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A battery of DNA effect biomarkers to evaluate environmental exposure of Flemish adolescents

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ABSTRACT: The present paper deals with the evaluation of a battery of genotoxicity biomarkers in healthy Flemish adolescents and their relation with common pollutants occurring in their life environment. DNA damage as reflected by the comet assay appeared to be most sensitive to ozone (partial $r^2 = 0.102$, $p < 0.00001$), and to a lesser extent to ortho-cresol (partial $r^2 = 0.055$; $p = 0.001$) and 1-hydroxy-pyrene (1-OH-pyrene, partial $r^2 = 0.031$; $p = 0.013$). 8-hydroxy-deoxyguanosine (8-OHdG) was only related to ortho-cresol ($r^2 = 0.069$; $p < 0.007$). Interestingly, the comet assay results and urinary 8-OHdG concentrations were positively correlated with a Pearson $r = 0.21$ ($p = 0.003$, $N = 200$). Logistic regression models revealed significant relations between chromatid breaks and 1-OH-pyrene (relative risk (RR): 1.58; $p = 0.008$), and t,t-muconic acid (RR: 1.71; $p = 0.014$). There was no correlation between micronucleus formation or occurrence of chromosomal or chromatid breaks on the one hand and comet or 8-OHdG results on the other hand. Thus, in this study the comet assay on whole blood samples and urine 8-OHdG measurements especially appeared sensitive biomarkers for assessing the genetic effects of environmental pollutants to which adolescents may be exposed. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS: adolescents; biomarker; cytogenetics; environmental exposure; genotoxic effect markers

Introduction

In 1999 a multidisciplinary biomonitoring study was conducted in 200 17–18 year old adolescents living in two suburbs of the city of Antwerp or in a rural area in the north-east of Flanders (Staessen *et al*., 2001). In these youngsters peripheral blood and urine samples were analysed for several environmental exposure and effect biomarkers. Children, and also those at age 17–18 years, are developing individuals, with possible increased susceptibility to environmental hazards. Indeed, adolescents were shown to be an interesting study group to observe exposure–effect relationships, without being affected by occupational exposures. The paper of Staessen *et al*. (2001) gives an overview of all the examined internal exposure markers, effect markers, their regional differences and the relationship between these exposure and effect markers.

To assess genetic effects a battery of genotoxicity markers was applied. This was useful because there is still a general need for intercomparison between genotoxicity biomarker results (Sram and Binkova, 2000). Of course the latter is especially true for children. This paper deals specifically with the evaluation of this battery of genotoxicity biomarkers in this age group. The alkaline comet assay of whole blood cells (Fairbairn *et al*., 1995) and the urinary 8-hydroxy-deoxyguanosine (8-OHdG) concentration (Loft *et al*., 1993; van Zeeland *et al*., 1999) were evaluated in all participants. The former is a measure for the loss of DNA integrity (Lebailly *et al*., 1998), while 8-OHdG results from a characteristic form of oxidative DNA damage and appears in the urine as a specific marker for oxidative stress (Floyd, 1990; Weitzman *et al*., 1994). Both biological effective dose markers reflect short-term (days) DNA damage. On the other hand, in 100 of the 200 individuals the 'early response' effect markers such as chromosome aberrations (chromosome and chromatid breaks)

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and/or micronucleus formation were examined. These cytogenetic effect markers may be related to rather persistent DNA effects (over years). This battery of different genotoxicity markers is frequently applied in epidemiological studies.

As was already reported by Staessen *et al*. (2001) the effect biomarkers were analysed in relation to each of the biomarkers of internal exposure to environmental pollutants. In the present paper all internal and external exposure markers were put simultaneously as independent variables in one multiple regression model per genotoxicity marker. In this way it was possible to relate the genetic effect markers to the cocktail of several exposure markers. The examined exposure markers were: (i) urinary concentrations of 1-hydroxypyrene (a pyrene metabolite) as a measure for polyaromatic hydrocarbons (PAHs) exposure, (ii) urinary concentrations of t,t′-muconic acid and o-cresol, metabolites of the volatile organic compounds (VOCs) benzene and toluene respectively, (iii) and outdoor ozone concentrations calculated from ozone measurements in a nearby measuring unit. These pollutants have a widespread occurrence and are emitted into the environment mainly by vehicle traffic and industry. They may enter the human organism by inhalation of polluted air and cigarette smoke, by uptake through the skin, and via the food chain (Perera, 2000). PAHs (Jongeneelen, 2001) and benzene (Duarte-Davidson *et al*., 2001) have well-documented DNA damaging potential and are human carcinogens (Perera, 2000; Golding and Watson, 1999). Toluene is thought to have little carcinogenic potential. However, it was suggested that some of its minor metabolites might damage DNA (Murata *et al*., 1999). Ozone is a pollutant of growing importance that may be related to the genetic effect biomarkers values. Exposure to ozone induces DNA damage in different cells (Haney *et al*., 1999).

In short, we examined the relation between several genetic effect biomarkers measured in 17–18 year old adolescents. These genetic effect markers were set in relation to some common pollutants occurring in the life environment of these youngsters.

