Pooled analysis of genotoxicity markers in relation to exposure in the Flemish Environment and Health Studies (FLEHS) between 1999 and 2018

Pooled analysis of genotoxicity markers in relation to exposure in the Flemish Environment and Health Studies (FLEHS) between 1999 and 2018

Koppen G, Franken C, Den Hond E, Plusquin M, Reimann B, Leermakers M, Covaci A, Nawrot T, Van Larebeke N, Schoeters G, Bruckers L, Govarts E

Abstract

Background. The Flemish Environment and Health Studies (FLEHS) are human biomonitoring surveys running in Flanders since 1999. Additionally to biomarkers of exposure, markers of genotoxicity and oxidative stress have been measured, including the alkaline comet assay on peripheral blood cells, the micronucleus (MN) assay in peripheral blood lymphocytes, and urinary concentrations of 8-oxo-2'-deoxyguanosine (8-oxodG).

Aim. Exposure-effect associations were explored in a pooled dataset of nine different cross-sectional FLEHS surveys in adolescents in a time frame of about 20 years (1999 to 2018). The aim of the study was to combine multiple study populations, with overall more variation in exposure, lifestyle and environmental factors, in order to examine if this would lead to more powerful and robust exposure-effect associations.

Materials & Methods. Genotoxicity markers and exposure markers were measured in 2,283 adolescents in the age range of 14-18 years. Biomarkers in blood or urine reflected exposure to polycyclic aromatic hydrocarbons (PAHs) (1-hydroxypyrene (1-OHP), benzene (tt'-muconic acid (TT-MA), metals (arsenic, cadmium, copper, nickel, thallium, lead, chromium), hexachlorobenzene (HCB), dichlorodiphenyldichloroethylene (*p,p'*-DDE), *trans*-nonachlor (TN), polychlorinated biphenyls (PCBs) and phthalates (metabolites of di(2-ethylhexyl) phthalate (DEHP), benzyl butyl phthalate (BzBP), di-butyl phthalate (DBP). Furthermore, outdoor air levels of particulate matter (PM_{10} and $PM_{2.5}$) at the residences of the youngsters were calculated. Pooled statistical analysis was done using a mixed model. To account respectively for possible study-specific differences in effect markers and for differences in the strength/direction of the association for the different studies, the random factor 'study' and a random study slope (if possible) were incorporated. The exposure markers were centered around the study specific means in order to correct for protocol changes over time. Causal directed acyclic graphs (DAGs) were made to illustrate potential confounding for the significant associations and Structural Equation Modelling (SEM) was used to examine these relationships.

Results. *A s*ignificant association was observed for the urinary oxidative stress marker 8-oxodG, which was positively associated with 1-OHP (5% increase for doubling of 1-OHP levels, p=0.001), and with urinary Cu (26% increase for doubling of Cu levels, p=0.001), a metal involved in the Fenton reaction in biological systems. 8-oxodG was also associated with the sum of the metabolites of the phthalate DEHP (3% increase for doubling of the DEHP levels, p=0.02). In the individual studies, similar associations were observed, although not always statistically significant. This means that pooling of the data increased the statistical power. However, some of the exposure markers were related with the genotoxicity outcomes in individual surveys, but this was not confirmed in the pooled analysis (such as comet assay and 8-oxodG vs. atmospheric PM; and 8-oxodG vs. urinary Ni). This may be due to inconsistencies in exposure-effect relations, or in some cases, pooling of studies may cause increased exposure 'misclassification' i.e. the exposure marker may represent different pollutant mixtures, especially in hot spot human biomonitoring.

Conclusion. Pooled analysis including a large population of 2,283 Flemish adolescents showed that 8-oxodG, a marker of oxidative DNA damage was consistently associated with biomarkers of exposure to daily life pollutants, such as PAHs, Cu and the phthalate DEHP.

Key words: human biomonitoring, Flemish Environment and Health Studies, pooled analysis, genotoxicity, 8-oxo-2'-deoxyguanosine, comet assay, micronucleus assay

1. Introduction

The Flemish Environment and Health Studies (FLEHS) are large human biomonitoring surveys that ran in Flanders since 2002, and were preceded by a pilot study in 1999 (Schoeters *et al.*, 2012, 2017). Markers of genotoxicity and oxidative stress have been measured, including the alkaline comet assay and the micronucleus (MN) assay in peripheral whole blood and urinary concentrations of 8-oxo-2'-deoxyguanosine (8-oxodG). The alkaline comet assay, originally developed by Ostling and Johanson (Ostling and Johanson, 1984) and later adapted by Singh *et al.* (Singh *et al.*, 1988), detects a broad spectrum of DNA lesions including single and double strand breaks as well as single strand breaks associated with incomplete excision repair sites and alkali-labile sites (Tice *et al.*, 2000; Collins *et al.*, 2014). 8-oxodG, an oxidized nucleoside of DNA, is the most frequently detected and studied DNA lesion. Upon DNA repair or oxidation of the DNA pool present in the cell, 8-oxodG is excreted in urine. 8-oxodG is an important biomarker of oxidative stress and DNA repair (Cooke, Olinski and Loft, 2008). Another way of detecting and quantifying genome anomalies is to count MN, which are small round bodies found in the cytoplasm outside the main nucleus. They arise from the encapsulation of chromosome fragments, as well as entire chromosomes that are not incorporated in the daughter cells' nuclei. Thus, the number of MN represents the amount of (unrepairable) double strand breaks and chromosome losses present in a cell (Fenech *et al.*, 1999; Fenech, 2007). A main difference between the three genotoxic biomarkers is the nature of damage measured. While the damage assessed by the comet assay or 8-oxodG reflects respectively mostly repairable or repaired DNA ('short-lived DNA damage'), the damage in the MN remains unrepaired and can persist in cells over several generations ('long-term DNA damage') (Bolognesi *et al.*, 1997; Collins, 2004; Azqueta *et al.*, 2014).

The three genotoxicity markers are interesting tools to study the pressure of environmental exposure on DNA integrity. They have been widely used in occupational and environmental biomonitoring studies on i.e. metal, persistent organic pollutants (POPs), volatile organic compounds, polycyclic aromatic compounds (PAHs), pesticides exposure in the age range of newborns to elderly (Koppen *et al.*, 2007; Costa *et al.*, 2011; Sughis *et al.*, 2012; Collins *et al.*, 2014; Franken, Koppen, *et al.*, 2017).

Within the FLEHS studies, these genotoxicity markers were measured together with a broad panel of exposure biomarkers, more specifically indicators of exposure to metals, persistent organic pollutants (POPs), perfluorinated compounds, plastic additives, volatile organic compounds, polycyclic aromatic hydrocarbons (PAHs), flame retardants, pesticides and personal care products (Koppen *et al.*, 2002; Den Hond *et al.*, 2013; Croes *et al.*, 2014; Geens *et al.*, 2014; Vrijens *et al.*, 2014; De Craemer *et al.*, 2016, 2017; Franken, Koppen, *et al.*, 2017; Schoeters *et al.*, 2017).

This paper includes a pooled statistical analysis of the exposure-effect associations for nine different cross-sectional FLEHS surveys performed in adolescents in a time frame of about 20 years (1999 to 2018). The aim of the study was to test exposure-effect associations with considerable statistical power using a study population of 2,283 individuals, with overall more variation in exposure, lifestyle and environmental factors, potentially leading to more powerful and profound analysis of the relationships.

