become biologically magnified in the food chain from soil and sediment to fish or animal feed, to dairy and meat products, and eventually to man.⁴⁴ Breastfeeding, as we noted, is an important source of PCB intake.^{21,45} Human milk also contains traces of dioxins.^{1,45} Absorption of PCAHs occurs via all possible routes, which include inhalation and skin contact.

The calux assay is sensitive to compounds that activate the aryl hydrocarbon receptor, such as dioxins, and coplanar and mono-ortho PCBs.^{21,22} We also measured di-ortho PCB congeners 138, 153, and 180, which frequently make up 40–60% of total PCB in human tissue.²² These di-ortho PCBs have little (congener 138) or no activity mediated via the aryl hydrocarbon receptor.²² Serum concentrations of dioxin-like compounds and PCBs were highest around the lead smelter and the waste incinerators, respectively, irrespective of whether concentrations were expressed in volumetric units or per g serum fat. At the time of our study the main waste incinerator in Wilrijk was not working. In middle-aged Belgian and Dutch women whose serum was analysed with the calux assay, the median concentrations of dioxin-like compounds were 37.4 and 100.1 pg of toxicity equivalents/g fat, respectively.⁴⁶ Marker PCB concentrations in our adolescents were lower than those in cord-blood samples from Düsseldorf, Germany.³¹ In 1995, median serum PCB concentrations in the general population of the USA were between 2 and 7 µg/L (about 6–21 nmol/L).⁵ These large between-study differences might not only show gradients in environmental exposure, but also differences in participants' diets and lifestyles, and investigators' preparation of biological matrices, handling and cleaning-up of biological samples, and analytical methods. Furthermore, in the more-developed world, exposure to PCBs has fallen since 1971.⁵

PCBs and dioxins accumulate in fat tissue and are endocrine disruptors.^{21,22,31,32} PCBs bind to oestrogen receptors and have oestrogenic and antiandrogenic

effects.^{21,22,31,32} Dioxins and dioxin-like compounds mainly disturb endocrine or cellular function by binding to the aryl hydrocarbon receptor and inducing enzymes involved in the synthesis, intracellular bioactivation, or degradation of hormones.^{31,32} In Wilrijk, compared with the other areas, a larger proportion of the adolescents had not yet matured into the adult stages of genital or breast development. Age at which adult genital characteristics are attained varies greatly between individuals. Normative data for Belgium are not available. However, around 1970, British boys reached the adult stage of genital development at a mean (SD) age of 14.9 (1.1) years,¹² and British girls reached the adult stage of breast development at 15.3 (1.8) years.¹¹ In boys, the probability of slowed genital development rose with higher serum concentrations of marker PCBs. In girls, the probability of slowed breast development was positively correlated with serum concentrations of dioxin-like compounds.

We also noted that testicular volume in boys was lower in the suburbs than in the rural control area. Testicular volume is dependent on the number of Sertoli cells.³³ Follicle stimulating hormone (FSH) causes the multiplication of Sertoli cells during fetal, neonatal, and prepubertal life. FSH secretion is under negative feed-back control of oestrogens produced by Sertoli cells. Multiplication of Sertoli cells stops before puberty. Thus, the main determinants of testicular volume (the number of Sertoli cells) is fixed before puberty.³³ Testicular volume was unrelated to serum concentrations of dioxins and PCBs. Because the two waste incinerators and the lead smelter were in full operation at the time of the boys' birth (1980–83), the smaller testes in the suburbs might have been caused by exposure to xeno-oestrogens in fetal, neonatal, or prepubertal life. Furthermore, xeno-oestrogens might decrease the male to female sex ratio and human fertility because of their sex-linked effects on fetal survival,^{44,47,48} and sperm quality.³³ In 1997, a Flemish government report⁴⁹ showed that the percentage of medically assisted conceptions was higher around the waste incinerators in Wilrijk than in Flanders, for singleton (5.6 *vs* 3.4%, respectively) and multiple (59.0 *vs* 33.4%, respectively) births. Although prognostic extrapolations are difficult to make from our findings, we note that the number of Sertoli cells and testicular volume correlate with sperm density, and with the total and percentage motile sperm per ejaculate.^{50,51}

Young people are very vulnerable to many noxious agents,^{52,53} and their protection is an important public health challenge. Feasibility of large-scale and long-term implementation of systematic biomonitoring in adolescents need to be assessed. Because we identified significant effects on sexual development, examination of younger people (aged 14–16 years) might be advisable. Environmental biomonitoring should be part of a health strategy, which could include screening for important cardiovascular risk factors, such as obesity, hypertension, and hypercholesterolaemia, and provide health education. Finally, our findings suggest that present environmental standards are insufficient to avoid measurable biological effects, which might cause disorders in adult life.

Contributors

J A Staessen and H A Roels developed the concept of environmental biomonitoring in adolescents, wrote the initial protocol, and drafted the manual of operations with the help of D Vanderschueren and G Schoeters. J A Staessen and V Nelen organised fieldwork. K Hoppenbrouwers trained the school doctors. G Koppen,

G Schoeters, H A Roels, and L Verschaeve supervised and managed the toxicological measurements. E Den Hond constructed and maintained the database and did the statistical analysis with T Nawrot and L Thijs. E Van Hecke did the spatial analysis and mapped the data. J A Staessen, E Den Hond, T Nawrot, and H A Roels wrote the paper. All authors read and commented on the paper.

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