Materials and Methods

Study Population and Sampling

The present investigation was carried out within the framework of the Flemish Environment and Health Study (FLEHS). A detailed description of the overall study protocol and of the characteristics of the study population has been published elsewhere (Staessen *et al*., 2001). Briefly, the study population was recruited from 524 potentially eligible adolescents born in 1980–1983 and living in two areas of Flanders. 169 youngsters were not invited to take part in the study, because they did not fulfil the requirement of lifelong residence in the study areas (n = 7), or because the sex quota by area had already been satisfied (n = 162). Of 355 invited subjects, 207 (58.3%) volunteered to be enrolled. Seven eligible adolescents were not examined because they recently moved out of the study area (n = 3) or were not immediately available for study due to illness (n = 2) or travel (n = 2). The resulting study population consisted of 200 adolescents (17–18 years of age) of whom 120 were female. Half of the study group was living in a rural area, whereas the other half was living in the urban region of Antwerp.

The adolescents were examined at their schools from May to December 1999. On each examination day, registered nurses collected spot urine samples and venous blood samples from eight adolescents. A self-administered questionnaire was completed to obtain information on individual characteristics (socio-economical status, diet, active and passive smoking and alcohol consumption). Trained school physicians recorded use of medicines, health status and medical history. The study protocol was approved by the Ethics Committee of the University of Leuven. Each participant gave written informed consent.

Biomarkers of Exposure: Internal Exposure to PAHs or VOCs, and External Exposure to Ozone

Analysis of urinary 1-OH-pyrene was performed according to Van Hummelen *et al*. (1993). Briefly, urine was enzymatically hydrolysed (β-glucuronidase/sulfatase, pH = 5 at 37 °C) overnight. Solid phase extraction on a C18-column was used to clean-up the urine before measuring 1-OH-pyrene with HPLC and fluorimetric detection. The detection limit was 0.01 μg l^{-1}. A modified Ducos *et al*. (1990) method was used to quantify t,t′-muconic acid in urine. The detection limit was 0.02 mg l^{-1} (Hotz *et al*., 1997). Ortho-cresol in urine was determined by gas chromatography with flame ionization detection (Pierce *et al*., 1998; Truchon *et al*., 1999). The detection limit was 0.05 mg l^{-1}. All urinary measurements were standardized to 1 mmol creatinine l^{-1} urine. Creatinine was determined by the colorimetric method of Jaffé (1986). The concentrations of the exposure markers were expressed in molar units rather than SI units, to allow comparison of the effects on a similar scale. Conversion factors are: t,t′-muconic acid, 1 mg = 7037 nmol; ortho cresol, 1 mg = 9246 nmol; 1-OH pyrene, 1 μg = 481 pmol. To standardise per mmol creatinine: creatinine, 1 g = 8.840 mmol; t,t′-muconic acid, 1 mg g^{-1} = 796 nmol $mmol^{-1}$; ortho cresol, 1 mg g^{-1} = 1046 nmol $mmol^{-1}$; 1-OH pyrene, 1 μg g^{-1} = 518 pmol $mmol^{-1}$.

Outdoor ozone values were obtained from sampling points of the Flemish Environment Agency (Vlaamse Milieu Maatschappij). Individual ozone exposure was estimated by taking the outdoor 8 h daily values of the

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nearest measuring unit, averaged over 7 days preceding blood and urine collection.

Biomarkers of Biological Effective Dose and Early Response

Loss of DNA integrity was evaluated by the alkaline comet assay (Singh *et al*., 1988). The whole blood cells were kept at room temperature for 1 day after the blood was taken. The comet assay methodology consisted of embedding the blood cells in agarose and layering on microscope slides. After lysis of the nuclei, the slides were put in a horizontal electrophoresis chamber filled with alkaline buffer to allow unwinding of the DNA. Electrophoresis in this buffer was followed by washing and staining with ethidium bromide. The slides were viewed using an image analysis system (Kinetic Imaging Inc.). For each person 50 cells were processed and the median percent of DNA migration in the tail area was determined and used as a measure of DNA damage. As a positive control one slide with nuclei from deep frozen whole blood was added to each electrophoresis chamber. DNA migration in those positive controls had to be at least 30%.

In 100 randomly selected adolescents, chromosome and/or chromatid breaks (including gaps) were counted in 100–200 cultured lymphocytes according to the method described by Schwartz (1990).

The cytokinesis-block micronucleus assay was performed on whole blood cultures from 100 individuals using standard procedures (Fenech, 2000). For each individual, 1000 binucleated cells were evaluated for the presence of micronuclei on a Zeiss Axioplan microscope with a 100× objective. Two blood cultures (from two individuals from the rural area) failed to give scoreable slides.

Urinary 8-OHdG was measured by high-performance liquid chromatography-electrochemical detection according to the method described in De Boeck *et al*. (2000).

Statistical Analysis

Measurements below the detection limit were set half of this value.

Statistical analyses were performed using Statistica for Windows ('99 Edition; StatSoft Inc.). Urinary concentrations of 8-OHdG, 1-OH-pyrene, t,t′-muconic acid and o-cresol were logarithmically transformed in order to accommodate the assumptions underlying the linear models. Covariables entered in the models were host factors such as micronutrients in plasma/serum (vitamins A and E; selenium), gender, educational level of parents (highest level of education of father or mother), smoking behaviour (current tobacco use), environmental tobacco smoke (hours in smoky environment), alcohol use and consumption of grilled food. Student's *t*-test was used to compare unpaired data, Chi-square test to compare proportions and Wilcoxon's rank test to compare non-normally distributed data. Differences in exposure or effect markers between smokers and non-smokers were analysed using analysis of variance (ANOVA) entering the above mentioned covariables (except smoking behaviour) and the covariable 'residence' in the models. Correlations between the biomarkers of genetic effect were expressed by Spearman rank order correlations. The relationship between biomarkers of exposure and effect was investigated using linear regression procedures. All exposure markers were put simultaneously as independent variables in one multiple regression model per genotoxicity marker. In addition to the common regression coefficients, standardized regression coefficients (SC) were also calculated. The magnitude of the SC reflect the relative contribution of each independent variable in the prediction of the dependent variable. For the frequencies of micronuclei, chromosome aberrations, chromosome breaks and chromatid breaks, the individuals were categorized into two groups: the individuals with no observed damage were classified in group 1, while those with one or more events of genetic damage were classified in group 2. This division allowed us to perform logistic regression for these biomarkers.