2. Materials & Methods

Study populations

Nine study populations of adolescents were included in the current analysis. Participants (N=197, 1999) of the pilot study were 17-18 years and were recruited in two industrial areas and one rural control area (Staessen *et al.*, 2001). In all following FLEHS studies, participants were 14-15 years old (N= number of participants in which minimal 1 genotoxicity marker has been measured). FLEHS-1

(N=434, 2003–2004) participants were recruited in eight pre-defined rural, urban or industrial regions. Flemish reference populations were recruited in FLEHS-2 (N=204, 2008–2009), FLEHS-3 (N=199; 2013–2014) and FLEHS-4 (N=547, 2017–2018, including N=182 recruited in a birth cohort of FLEHS-1) through a stratified clustered multi-stage design. Further, specific populations around industrial sites were recruited in hotspot biomonitoring studies around an industrial site in Genk-Zuid (FLEHS-2 GZ, N=192 2009-2010), a shredder in Menen (FLEHS-2 M, N=196, 2010-2011), the industrial zone around Ghent harbor (FLEHS-3 GKZ, N=193, 2013-2014) and a follow-up study in Genk-Zuid (GZ, N=121, 2016). More details about the study design and recruitment strategy of the different studies (except for FLEHS-4) have been previously reported (Staessen *et al.*, 2001; Koppen *et al.*, 2007; Den Hond *et al.*, 2009; Baeyens *et al.*, 2014; De Craemer *et al.*, 2016; Franken, Koppen, *et al.*, 2017; Schoeters *et al.*, 2017). For the current pooled analysis, all adolescents in which at least one genotoxicity marker was measured, and of which information on the covariates was available, were included, resulting in N=2,283 individuals.
In each study, a questionnaire on relevant covariates was included. Length and weight were assessed by the study nurses. Cut-off values for body mass (BMI) classes per age category of half a year, were based on the Belgian growth curves, specific for age and sex (http://www.vub.ac.be/groeicurven/groeicurven.html) (see also Supplementary Table 1). All study protocols were approved by the Ethical Committee of the University of Antwerp, and informed consent was obtained from all participants and their parents.

Genotoxicity biomarkers

Biomarkers of effect, including the alkaline comet assay, MN frequency and urinary levels of 8-oxodG, were analyzed in the same laboratory. Over the time frame of the different studies, the protocols were slightly adapted. The main differences were for: (i) comet assay: fresh as well as frozen blood cells were used, lysis duration, stain used and scoring system; (ii) MN assay: change in stain used, and scoring system; (iii) 8-oxodG: in the pilot study assessed via HPLC and in all later studies via a same type of ELISA kit.

DNA strand breaks were evaluated by the alkaline comet assay according to Singh *et al.* (Singh *et al.*, 1988), with a few modifications. Whole blood samples were kept at a maximum of 48 h at room temperature. In all studies, this fresh blood was used in the comet assay, except for the FLEHS-4 survey, in which whole blood was frozen according to the protocol described in Koppen *et al.* (Koppen *et al.*, 2018). For each individual, 5 µL fresh whole blood (or 5 µL of rinsed frozen cells) was mixed with 120 µL 0.8% Low Melting Point Agarose (Life Technologies, Foster City, CA, USA). Of this mixture, 100 µL was placed onto a Gelbond® Film (Lonza, Basel, Switzerland) with the size of a conventional glass microscope slide. Two gels were made from each sample and considered as technical replicates. Gels were immersed in a cold lysis solution (2.25 M NaCl, 90 mM Na2EDTA, 9 mM Tris, 1% Triton X-100, 10% DMSO) and kept at 4°C overnight until maximum 3 months depending on the study. Before electrophoresis, slides were placed for 40 min in the electrophoresis tank in a precooled (4°C) alkaline solution (300 mM NaOH, 1 mM Na_2EDTA) to unwind the DNA. Electrophoresis was performed for 20 min at 4°C and 1 V/cm (0.8 V/cm across the elevated platform of the electrophoresis tank). The electrophoresis buffer was recirculated with a peristaltic pump from anode to cathode at a flow of 100 mL/min. The tank was cooled and the electrophoresis solution had a temperature below 15°C during electrophoresis. After electrophoresis, gels were washed and stained with ethidium bromide (until FLEHS-2) or SYBR® Gold Nucleic Acid Gel Stain (Life Technologies, Foster City, CA, USA) (from FLEHS-3 onwards). Per slide, N=300 cells were scored. The data were analyzed by using a computerized image analysis system (Metafer of MetaSystems, Althussheim, Germany; except for the pilot study: Kinetic Imaging Ltd., Liverpool UK). A scorer performed a visual check of the automatic scored 'comets' (the scorer changed over the time frame of the studies). The relative amount of DNA damage, i.e. percentage DNA in the tail compared to the total amount in the whole comet, was assessed (tail

intensity). The classifier protocol (algorithm for quantification of the comet tail content) of the software was optimized over the time period of the 9 surveys.

The MN assay has previously been described in detail (De Coster *et al.*, 2008). Briefly, the cytokinesis-block micronucleus assay was performed within 12h after sampling on heparinized fresh peripheral whole blood using standard procedures as described by Fenech (Fenech, 2007). For each subject, 1,000 binucleated (BN) cells were evaluated for the presence of MN semi-automatically using Metacyte of Metasystems. In the pilot study, however, counting was performed manually using a microscope (Zeiss, Germany). The applied staining also changed over time. From FLEHS-3 on the fluorescent stain DAPI was used, whereas earlier, the slides were stained using Giemsa-May-Grünwald. MN frequency was reported as the number of MN per 1000 BN cells. Over the studies, the scorer was changing.

Urine samples were frozen and stored at -80°C until 8-oxodG analysis. After thawing, they were centrifuged at 2000×g for 15 min. Fifty µL of the supernatant was used for the determination of 8-oxodG with a commercial competitive enzyme-linked immunosorbent assay kit (New 8-oxodG check ELISA kit; Japan Institute for the Control of Aging, Shizuoka, Japan) according to the manufacturer's instructions. The determination range was 0.5-200 ng/mL. The anti-8-oxodG mouse monoclonal antibody (clone N45.1), with an established specificity, was used as a primary antibody (Toyokuni *et al.*, 1997). The values from each urine sample were calculated based on calibration sigmoid plots of absorbance (450 nm) of an 8-oxodG standard at various concentrations. The same type of kit was used from FLEHS-1 on, and the assay was performed mainly by the same technician. However, in the pilot study of 1999, 8-oxodG was measured with a different technique. There, urine was enzymatically hydrolysed (β-glucuronidase/sulfatase, pH=5 at 37 °C) overnight. Solid phase extraction on a C18-column was used for clean-up of the urine before measuring 8-oxodG with HPLC using fluorimetric detection. The detection limit was 0.01 ng/mL (Koppen *et al.*, 2007).
Validation dossiers were assembled for all effect biomarkers as well as results from participation to international ring tests, when available.

Exposure biomarkers

From the different studies, the exposure biomarkers that were known to cause oxidative stress, DNA damage, or loss of DNA integrity, were selected. This included the following biomarkers: the PAH metabolite 1-hydroxypyrene (1-OHP), the benzene metabolite *tt'*-muconic acid (TT-MA), persistent organochlorine pollutants hexachlorobenzene (HCB), dichlorodiphenyldichloroethylene (*p,p'*-DDE), polychlorinated biphenyl (PCB) congeners (PCB 138, 153 and 180), *trans*-nonachlor (TN), and metals (arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), nickel (Ni), thallium (Tl)).
The methodology of the measurements was described previously (Schoeters *et al.*, 2017). 1-OHP was enzymatically released overnight, followed by on-line solid phase extraction and HPLC-fluorescence detection. TT-MA was separated from urine by ion chromatography (SPE-SAX), and further separated and detected by high performance liquid chromatography (HPLC) coupled with diode array detection. PCB 138, PCB 153, PCB 180, *p,p'*-DDE, HCB and TN were measured in serum using solid-phase extraction and gas chromatography coupled to electron capture ionization mass spectrometry. Metals in whole blood (As, Cd, Cu, Pb, Tl) were determined using a high resolution inductively coupled plasma mass spectrometer (HR-ICP-MS). Cr and Ni analyses were performed with a dynamic reaction cell (DRC)-ICP-MS. Metals in urine (Cd, Cu, Ni, Tl) were determined using a HR-ICP-MS. As in urine was measured by DRC-ICP-MS. In FLEHS-2, toxic relevant organic and inorganic arsenic (TRA) in urine was determined as a sum parameter using flow injection hydride generation atomic fluorescence spectrometry. In FLEHS-3 and FLEHS-4, the individual arsenic metabolites of the TRA mixture (As(III), As(V), monomethyl arsenic (MMA), dimethyl arsenic (DMA)) were measured separately using High Performance Liquid Chromatography-ICP-MS, with a Dynamic Reaction Cell. TRA was calculated as the

sum of these markers. The phthalate metabolites mono-2-ethylhexyl phthalate (MEHP), mono-2-ethyl-5-hydroxyhexyl phthalate (5-OH MEHP), mono-2-ethyl-5-oxohexyl phthalate (5-oxo MEHP), mono-n-butyl phthalate (MnBP), mono-benzyl phthalate (MBzP) were measured in urine. The methodology was described in detail in Geens *et al.* (Geens *et al.*, 2014). The sum of three DEHP metabolites MEHP + 5-oxo MEHP + 5-OH MEHP was further indicated as ∑DEHP.
All samples were handled and stored at the central laboratory within one day after sampling. Exposure biomarkers were measured according to international standard quality assurance and quality control (QA/QC), guaranteeing linearity, reproducibility, repeatability, accuracy and precision of the results. Validation dossiers were assembled for the exposure and as well as results from participation to international ring tests, when available.

Particulate Matter (PM) air quality levels

PM_{10} and $PM_{2.5}$ (the latter only available since 2005) were measured on 36 air quality measurement stations all over Flanders. The RIO interpolation model was used to interpolate the levels for areas of 1x1 km based on the available measurements from the fixed measuring stations taking into account information on land use (Janssen *et al.*, 2008). For the individual adolescents, averages were calculated for a period of 7 days before the urine/blood collection day. The averages of shorter and longer exposure periods were also assessed but did not show different results. These calculations were performed by the Belgian air quality center IRCEL. The home addresses of all adolescents were transformed to GPS codes and via GIS software layered on the RIO modeled air quality grids.

Statistical analysis

Database building – pooling
Data of the biomarkers of effect and exposure were selected from the individual databases of the nine surveys and integrated into one database. If needed, the biomarkers of the different studies were converted to the same unit. The method to quantify TRA in urine used in FLEHS-2 differed from the method used in later studies (hybride-AAS versus HPLC). Control samples, quantified with both methods, showed a strong correlation between the old and new method but the new method resulted in measurements that were systematically 25% lower. Therefore, the measurements from FLESH-2 were adjusted to the new method by means of a linear regression model on the ln-transformed measurements. Idem for 1-OHP, where the measurement method of FLESH-4 differed from the one used in the earlier studies. For this marker, the measurements of the studies before FLESH-4 were calibrated towards the measurement method of FLESH-4 by means of a linear regression model of the ln-transformed measurement of control samples.

In every study, a detailed questionnaire about lifestyle was conducted. Since the surveys span a period of 20 years, changes were made to these questionnaires. The following information with respect to lifestyle and environmental factors was uniformly available for all nine studies and known to be associated with the genotoxicity biomarkers: gender, smoking behavior (yes/no), highest educational level of the family (3 levels), body mass index (BMI class: underweight, normal weight, overweight), and average temperature 7 days before the blood/urine collection. These variables were used as covariates (explanatory variables) in the exposure-effect models.

Descriptive statistics
Descriptive statistics to characterize the study population and the biomarkers are based on the adolescents that had at least one marker of genotoxicity and information on the covariates to be included in the regression models (N= 2283). For the exposure markers the median and interquartile range was presented. For the effect markers the (geometric) mean and 95% confidence interval was given.

Study specific exposure-effect associations

For every separate study, exposure-effect associations were investigated by means of a linear regression model. All biomarkers of exposure and 8-oxodG per g creatinine were ln-transformed. Values below the limit of detection (LOD) or limit of quantification (LOQ) were replaced by half of the LOD/LOQ. In case, in a study, more than 60% of the samples had a value below the LOD/LOQ, the biomarker was not taken up in the statistical analysis of the exposure-effect relationship. The urinary exposure markers and 8-oxodG were expressed per gram creatinine. The fat-soluble exposure markers determined in blood were expressed per gram serum lipids. Additionally to the pre-defined covariates, the models with urinary exposure markers were corrected for creatinine and the models with exposure markers measured in serum lipids were corrected for serum lipids (O'Brien *et al.*, 2016).

Based on these regression models, an estimate (slope) and 95% confidence interval for the (corrected) exposure-effect association were obtained. For the comet and MN assay, the increase in average effect (additive effect) for a doubling in the exposure is given. For 8-oxodG, the ratio of the average effect (multiplicative effect , % increase) for a doubling in exposure is presented. A forest plot was used to graphically display the results of these study specific exposure-effect associations.

Pooled analysis

The statistical models used for the pooled analysis (i.e. on the combined data of the nine surveys) were the same as the study-specific models, i.e. the same set of explanatory variables was considered and the same transformations were used.

However, to account for differences in the study design (e.g. changes in measurement technique) over the 20 years period, the ln-transformed exposure biomarkers were first centered by subtracting the study specific mean. To correct for a possible correlation between the effect marker values of participants of the same survey and to allow for differences in the level of the effect marker of the nine surveys, the random factor 'study' was introduced in the model. If necessary, the study specific (random) slope for the exposure biomarker was also used. A random slope would indicate that the strength or even possibly the direction of the association significantly differed between the surveys and that the strength of the exposure-effect association could not be quantified by one estimate; or that pooling was not advisable. Finally, the model allowed for study specific error variability. Exposure-effect associations were fitted when a minimum of three individual surveys could be included in the pooled analysis. Model selection techniques were not applied. This implied that the complete set of explanatory variables was used in every exposure-effect model.

Sensitivity analyses of the mixed model pooled analysis were performed by looking at (i) fixed effects models where a fixed study effect and the interaction term between study and biomarker of exposure were included, (ii) mixed model in which each time one study was left out, (iii) mixed model in which the exposure parameters were not mean-centered and (iv) meta analyses, using as input the corrected estimates of the exposure-effects associations of the different surveys. For multiple testing corrected p-values are obtained using the false discovery rate (FDR) approach of Benjamini and Hochberg (Benjamini and Hochberg, 1995). Effect modification by sex was examined by looking into the exposure-effect association separately in boys and girls. All data manipulation and the mixed/fixed effects models were done in SAS version 9.4.