Results

Levels of Biomarkers

The study population consisted of 200 adolescents (120 girls and 80 boys) with a mean age of 17.4 years old. The levels of micronutrients examined as plasma/serum concentrations of vitamin A, E and selenium were well within expected ranges. There were no sex differences in those parameters, neither in life style factors nor social class. Only alcohol use was higher in boys (65%) compared with girls (29%) (Table 1).

The mean urinary concentrations of t,t′-muconic acid (40.0 nmol $mmol^{-1}$ creatinine), o-cresol (62.9 nmol $mmol^{-1}$ creatinine) and 1-OH-pyrene (33.9 pmol $mmol^{-1}$ creatinine) are shown in Table 2. No gender differences in exposure were observed (data not shown), but there was a difference between smokers and non-smokers. About 26% ($n = 31$) of the girls and 24% of the boys ($n = 19$) were smokers at the time of the study. Smokers had higher 1-OH pyrene (56.3 pmol $mmol^{-1}$ creatinine) and t,t′-muconic acid (55.2 nmol $mmol^{-1}$ creatinine) values compared with non-smokers (respectively 30.0 and 36.1) (Table 2).

There were, however, no differences in the genetic effect markers between smokers and non-smokers or between boys and girls. The mean % chromosomal

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Table 1. Characteristics of the study population

	Girls (n = 120)	Boys (n = 80)	P value	Total (n = 200)
Host factors				
Age	17.3 (0.7)	17.4 (0.8)	NS	17.4 (0.8)
Vitamin A in plasma (μmol l^{-1})	1.9 (0.4)	1.9 (0.3)	NS	1.9 (0.4)
Vitamin E in plasma (μmol l^{-1})	23.4 (3.9)	21.1 (4.9)	NS	22.4 (4.4)
Selenium in serum (μmol^{-1} l^{-1})	0.9 (0.1)	0.9 (0.1)	NS	0.9 (0.1)
Life style				
% current smokers	26	24	NS	25
% current alcohol users	29	65	0.001	44
Median (IQR) grilled food (servings/month)	1 (1–8)	3 (1–8)	NS	3 (1–8)
Social class				
Highest educational level parents:				
% workers	28	16	NS	24
% middle class	63	68	NS	64
% learned professionals	9	16	0.08	12

Data that are normally distributed are presented as arithmetic means (SD) and were compared by Student's t-test. Percentages are compared by Chi-square test. Data that are not normally distributed are presented as medians and interquartile range (IQR) and were compared by Wilcoxon's rank test.

Table 2. Exposure and genetic effect markers measured in 200 Flemish adolescents

Biomarker	Non-smokers (n = 150)	Smokers (n = 50)	P value	Total (n = 200)
Exposure marker[a]				
1-OH pyrene	30.0 (25.7–35.0)	56.3 (42.9–73.9)	<0.001	33.9 (29.4–39.1)
t,t′-Muconic acid	36.1 (32.1–40.5)	55.2 (44.4–68.6)	<0.001	40.0 (35.9–44.3)
O-cresol	62.2 (52.3–74)	80.0 (58.6–109.3)	0.10	62.9 (54.1–73.2)
Outdoor ozone[b]	—	—	—	44.5 (25.7)
Genetic effect marker[c]				
% Comet tail DNA	1.19 (0.48)	1.15 (0.60)	NS	1.16 (0.51)
8-OHdG	0.49 (0.46–0.52)	0.50 (0.44–0.56)	NS	0.48 (0.45–0.51)
	Non-smokers (n = 68)	Smokers (n = 32)	P value	Total (n = 100)
‰ Micronuclei	1.34 (1.58)	2.09 (2.66)	NS	1.51 (1.91)
% Chromatid breaks	0.76 (0.92)	0.83 (0.73)	NS	0.79 (0.86)
% Chromosome breaks	0.61 (0.75)	0.53 (0.87)	NS	0.58 (0.78)
% Chromosome aberrations	1.36 (1.26)	1.26 (1.22)	NS	1.32 (1.24)

Geometric mean (95% confidence limits) is shown for normalised data. Data that are not normalised, are presented as arithmetic mean (SD). ANOVA analysis of difference between smokers and non-smokers was done entering in the model the covariables residential area, educational level parents and gender.
[a] Units for the exposure markers measured in urine: pmol $mmol^{-1}$ creatinine for 1-OH pyrene, nmol $mmol^{-1}$ creatinine for t,t′-muconic acid and o-cresol.
[b] The individual outdoor ozone concentration is expressed as: mean of 8-h daily ozone values in μg m^{-3}, during 7 days before blood sampling.
[c] The urinary 8-OHdG was expressed in μg $mmol^{-1}$ creatinine. Micronuclei were counted per 1000 binucleated blood cells. Chromosome aberrations, chromatid and chromosome breaks are indicated inclusive gaps.

aberrations (1.32%) and the micronucleus frequency (1.5‰) were low. On average the DNA of the adolescents showed only 1.16% migration during electrophoresis in the comet assay, and the urinary 8-OHdG concentration was 0.48 μg $mmol^{-1}$ creatinine.

Correlation between Genetic Effect Markers

There was no correlation between the biomarkers of biological effective dose (comet assay and urinary 8-OHdG concentrations) and the biomarkers of 'early response' (micronucleus, chromosome and chromatid breaks). The comet results, however, were positively correlated to the 8-OHdG results (Pearson $r = 0.21$, $P = 0.003$, $n = 200$). On the other hand, for the 36 adolescents, from whom both micronucleus and chromosomal aberrations were measured, there appeared to be a negative correlation between both measurements (Pearson $r = -0.34$, $P = 0.04$, $n = 36$) (Table 3).

Dose-effect Relations between Biomarkers of Exposure and Biomarkers of Biological Effective Dose or Biomarkers of Early Response

In order to evaluate to what extent the different biomarkers of exposure had influenced the biomarkers of effect, a linear multiple regression analysis was performed, with the genotoxic biomarker (comet or 8-OHdG) as the dependent variable and the exposure markers (t,t′-muconic acid, o-cresol and 1-OH-pyrene in urine, and average ozone concentration) put simultaneously as independent variables in the models. In these models, none of the

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Table 3. Spearman rank order correlations between biomarkers of genetic effect

	Comet assay	Micronuclei	Chromosome aberrations	Chromosome breaks	Chromatid breaks
8-OH dG	**0.21**[b]	0.03	0.11	0.02	0.14
	$n = 200$	$n = 98$	$n = 100$	$n = 100$	$n = 100$
Comet assay	—	0.06	0.07	−0.02	0.13
		$n = 98$	$n = 100$	$n = 100$	$n = 100$
Micronuclei		—	−0.24	**−0.34**[a]	−0.15
			$n = 36$	$n = 36$	$n = 36$
Chromosome aberrations			—	**0.75**[c]	**0.81**[c]
				$n = 100$	$n = 100$
Chromosome breaks				—	**0.27**[b]
					$n = 100$

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$.
The urinary 8-OHdG was expressed in μg mmol^{-1} creatinine. DNA migration in the comet assay was given as % DNA in the tail. Micronuclei were counted per 1000 binucleated blood cells. Chromosome aberrations, chromatid and chromosome breaks are indicated inclusive gaps.

Table 4. Exposure/genetic effect relationships in 200 adolescents (stepwise multiple linear regression)

Dependent effect variable	Independent exposure variable	Regression coefficient[a]	Standardised coefficient (SC)[a]	Partial r^2	Overall R^2	P
Comet tail DNA content (%) in blood cells	1-OH-pyrene in urine	18.9 (3.9/33.9)	16.5	0.031		0.013
	ortho-cresol in urine	24.9 (10.2/39.6)	23.0	0.055		0.001
	ozone outdoor (7 days)[b]	0.62 (0.36/0.88)	31.4	0.102		<0.001
					0.294	
8-OHdG in urine (μg mmol^{-1} CRT)	ortho-cresol in urine	11.8 (6.7/16.9)	31.0		0.069	0.007

[a] Percentage change (95% confidence interval) associated with a one-unit change in the independent variable.

Table 5. Exposure/genetic effect relationship in 100 adolescents (logistic regression)

Dependent effect variable	Independent exposure variable	Relative risk[a] (95% CI interval)[b]	P
Chromatid breaks	1-OH-pyrene in urine	1.58 (1.13–2.20)	0.008
(Breaks per 100 cells)	t,t′-muconic acid in urine	1.71 (1.12–2.60)	0.014
Chromosome aberrations	1-OH-pyrene in urine	1.39 (0.98–1.95)	0.064
(Aberrations per 100 cells)			

[a] Relative risk of increase in genetic effect marker for a twofold increase of the exposure marker.
[b] 95% confidence interval.

covariates (gender, educational level of parents, smoking behaviour, environmental tobacco smoke, alcohol use, consumption of grilled food, vitamins and plasma/serum levels of micronutrients such as vitamin A, E and Se) showed a significant relationship with the genotoxicity biomarkers. Still, gender, educational level of parents, smoking behaviour and levels of micronutrients were entered in the regression models, as they might have had an influence in multivariable analysis.

For both genotoxic endpoints, some of the variance was explained by one or a combination of exposure biomarkers (Table 4). Three were found significantly to contribute to DNA damage measured by the comet assay (overall $R^2 = 0.294$; $P < 0.001$). Most of the variance was explained by ozone exposure (partial $r^2 = 0.102$; $P < 0.001$), followed by exposure to toluene (urinary o-cresol: partial $r^2 = 0.055$; $P = 0.001$) and PAHs (urinary 1-OH-pyrene: partial $r^2 = 0.031$; $P = 0.013$). Considering the SCs, the comet assay appeared to be most sensitive to the atmospheric ozone concentration (SC = 31.4), followed by o-cresol (SC = 23.0) and 1-OH-pyrene (SC = 16.5) (Table 4).

Only o-cresol in urine ($r^2 = 0.069$; $P = 0.007$; SC = 31.0) significantly correlated with urinary 8-OHdG, which reflects DNA damage through oxidative stress.

Using logistic regression, the relative risk of chromatid breaks for a twofold increase of the urinary concentration of 1-OH-pyrene was 1.58 (95% CI: 1.13–2.20; $P = 0.008$) and 1.71 (95% CI: 1.12–2.60; $P = 0.014$) for a twofold increase of the urinary concentration of t,t′-muconic acid (Table 5). The relative risk for chromosome aberrations was 1.39 (95% CI: 0.98–1.95; $P = 0.064$) for a doubling of the urinary 1-OH-pyrene concentration. None of the biomarkers of exposure were found to be related to the frequencies of micronuclei and chromosome breaks.