Covariates/confounders tested

For each of the effect biomarkers, the significance of the selected explanatory variables (smoking, age, sex, BMI class, highest education level of the family, and outdoor temperature 7 days before blood/urine collection) was tested in a mixed multiple regression model with random study effect. Furthermore, for the associations observed in the pooled analysis, which also showed consistent trends in the individual studies (8-oxodG vs. 1-OHP, urinary Cu and ∑DEHP metabolites), a causal

directed acyclic graph (DAG) was adopted as a visual aid to check for relevant confounders and for completeness of the model used in the pooled analysis.

DAGs facilitate the choice of minimal sufficient adjustment sets required to minimize confounding bias and assist in the decision if the adjustment for additional variables is appropriate or harmful. In a first step covariates were identified by literature search for associations with 8-oxodG and respectively 1-OHP, Cu or ∑DEHP. Graphical models of the hypothesized causal relationships were designed using directed acyclic graphs (DAGs) constructed with the online tool DAGitty 2.3 (www.dagitty.net) (Textor *et al.*, 2016). In a next step, structural equation modeling (SEM) was used to model all hypothesized relations between the variables displayed in the DAGs on the pooled data. This pathway analysis was done for all three associations. To preserve the initial direction of associations, the measurements of the four biomarkers 8-oxodG, 1-OHP, urinary Cu and ∑DEHP were not additionally corrected for creatinine but creatinine levels were included in the path. To evaluate the goodness of fit of the model the normed chi-square (ratio of chi-square to degrees of freedom, $\chi 2/df$) was calculated and a value lower than 3 was deemed acceptable (Carmines and McIver, 1981). Furthermore, the root mean square error of approximation (RMSEA) was determined, with smaller values implying a better fit. A model with an RMSEA value of less than 0.08 was considered as an adequate model (Browne and Cudeck, 1992). Additionally, for the standardized root mean square residual (SRMR) a cut-off of <0.05 (Byrne, 1998) and for the comparative fit index (CFI) a value larger than 0.95 (Schermelleh-Engel, Moosbrugger and Müller, 2003) constituted an acceptable fit. To accomplish the path analysis the Lavaan package, version 0.6-5 (Rosseel, 2012) was used within the RStudio 3.4.2 environment.

3. Results

Characteristics of the study populations

In the total population of N=2,283 adolescents (of which information on minimal one genotoxicity marker and the covariates were available), the age ranged between 14 and 18 years, an equal number of girls and boys were included (Table 1). The overall percentage of smokers among this population was 9.2% and varied over the individual studies between 0.8% and 24.9%. The majority of the teenagers (75.8%) had a normal BMI, 10.5% were underweight and 13.7% overweight. Thirteen percent of the children had parents of which the highest education level was lower secondary level, whereas 35.8% of the families had at least a higher secondary education and 51.3% a higher education level.

Biomarkers of exposure and effect

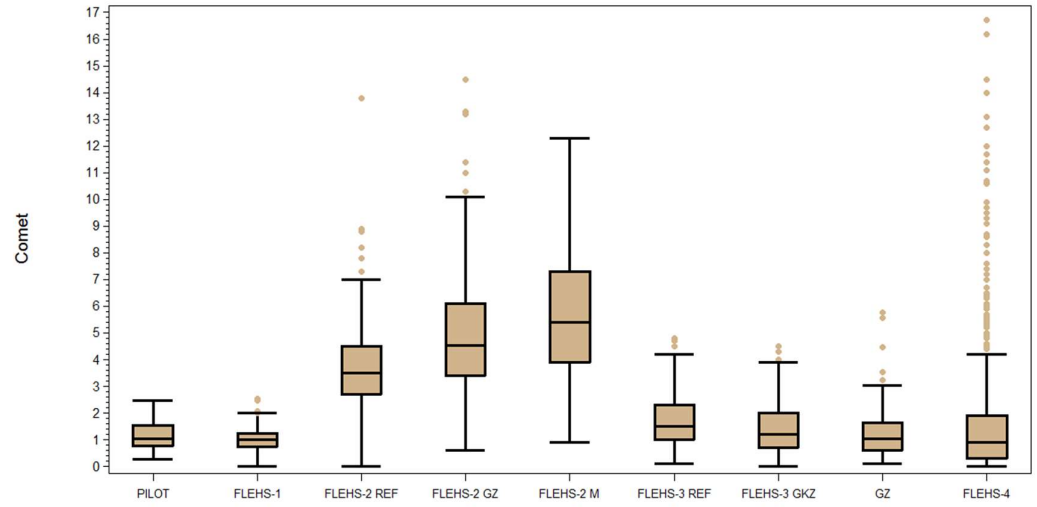
The levels of the genotoxicity biomarkers in the different surveys are given in Figure 1 and Supplementary Table 2. For the total study population, the mean (95% CI) levels equaled 2.4 (2.3-2.5) % DNA migration for the comet assay, 2.0 (1.8-2.1) micronuclei/1000 binucleated cells for the MN assay and 9.0 (8.8-9.2) µg 8-oxodG/g creatinine. The genotoxicity biomarkers were weakly, but significantly ($p<0.001$) correlated, i.e. Pearson correlation was $r=0.24$ for 8-oxodG vs. comet assay, $r=0.12$ for 8-oxodG vs. MN, $r= 0.13$ for the comet assay vs. MN assay results. Descriptive statistics for the exposure biomarkers in the total study population is given in Table 2, and for the different surveys in Supplementary Table S3.

***Table 1**: Characteristics of the adolescents included in the different surveys (except for age, data are numbers and percentages N (%)), adolescents with at least one genotoxicity marker and info for the covariates.*