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Discussion

In the past years there has been an increasing interest in biomonitoring and especially monitoring of pollutants and their effects on children (age range 0–18 years) (e.g. WHO 'Children Environment and Health Action Plan for Europe — CEHAPE' and EU initiative called: 'Science, focused on Children, aiming at raising Awareness, using Legal instruments and including constant Evaluation — SCALE'). In 1999 the Flemish Government decided to set up a pilot biomonitoring study of children to try out the feasibility of setting up biomonitoring campaigns on a regular basis. In this context, several genotoxic endpoints were measured in adolescents with lifetime residence in two different areas of Flanders and these were related to urinary concentrations of biomarkers of exposure to common environmental pollutants, i.e. benzene, toluene and PAHs.

Environmental Exposure Markers

Staessen *et al*. (2001) reported that the 1-OH pyrene, t,t′-muconic acid and o-cresol concentrations were higher in the urban compared with the rural area. However, the mean urinary concentrations of t,t′-muconic acid (40.0 nmol mmol^{-1} creatinine), o-cresol (62.9 nmol mmol^{-1} creatinine) and 1-OH-pyrene (33.9 pmol mmol^{-1} creatinine) were far below reference values for the general population (Lauwerys and Hoet, 2001), which are 396 nmol, 314 nmol and 1036 pmol mmol^{-1} creatinine. Compared with those reference values, two individuals were observed who had higher t,t′-muconic acid concentrations, 21 had higher o-cresol values, and none of the individuals exceeded the 1-OH pyrene values. No gender difference was observed in exposure markers. On the other hand, the 1-OH pyrene and t,t′-muconic acid concentrations were higher in smokers compared with non-smokers (Table 1). This was not surprising. Across five studies in Europe, the median urinary concentration of 1-OH-pyrene ranged from 80 to 270 pmol mmol^{-1} creatinine in non-smokers and from 170 to 510 pmol mmol^{-1} creatinine in smokers (Van Rooij *et al*., 1994).

Genetic Effect Biomarkers

The levels of genotoxic effect biomarkers were within values observed in other studies. The frequency of chromosomal aberrations was on average 1.32%. In 1998, 0.95% and 1% chromosomal aberrations were found in 44 children of 10–12 years old, who resided in two areas of Antwerp (Nouwen *et al*., 2001). Steenland *et al*. (1986) and Bender *et al*. (1989) reported 0.98% and 1.49% cells with chromosomal aberrations for controls below 30 years of age or with a mean age of 10.6 years, respectively. Similar values (1.08% aberrant cells) were found in a group of Czech adolescents (16–19 years) examined in the period 1994–1999 (Rössner *et al*., 2002).

To our knowledge, no study has ever shown micronuclei frequencies in such a large group of similarly aged adolescents reviewed in Neri *et al*. (2003). The mean micronucleus frequency observed in our study was low (1.5‰), and is certainly within the range that can be expected for this age group (Bonassi *et al*., 2001). Surprisingly, the micronucleus data in these youngsters were negatively correlated to the chromosomal aberrations observed (Pearson $r = -0.34$, $P = 0.04$) (Table 3). This might indicate that the observed micronuclei formation could be caused by aneuploidy rather than DNA breaks.

The mean value of the alkaline comet assay (1.16% DNA migration) was similar to the mean value of 1.20% obtained from a control group of non-smokers from our institute (on average 32 years old; unpublished result). The average urinary 8-OHdG concentration in the present study was higher (0.48 μg mmol^{-1} creatinine) than reported values, i.e. 0.36 μg mmol^{-1} creatinine (Lagorio *et al*., 1994) and 0.29 μg mmol^{-1} creatinine (Tagesson *et al*., 1993).

Contrary to some of the exposure markers, there was no difference in genetic effect markers between smokers and non-smokers. This could be because the study examined school boys and girls, who started smoking on average only 2 to 3 years ago. Nevertheless, conflicting reports exist on the smoking influence on genotoxicity results. Hong *et al*. (2000) reported a 48% higher concentration of urinary 8-OHdG in adult smokers compared with non-smokers. On the other hand, van Zeeland *et al*. (1999) found less 8-OHdG in the leukocytes of smokers (on average 17 years of smoking) compared with non-smokers. Møller *et al*. (2000) reported that several studies finding differences in DNA damage assessed by the comet assay, showed only slightly higher levels in DNA damage for smokers compared with non-smokers. However, Hininger *et al*. (2004) found a 56% higher level in DNA damage assessed by the comet assay for individuals smoking for 18 ± 9 years compared with non-smokers.

Despite a few reports showing a positive association between smoking and micronuclei, the large majority of studies (reviewed in Bonassi *et al*., 2003) did not find any association. Also, in 5710 adults (among them 1409 smokers) examined in 24 laboratories of 16 different countries smoking caused an increase in micronuclei frequency, only when the interaction with occupational exposure was taken into account (Bonassi *et al*., 2003).

Relationship between Markers of Exposure and Markers of Genetic Effect

Despite the low levels of genetic damage, the relationships between the genotoxic biomarkers and internal/

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external biomarkers of exposure to common environmental pollutants (benzene, toluene, PAHs, ozone) were investigated. No influence of plasma/serum concentrations of vitamin A, E and/or selenium on any of the DNA-damage biomarkers was found, possibly because of the low levels of DNA damage or the adequate nutritional status for these antioxidants.

DNA migration measured with the comet assay as well as chromatid and chromosome breaks (the latter borderline non-significant) correlated with urinary 1-OH-pyrene (Tables 4, 5). This finding suggests continuous exposure to this pollutant. 1-OH-pyrene in urine originates from the metabolism of pyrene, which is present in all PAH mixtures. Thus, 1-OH-pyrene in urine can be considered as a biomarker that indirectly reflects exposure to PAHs (Jongeneelen, 2001). In the general population, smoking and diet are considered the most important sources of PAH intake (IARC, 1983). Inhalation of polluted urban air may be an additional source. The mutagenic properties of PAHs have been extensively described in the literature. In spite of the relative rapid clearance of 1-OH-pyrene from the body (6–35 h), it was still detectable in the urine of 80% of the study group, suggesting continuous exposure to PAHs. Therefore, it is plausible that short-term as well as long-term DNA effects were observed.

The comet assay and also urinary 8-OHdG concentrations correlated well with urinary o-cresol (Table 4). Murato *et al*. (1999) reported that metabolites of toluene induce formation of 8-OHdG in calf thymus DNA treated with the metabolites in the presence of Cu(II) and NADH. It is not entirely clear by which mechanisms toluene exposures may contribute to the observed short-term DNA damage in peripheral blood cells. The presence of minor toluene metabolites might be a possible explanation. Also interaction with unknown factors, might result in the occurrence of DNA damaging components in blood cells.

Besides the above mentioned correlations with 1-OH pyrene and o-cresol, DNA migration in the comet assay was most influenced by ozone exposure (Table 4). Of course, the relative contribution of the exposure biomarkers to the comet-regression-model may reflect (i) the relative abundance of the respective environmental pollutants, and/or (ii) it may reflect the difference in potency of these specific environmental pollutants to cause the specific genotoxic endpoint. Oxidative DNA damage caused by ozone could even have been assessed more specifically by the application of enzymes such as: endonuclease III (converts oxidized pyrimidines to strand breaks) or formamidopyrimidine glycosylase (FPG, recognizes altered purines) in the comet assay (Gedik *et al*., 1998). Unfortunately this was not applied here. Nevertheless, also in *in vivo* exposed mice, ozone doses of 250 $\mu g\ m^{-3}$ induced single strand breaks in murine bronchoalveolar lavage cells measured with the comet assay (Haney *et al*., 1999). Calderon-Garciduenas *et al*. (1999) reported that the combination of both the comet assay and 8-OHdG in nasal biopsies might be useful for monitoring oxidative damage in individuals exposed to polluted atmospheres. Our study observed a good correlation between the comet assay results and urinary 8-OHdG (Pearson $r = 0.21$, $P = 0.003$). 8-OHdG urinary excretion is known to reflect current oxidative DNA damage and repair (Hong *et al*., 2000).

In our study the best measure for ozone exposure in relation to the genetic effect markers appeared to be the 8 h daily values averaged over the week before blood and urine collection. The maximum 8 h daily ozone concentration of 1 day before sampling correlated less, or not, with the comet assay (data not shown). This could indicate that this effect biomarker mainly reflects oxidative DNA damage caused during some days, and not (only) hours, before sampling. In contrast, Lebailly *et al*. (1998) reported that DNA damage detected by the alkaline comet assay seems to reflect mainly ongoing exposure to genotoxic agents, but not an accumulation of damage. For practical considerations, our study examined DNA migration in the comet assay only 24 h after blood collection. In this time, the DNA repair of lesions detected by the comet assay is considered to be complete (Collins *et al*., 1997). Nevertheless, a good correlation was observed between DNA migration in the comet assay and the exposure parameters. Moreover, there was a correlation between the comet assay results and urinary 8-OHdG. It is possible that accumulation of low DNA damage over a longer time period, may be decisive for the detection of mutagen exposure with the comet assay. Plappert *et al*. (1994) concluded this, based on the exposure of mice in inhalation chambers to benzene and/or toluene for up to 8 weeks, followed by 48 h residence in a normal atmosphere. After this repair period, the persistence of DNA damage was higher at 8 weeks of exposure compared with 2 weeks. To increase the sensitivity of the comet assay, the use of repair inhibitors (aphidicolin or ara-C) has been proposed for human biomonitoring studies (Crebelli *et al*., 2002; Speit *et al*., 2003). Nevertheless, conflicting results with respect to the enhancement of the sensitivity of the comet assay, led Speit *et al*. (2005) to conclude that an appropriate experimental protocol still has to be defined.

In conclusion, the alkaline comet assay on blood cells, chromatid breaks in blood and urinary 8-OHdG appeared to be sensitive biomarkers of environmentally induced genetic damage in 17–18 year old youngsters. Alternatively, the nature and level of the pollution in their life environment might have been such that induced genetic damage was most readily detectable by those assays. Interestingly, the comet assay results correlated well with urinary 8-OHdG, a marker of oxidative stress. Because there is still a great need for knowledge on the biological/clinical importance of increased genetic damage of the

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latter biological effective dose markers, this study also examined some markers of early effect. There was no correlation between the appearance of micronuclei, chromosome or chromatid breaks and blood cell DNA damage in the comet assay or urinary 8-OHdG.

In children biomonitoring studies, non-invasive biomarkers are recommended. In this context, the comet assay on a blood sample of a single finger prick or urinary markers such as 8-OHdG, seem to be the most interesting. Our data suggest that the comet assay and 8-OHdG measured *in vivo* have at least a good capacity for measuring genetic effects of pollutants appearing in the life environment of school boys and girls.

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References

Bender MA, Preston RJ, Leonard RC, Pyatt BE, Gooch PC. 1989. Chromosomal aberrations and sister chromatid exchange frequencies in peripheral blood lymphocytes of a large human population sample. II. Extension of age range. *Mutat. Res.* **212**: 149–154.