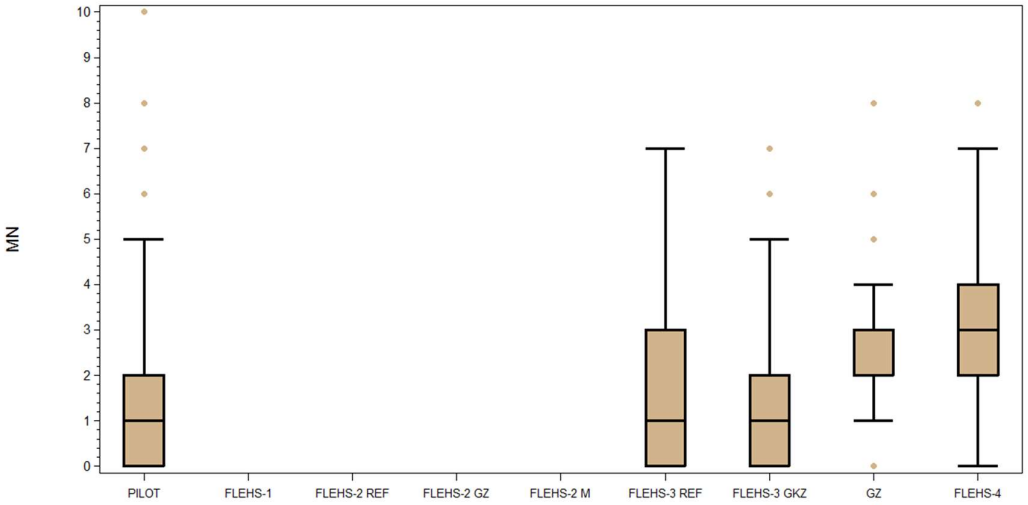
Characteristics	***PILOT***	***FLEHS-1***	***FLEHS-2 REF***	***FLEHS-2 GZ***	***FLEHS-2 MENEN***	***FLEHS-3 REF***	***FLEHS-3 GKZ***	***GZ***	***FLEHS-4***	***POOLED***
Year of recruitment	***1999***	***2003-2004***	***2008-2009***	***2009-2010***	***2010-2011***	***2013-2014***	***2013-2014***	***2016***	***2017-2018***	***1999-2018***
Number of participants	197	434	204	192	196	199	193	121	547	2,283
Age (years)[a]	17.35 ± 0.82	14.74 ± 0.49	14.77 ± 0.50	14.97 ± 0.67	15.06 ± 0.78	14.84 ± 0.51	14.94 ± 0.67	14.44 ± 0.64	14.77 ± 0.47	15.03 ± 0.94
Gender										
Boys	79 (40.1)	256 (59.0)	118 (57.8)	87 (45.3)	111 (56.6)	92 (46.2)	97 (50.3)	58 (47.9)	261 (47.7)	1,159 (50.8)
Girls	118 (59.9)	178 (41.0)	86 (42.2)	105 (54.7)	85 (43.4)	107 (53.8)	96 (49.7)	63 (52.1)	286 (52.3)	1,124 (49.2)
Current smoking	49 (24.9)	54 (12.4)	18 (8.8)	11 (5.7)	15 (7.7)	29 (14.6)	13 (6.7)	1 (0.8)	21 (3.8)	211 (9.2)
BMI class[b]										
Underweight	22 (11.2)	26 (6.0)	19 (9.3)	20 (10.4)	20 (10.2)	42 (21.1)	30 (15.5)	10 (8.3)	51 (9.3)	240 (10.5)
Normal weight	155 (78.7)	349 (80.4)	163 (79.9)	131 (68.2)	159 (81.1)	136 (68.3)	153 (79.3)	89 (73.6)	395 (72.2)	1,730 (75.8)
Overweight	20 (10.2)	59 (13.6)	22 (10.8)	41 (21.4)	17 (8.7)	21 (10.6)	10 (5.2)	22 (18.2)	101 (18.5)	313 (13.7)
Educational level parents										
Lower secondary school	46 (23.4)	69 (15.9)	24 (11.8)	38 (19.8)	30 (15.3)	23 (11.6)	17 (8.8)	22 (18.2)	27 (4.9)	296 (13.0)
Higher secondary school	127 (64.5)	148 (34.1)	66 (32.4)	61 (31.8)	54 (27.6)	75 (37.7)	79 (40.9)	35 (28.9)	172 (31.4)	817 (35.8)
Higher education	24 (12.2)	217 (50.0)	114 (55.9)	93 (48.4)	112 (57.1)	101 (50.8)	97 (50.3)	64 (52.9)	348 (63.6)	1,170 (51.3)

[a] Mean ± standard deviation, [b] Cut-off values for body mass index (BMI) classes per age category of half a year, based on Belgian growth curves, specific for age and sex (http://www.vub.ac.be/groeicurven/groeicurven.html).

(a) *Alkaline comet assay in whole blood (% DNA migration= tail intensity)*



(b) *MN assay (number of MN/1000 binucleated cells)*



(c) *Urinary 8-oxodG (µg/g creatinine)*

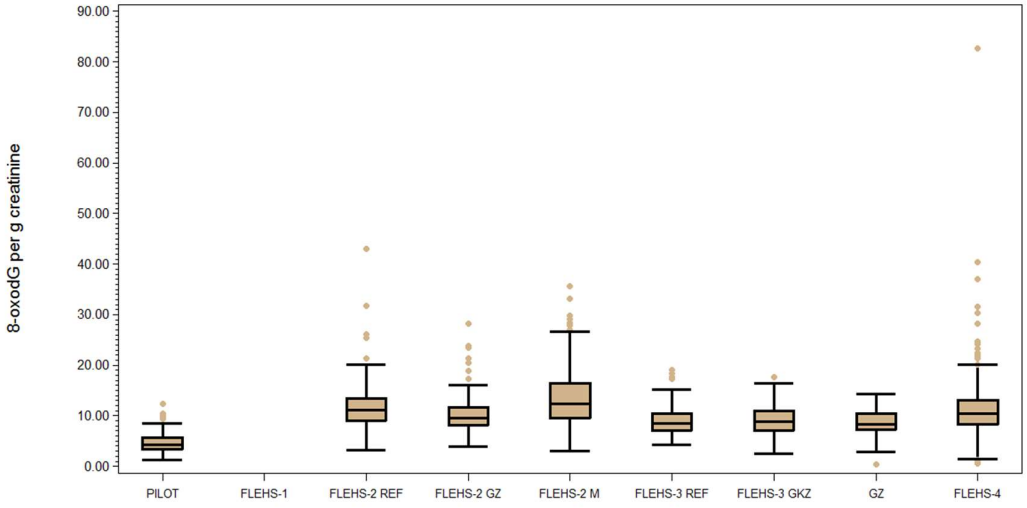


Figure 1: *Boxplots of the genotoxicity biomarker levels in the different study populations: (a) alkaline comet assay (% DNA migration); (b) MN frequency (#MN/1000 BN cells); (c) urinary 8-oxodG (µg/g creatinine).(MN: micronucleus, 8-oxodG: 8-oxo-2'-deoxyguanosine, PILOT: pilot study, FLEHS: Flemish Environment and Health Study; REF: reference population; GZ: Genk-Zuid; M: Menen; GKZ: Ghent harbor). The vertical line inside the box is the median (50th percentile). The two vertical lines that constitute the top and bottom of the box are the 25th and 75th percentiles respectively. The whiskers are calculated as 1.5 IQR. Outliers are indicated as dots.*

Table 2: *Descriptive statistics of the biomarkers of exposure in the pooled dataset (non-centered original data)*