Bonassi S, Fenech M, Lando C, Lin Y, Ceppi M, Chang WP. 2001. Human Micronucleus Project: international database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes: I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei. *Environ. Mol. Mutagen.* **37**: 31–45.

Bonassi S, Neri M, Lando C, Ceppi M, Lin YP, Chang WP, Holland N, Kirsch-Volders M, Zeiger E, Fenech M. 2003. Effect of smoking habit on the frequency of micronuclei in human lymphocytes: results from the Human Micronucleus project. *Mutat. Res.* **543**: 155–166.

Calderon-Garciduenas L, Wen-Wang L, Zhang YJ, Rodriguez-Alcaraz A, Osnaya N, Villarreal-Calderon A, Santella RM. 1999. 8-Hydroxy-2′-deoxyguanosine, a major mutagenic oxidative DNA lesion, and DNA strand breaks in nasal respiratory epithelium of children exposed to urban pollution. *Environ. Health Perspect.* **107**: 469–474.

Collins A, Dušinská M, Franklin M, Somorovská M, Petrovská H, Duthie S, Fillion L, Panayiotidis M, Rašlová K, Vaughan N. 1997. Comet assay in human biomonitoring studies: Reliability, validation, and applications. *Environ. Mol. Mutagen.* **30**: 139–146.

Crebelli R, Carta P, Andreoli C, Aru G, Dobrowolny G, Rossi S, Zijno A. 2002. Biomonitoring of primary aluminium industry workers: detection of micronuclei and repairable DNA lesions by alkaline SCGE. *Mutat. Res.* **516**: 63–70.

De Boeck M, Lardau S, Buchet JP, Kirsch-Volders M, Lison D. 2000. Absence of significant genotoxicity in lymphocytes and urine from workers exposed to moderate levels of cobalt-containing dust. *Environ. Mol. Mutagen.* **36**: 151–160.

Duarte-Davidson R, Courage C, Rushton L, Levy L. 2001. Benzene in the environment: an assessment of the potential risk to the health of the population. *Occup. Environ. Med.* **58**: 2–13.

Ducos P, Gaudin R, Robert A, Francin JM, Maire C. 1990. Improvement in HPLC analysis of urinary *trans*, *trans*-muconic acid, a promising substitute for phenol in the assessment of benzene exposure. *Int. Arch. Environ. Health* **62**: 529–534.

Fairbairn DW, Olive PL, O'Neill KL. 1995. The comet assay: a comprehensive review. *Mutat. Res.* **339**: 37–59.

Fenech M. 2000. The *in vitro* micronucleus assay. *Mutat. Res.* **455**: 81–95.

Floyd RA. 1990. The role of 8-hydroxyguanine in carcinogenesis. *Carcinogenesis* **11**: 1447–1450.

Gedik CM, Wood SG, Collins AR. 1998. Measuring oxidative damage to DNA; HPLC and the comet assay compared. *Free Radic. Res.* **29**: 609–615.

Golding BT, Watson WP. 1999. Possible mechanisms of carcinogenesis after exposure to benzene. *IARC Sci. Publ.* **150**: 75–88.

Haney JT, Connor TH, Li L. 1999. Detection of ozone-induced DNA single strand breaks in murine bronchoalveolar lavage cells acutely exposed *in vivo*. *Inhal. Toxicol.* **11**: 331–341.

Hininger I, Chollat-Namy A, Sauvaigo S, Osman M, Fure H, Cadet J, Favier A, Roussel A-M. 2004. Assessment of DNA damage by comet assay on frozen total blood: method and evaluation in smokers and non-smokers. *Mutat. Res.* **558**: 75–80.

Hong Y-C, Park H-S, Ha E-H. 2000. Influence of genetic susceptibility on the urinary excretion of 8-hydroxydeoxyguanosine of firefighters. *Occup. Environ. Med.* **57**: 370–375.

Hotz P, Carbonnelle P, Haufroid V, Tschopp A, Buchet JP, Lauwerys R. 1997. Biological monitoring of vehicle mechanics and other workers exposed to low concentrations of benzene. *Int. Arch. Environ. Health* **70**: 29–40.

IARC (International Agency for Research on Cancer). 1983. *Polynuclear Aromatic Compounds: Part 1. Chemical Environmental and Experimental Data*. IARC Monograph Volume 32. IARC: Lyon.

Jaffé M. 1986. Ueber den Niederslag welchen Pikrinsäure in normalen Harn erzeugt und über eine neue Reaction des Kreatinins. *Z. Physiol. Chem.* **10**: 391–400.

Jongeneelen FJ. 2001. Benchmark guideline for urinary 1-hydroxypyrene as biomarkers of occupational exposure to polycyclic aromatic hydrocarbons. *Ann. Occup. Hyg.* **45**: 3–13.

Lagorio S, Tagesson C, Forastiere F, Lavarone I, Axelson O, Carere A. 1994. Exposure to benzene and urinary concentrations of 8-hydroxydeoxyguanosine, a biological marker of oxidative damage to DNA. *Occup. Environ. Med.* **51**: 739–743.

Lauwerys RR, Hoet P. 2001. *Industrial Chemical Exposure: Guidelines for Biological Monitoring*, 3rd edn. Lewis Publishers CRC Press LLC: Boca Raton, FL.