Exposure marker	# Studies	# Adolescents	Median (P_{25} – P_{75})
Benzene metabolite in urine			
TT-MA *(µg/g creatinine)*	8	2,010	55.62 (31.75 – 106.67)
Polycyclic aromatic hydrocarbon (PAH) metabolite in urine			
1-OHP *(ng/g creatinine)*	9	2,113	61.98 (40.81 – 89.34)
Persistent organochlorine pollutants in serum			
HCB *(ng/g serum lipids)*	5	1,412	10.16 (6.85 – 16.90)
p,p'-DDE *(ng/g serum lipids)*	7	1,799	54.33 (30.80 – 100.11)
PCB138 *(ng/g serum lipids)*	8	1,994	11.27 (6.68 – 18.94)
PCB153 *(ng/g serum lipids)*	8	1,995	18.90 (10.76 – 31.48)
PCB180 *(ng/g serum lipids)*	8	1,994	10.38 (5.23 – 18.41)
TN *(ng/g serum lipids)*	3	779	0.81 (0.53 – 1.21)
Phthalates in urine			
∑DEHP metabolites *(µg/g creatinine)*	3	832	12.16 (6.88 – 26.82)
MnBP *(µg/g creatinine)*	3	832	16.72 (10.37 – 26.72)
MBzP *(µg/g creatinine)*	3	832	3.54 (1.43 – 13.36)
Metals in urine			
As *(µg/g creatinine)*	3	591	6.30 (4.00 – 13.80)
TRA *(µg/g creatinine)*	7	1,322	2.80 (2.03 – 4.02)
Cd *(µg/g creatinine)*	7	1,816	0.19 (0.14 – 0.26)
Cu *(µg/g creatinine)*	3	518	7.77 (6.64 – 8.96)
Ni *(µg/g creatinine)*	4	640	1.50 (1.04 – 2.27)
Tl *(µg/g creatinine)*	5	1,185	0.20 (0.15 – 0.26)
Metals in blood			
As *(µg/L)*	3	594	0.65 (0.39 – 1.23)
Cd *(µg/L)*	9	2,266	0.20 (0.15 – 0.30)
Cr *(ng/L)*	3	497	352.91 (233.14 – 527.56)
Cu *(µg/L)*	6	1,514	824.50 (750.90 – 909.00)
Ni *(ng/L)*	3	492	1074.35 (881.05 – 1305.55)
Pb *(µg/L)*	8	2,145	12.12 (8.18 – 18.82)
Tl *(ng/L)*	7	1,635	28.00 (24.12 – 33.10)
Particulate matter (PM) in ambient air			
$PM_{2.5}$ *($\mu g/m^3$)*	7	1,652	14.80 (8.90 – 19.00)
PM_{10} *($\mu g/m^3$)*	8	2,085	23.00 (17.30 – 27.50)

TT-MA: tt'-muconic acid; 1-OHP: 1-hydroxypyrene; HCB: hexachlorobenzene, *p,p'*-DDE: dichlorodiphenyldichloroethylene, PCB: polychloro biphenyls; TN: *trans*-nonachlor; DEHP: di(2-ethylhexyl) phthalate; MnBP: mono-n-butyl phthalate; MBzP: mono-benzyl phthalate; As: arsenic; TRA: toxic relevant arsenic; Cd: cadmium; Cu: cupper; Ni; nickel; Tl: thallium; Cr: chromium; Pb: lead; PM: particulate matter

Pooled analysis of the exposure-effect associations

Based on the pooled database, exposure-effect associations were assessed between the three genotoxicity markers and the exposure biomarkers or ambient particulate matter levels. A 5% level of significance was used and the p-values presented in Table S4 are unadjusted for multiple testing. Significant associations were observed for the urinary oxidative stress marker 8-oxodG, which was positively associated with urinary 1-OHP, urinary Cu, and ∑DEHP (Figure 2, Supplementary Table S4). For the association of 8-oxodG and 1-OHP, the pooled analysis showed significance, whereas the positive trend was only significant in one out of eight of the included individual studies (Figure 2, a, Supplementary Table S4). The association of 8-oxodG and urinary Cu was consistently observed in the three individual surveys, that ran in the period of 2008-2011 (Figure 2, b, Supplementary Table S4). 8-oxodG correlated significantly with DEHP metabolites in one of three individual studies, and significant in the pooled analysis (Figure 2, c).

For 1-OHP and Cu, the association was still statistically significant after FDR multiple testing correction. Those associations were furthermore confirmed using meta-analysis of the individual studies. A further sensitivity analysis in which one of the studies was systematically left out from the pooled analysis, showed a similar significance throughout all pooled analyses done for 8-oxodG in relation to 1-OHP. The pooled analysis of 8-oxodG vs. urinary Cu and DEHP, showed roughly the same estimate for the complete pooled analysis compared to the pooled analysis with exclusion of one of the studies (data not shown). However, excluding one of the studies (respectively FLEHS-2 REF for urinary Cu and FLEHS-3 REF for ∑DEHP) from the pooled analysis, caused loss of the significance due to loss of power.

For all other tested exposure-effect associations the pooled association showed to be: not significant, as was the case in the individual surveys or significant in at least one of the individual surveys, but not in the pooled analysis (Supplementary Table 4). The latter was the case for:
(i) 8-oxodG was positively related to the urinary metal levels of total As, Ni, Tl, and the blood levels of Tl and Pb, the urinary phthalate metabolite MnBP, and the 7d-averaged PM_{10} and $PM_{2.5}$. However, negatively associated with the serum levels of PCB138, PCB180 and blood Cu;
(ii) comet assay was positively associated with blood metals (Cd, total As, Ni, Pb), serum PCB138, serum TN, urinary TT-MA, negatively with urinary Cd and TRA, ∑DEHP, and blood Tl, and either positively or negatively with urinary 1-OHP and 7d-averaged PM_{10} and $PM_{2.5}$;
(iii) micronucleus results, fewer significant and less consistent associations were seen. Indeed, in some of the studies the MN levels were positively associated with blood Cd, negatively with urinary TRA and 1-OHP, the three marker PCBs in serum, and blood Tl. Conflicting positive and negative associations were seen for TN and 7d-averaged PM_{10} and $PM_{2.5}$. Remarkably in one and the same survey (FLEHS-4) quite some negative associations (sometimes conflicting with the other studies) were observed.
More specifically ambient PM_{10} (and similarly $PM_{2.5}$) and urinary Ni were two compounds related to the genotoxicity markers in several of the individual studies, but not in the pooled analysis. PM_{10} was significantly positively associated with the comet assay (Figure 2, d) and 8-oxodG in respectively three out of eight and two out of seven of the studies. The relationship of urinary Ni with 8-oxodG was significant in two out of four individual studies, but again not in the pooled analysis (Figure 2, e). A sensitivity analysis excluding one of the studies (indicated as 'GZ' in Figure 2e) from the latter pooled analysis , showed that the association became nearly significant in that case (estimate (95%CI) = 1.06 (0.99-1.14), p=0.06).

The relationships in the pooled analysis were also tested for boys and girls separately, which obviously decreases the power of the analysis. Pooling of the data of girls did not show the overall observed significant association between 1-OHP and 8-oxodG (estimate i.e. ratio of average effect for doubling of exposure in girls was 1.02 (0.99-1.06), p=0.20), whereas it was significant in boys (1.07 (1.03-1.09),

p<0.001). On the other hand, the significant pooled association between urinary Cu and 8-oxodG was observed in both sexes separately, with the estimate being similar: 1.27 (1.14-1.42) for doubling of urinary Cu, p<0.001 in girls, and 1.25 (1.10-1.41), p=0.0007 in boys. The estimates of the association of 8-oxodG with the sum of the DEHP metabolites were similar between boys and girls. They were significant in boys (1.04 (1.00-1.08), p=0.03), however not in girls (1.02 (0.98-1.06), p=0.25).

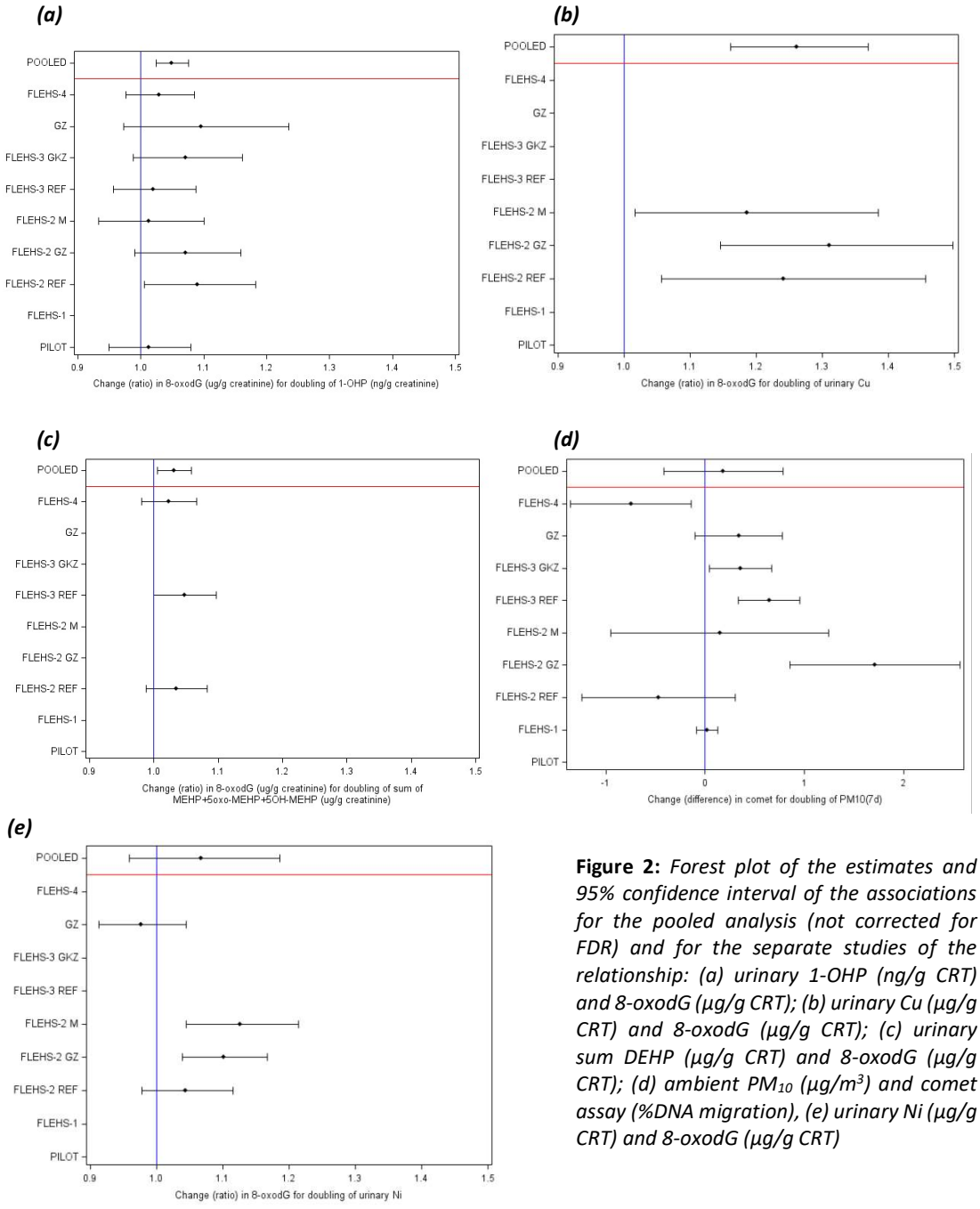


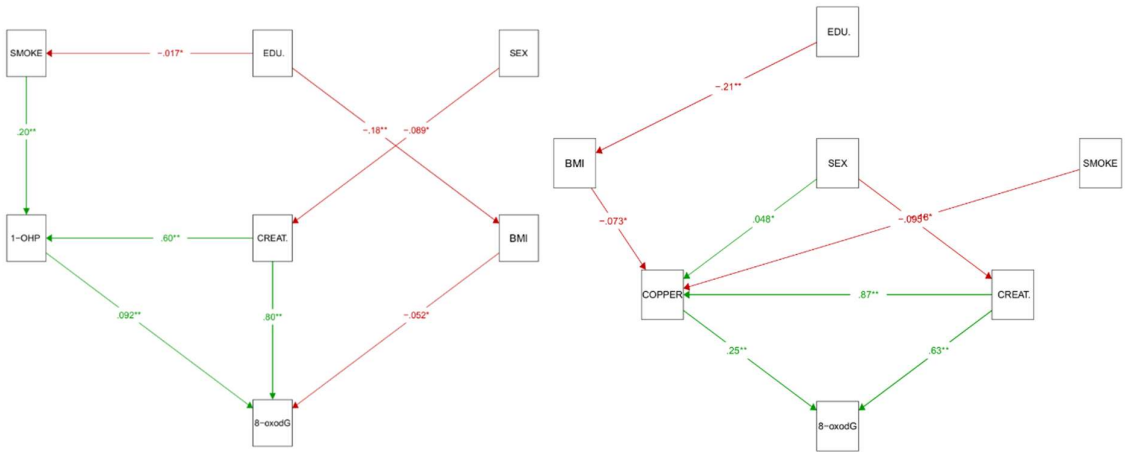
Figure 2: *Forest plot of the estimates and 95% confidence interval of the associations for the pooled analysis (not corrected for FDR) and for the separate studies of the relationship: (a) urinary 1-OHP (ng/g CRT) and 8-oxodG (µg/g CRT); (b) urinary Cu (µg/g CRT) and 8-oxodG (µg/g CRT); (c) urinary sum DEHP (µg/g CRT) and 8-oxodG (µg/g CRT); (d) ambient PM_{10} (µg/m^3) and comet assay (%DNA migration), (e) urinary Ni (µg/g CRT) and 8-oxodG (µg/g CRT)*

Covariates of influence

The association between the genotoxicity markers and covariates was examined by means of a multiple mixed effects regression model. Overall, for all adolescents in the study, the ambient temperature averaged over 7 days before blood or urine collection was of influence on the comet and MN assay results. For a ten-degree Celsius increase of temperature, the comet result increased with 0.1 (95%CI: 0.05-0.20, p=0.001) in %DNA migration, and the number of MN with 0.6 (0.3-0.9, p<0.001). Children of families with higher secondary school education had on average a 0.1 (0.03-0.2, p=0.008) higher %DNA migration in the comet assay, compared to those with lower and higher education. Furthermore, we observed the following significant associations: boys compared to girls had 0.1 (0.02-0.2, p=0.02) increase in % DNA migration in the comet assay. Adolescents with underweight and normal weight had respectively 10% (95%CI=6%-14%, p=0.005) and 5% (2%-7%, p=0.08) higher 8-oxodG levels compared to overweight adolescents. Current smokers (9.2 % of the total population) showed on average a decrease of 0.12 (0.02-0.21, p=0.01) in %DNA migration in the comet assay. As mentioned in the M&M, all these explanatory covariates were included in each of the exposure-effect models, even if they were not statistically significant.