Lebailly P, Vigreux C, Lechevrel C, Ledemeney D, Godard T, Sichel F, LeTalaer JY, Henry-Amar M, Gauduchon P. 1998. DNA damage in mononuclear leukocytes of farmers measured using the alkaline comet assay: discussion of critical parameters and evaluation of seasonal variations in relation to pesticide exposure. *Cancer Epidemiol. Biomarkers Prev.* **7**: 917–927.

Loft S, Fischer-Nielsen A, Jeding IB, Vistisen K, Enghusen Poulsen HB. 1993. 8-Hydroxydeoxyguanosine as a urinary biomarker of oxidative DNA damage. *J. Toxicol. Environ. Health* **40**: 391–404.

Møller P, Knudsen LE, Loft S, Wallin H. 2000. The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors. *Cancer Epidemiol. Biomarkers Prev.* **9**: 1005–1015.

Murata M, Tsujikawa M, KawanishiI S. 1999. Oxidative DNA damage by minor metabolites of toluene may lead to carcinogenesis and reproductive dysfunction. *Biochem. Biophys. Res. Commun.* **261**: 478–483.

Neri M, Fucic A, Knudsen LE, Lando C, Merlo F, Bonassi S. 2003. Micronuclei frequency in children exposed to environmental mutagens: a review. *Mutat. Res.* **544**: 243–254.

Nouwen J, Cornelis C, De Fré R, Wevers M, Viaene P, Mensink C, Patyn J, Verschaeve L, Hooghe R, Maes A, Collier M, Schoeters G, Van Cleuvenbergen R, Geuzens P. 2001. Health risk assessment of dioxin emissions from municipal waste incinerators: the Neerlandquarter (Wilrijk, Belgium). *Chemosphere* **43**: 909–923.

Perera FP. 2000. Molecular epidemiology: on the path to prevention? *J. Natl Cancer Inst*. **92**: 602–612.

Pierce CH, Dills RL, Morgan MS, Vicini P, Kalman DA. 1998. Biological monitoring of controlled toluene exposure. *Int. Arch. Occup. Environ. Health* **71**: 433–444.

Plappert U, Barthel E, Seidel HJ. 1994. Reduction of benzene toxicity by toluene. *Environ. Mol. Mutagen*. **24**: 283–292.

Rössner P, Bavarova H, Ocadlikova D, Svandova E, Sram RJ. 2002. Chromosomal aberrations in peripheral lymphocytes of children as biomarkers of environmental exposure and life style. *Toxicol. Lett*. **134**: 79–85.

Schwartz GG. 1990. Chromosome aberrations. In *Biological Markers in Epidemiology*, Hulka BS, Wilcosky TC, Griffith JD (eds). Oxford University Press: New York, 147–172.

Singh NP, McCoy MT, Tice RR, Schneider EL. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res*. **175**: 184–191.

Speit G, Schütz P, Hoffmann H. 2005. Enhancement of genotoxic effects in the comet assay with human blood samples by aphidicolin. *Mutat. Res*. **581**: 105–114.

Speit G, Witton-Davies T, Heepchantree W, Trenz K, Hoffman H. 2003. Investigations on the effect of cigarette smoking on the comet assay. *Mutat. Res*. **542**: 33–42.

Sram RJ, Binkova B. 2000. Molecular epidemiology studies on occupational and environmental exposure to mutagens and carcinogens, 1997–1999. *Environ. Health Perspect*. **108**: 57–70.

Staessen JA, Nawrot T, Den Hond E, Thijs L, Fagard R, Hoppenbrouwers K, Koppen G, Nelen V, Schoeters G, Vanderschueren D, Van Hecke E, Verschaeve L, Vlietinck R, Roels HA. 2001. Renal function, cytogenetic measurements, and sexual development in adolescents in relation to environmental pollutants: a feasibility study of biomarkers. *Lancet* **357**: 1660–1669.

Steenland K, Carrano A, Ratcliffe J, Clapp D, Ashworth L, Meinhardt TA. 1986. Cytogenetic study of papaya workers exposed to ethylene dibromide. *Mutat. Res*. **170**: 151–160.

Tagesson C, Chabiuk D, Axelson O, Baranski B, Palus J, Wyszynska K. 1993. Increased urinary excretion of the oxidative DNA adduct, 8-hydroxydeoxyguanosine, in the asbestos, rubber, and azo-dyes industries. *Pol. J. Occup. Med. Environ. Health* **6**: 357–368.

Truchon G, Tardif R, Brodeur J. 1999. o-Cresol: a good indicator of exposure to low levels of toluene. *Appl. Occup. Environ. Hyg*. **14**: 677–681.

Van Hummelen P, Gennart JP, Buchet JP, Lauwerys R, Kirsch-Volders M. 1993. Biological markers in PAH exposed workers and controls. *Mutat. Res*. **300**: 231–239.

Van Rooij JGM, Veeger MMS, Bodelier-Bade MM, Scheepers PTJ, Jongeneelen FJ. 1994. Smoking and dietary intake of polycyclic aromatic hydrocarbons as sources of interindividual variability in the baseline excretion of 1-hydroxypyrene in urine. *Int. Arch. Occup. Environ. Health* **66**: 55–65.

van Zeeland AA, de Groot AJL, Hall J, Donato F. 1999. 8-Hydroxydeoxyguanosine in DNA from leukocytes of healthy adults: relationship with cigarette smoking, environmental tobacco smoke, alcohol and coffee consumption. *Mutat. Res*. **439**: 249–257.

Weitzman SA, Turk PW, Milkowski DH, Kozlowski K. 1994. Free radical adducts induce alterations in DNA cytosine methylation. *Proc. Natl Acad. Sci. USA* **91**: 1261–1264.

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