For the significant associations observed in the pooled analyses (1-OHP, urinary Cu and DEHP vs. 8-oxodG), a causal DAG was adopted as a visual aid to check for relevant influencing factors and for the completeness of the model used in the pooled analysis (Supplementary Figure 1-3). In the SEM model representing the causal assumptions represented in the DAG of 1-OHP vs. 8-oxodG, the chi-square test showed a value of 52.81 with 23 degrees of freedom defining a ratio of 2.21. The value of RMSEA=0.03, as well as SRMR=0.014 and CFI=0.99 gave evidence of a good model fit. Assuming all other variables with a direct effect on 8-oxodG are held constant, creatinine had the strongest direct effect on 8-oxodG with an increase of 0.80 standard deviations (SD) in 8-oxodG for each SD increase in creatinine (p<0.001). Under the same assumption an increase of one SD 1-OHP was associated with an increase of 0.092 SD in 8-oxodG (p<0.001), and a 1 SD increase in BMI with a decrease in 8-oxodG of -0.052 SD (p<.05). The strongest effect on the 1-OHP values was also exerted by creatinine, followed by smoking and BBQ. No statistically significant (p<0.05) associations for smoking, education, outdoor temperature and sex with 8-oxodG, nor between open fireplace and traffic on 1-OHP were found. Additionally, also the associations between BMI and creatinine as well as smoking and BMI were statistically not significant (Figure 3**Error! Reference source not found.**a).

(a) ***(b)***



(c)

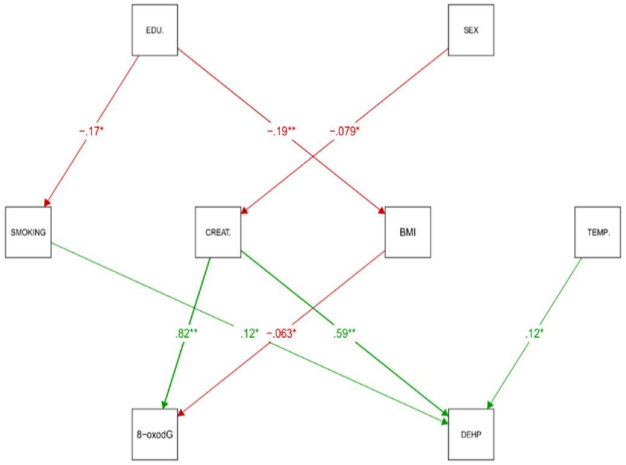


***Figure 3**: Graphic display of the estimated path analysis model for the association between: **(a)** 1-OHP, **(b)** Cu or **(c)** DEHP vs. 8-oxodG, based on the causal directed acyclic graphs (only p<0.05 associations shown). The arrows represent standardized regression coefficients (red = negative and green = positive association). 1-OHP = 1-hydroxypyrene, DEHP = sum of Di(2-ethylhexyl) phthalate metabolites, 8-oxodG = 8-oxo-7,8-dihydro-2'-deoxyguanosine, BBQ = BBQ in last 3 days, BMI = Body mass index, CREAT = urinary creatinine, EDU. = highest educational degree in family, FIRE = indoor open fireplace, SEX = gender participant, SMOKE = smoking, TEMP = temperature averaged over 7 days before urine collection* p <0.05, ** p <0.001*

In the SEM model representing the causal assumptions between urinary Cu and 8-oxodG represented in the DAG, the chi-square test showed a value of 13.66 with 9 degrees of freedom defining a ratio of 1.52 and a chi-square p-value of 0.14, and also other indices of the model fit were good (RMSEA=0.032, SRMR=0.032 and CFI= 1). Again creatinine had the strongest direct effect on 8-oxodG and also on Cu, with an increase of 0.63 and 0.87 standard deviations (SD) respectively for each SD increase in creatinine (p<.001). Under the assumption that all other variables are held constant, an increase of one SD Cu was associated with a 0.25 SD increase in 8-oxodG (p<0.001). None of the other variables had a significant direct influence on 8-oxodG in the path analysis model. Additionally, smoking, BMI and female sex had a statistically significant influence on the measured Cu values (β=-0.18, β=-0.073, β= 0.048, all p<0.05) (Figure 3b).

For the path analysis model based on the causal assumptions between DEHP and 8-oxodG found in the corresponding DAG, the chi-square test showed a value of 22.73 with 9 degrees of freedom defining a ratio of 2.53 and a chi-square p-value of 0.007. Concerning other indices of the model fit, the RMSEA reached a value of 0.044, the SRMR and CFI were calculated to be 0.011 and 0.990 respectively. Also here, creatinine had the strongest direct effect on 8-oxodG as well as on ∑DEHP metabolites, [0.82 and 0.59 standard deviations (SD) respectively for each SD increase in creatinine, both p < 0.001]. The direct effect of one SD increase in ∑DEHP on 8-oxodG was 0.043 SD (p = 0.088), and an increase in one SD of the BMI resulted in an 0.063 SD decrease in 8-oxodG (p = 0.001). None of the other variables had a significant direct influence on 8-oxodG in this path analysis model. ∑DEHP was also significantly affected by temperature and smoking (β = 0.12, p = 0.001, and β = 0.12, p = 0.028 respectively) (Figure 3c). It should be mentioned that the direct effect of DEHP on 8-oxodG was not significant (p=0.09), in case of non-creatinine corrected ∑DEHP. In an analysis using creatinine-corrected values, the association between both biomarkers was significant (β = 0.075, p=0.03).

4. Discussion

In 2,283 adolescents between 14 and 18 years old, recruited in Flanders in nine separate studies spread over time between 1999 to 2018, the association between daily-life-level environmental exposures and (mainly oxidative) damage to DNA was examined. Pooling of data may be cumbersome in case different studies of different labs and recruitment strategies are combined. The current pooled analysis was of large scale and rather unique, as it allowed to combine exposure, genotoxicity and covariate info, rather uniformly collected over a 20-years' time period.

Exposure ranges in pooled population

The exposure biomarker levels were within the normal range of environmental exposures. For the vast majority of the teenagers, the levels were below currently available health-based guidance values. Compared to recent guidance values available, the following percentage of teenagers participating in the different surveys, had a level above the guideline: 27.8% for TRA of 6.4 µg/L (Aylward *et al.*, 2013), 2.7% for blood Cd of 1.7µg/L (Hays *et al.*, 2008), 44.6% had a level above the urinary Cd 0.2 µg/g creatinine guideline adopted within the European Human Biomonitoring (HBM4EU) program (concept note, in preparation) , 0% above the HBM-1 value of 5 µg/L for urinary Tl (Schulz *et al.*, 2012), 4% for blood Pb guideline of 50 µg/L, 61.1% for the blood Pb level of 12µg/L of the European Food Safety Association (Efsa, 2010), 0% had a level of the sum of marker PCBs (PCB138+153+180) above 3500 ng/L (HBM-I, below no risk for adverse health effect is supposed) or 7000 ng/L (HBM-II, above a risk for adverse health effect is anticipated) (Umweltbundesamt, 2017), 17.9% had a serum level above 25 ng/g lipid for HCB (Aylward *et al.*, 2010), 65.5% had a serum level of *p,p'*-DDE above 50 ng/g lipid (Kirman *et al.*, 2011), 0.24% had a level of sum of 5-oxo MEHP and 5-OH MEHP above HBM-I value of 500 µg/L (Umweltbundesamt, 2017) and above the biomonitoring equivalent value of 300 µg/L for MBzP in adults, adopted by HBM4EU (concept note, in preparation), and 0.96% had more than 200 µg/L MnBP in their urine (Aylward *et al.*, 2009).
Considering the trends of the exposure biomarkers over time in the different surveys, the urinary concentration of 1-OHP, and the urinary and/or blood levels of most metals were rather stable over this period of 20 years (also discussed in: Schoeters *et al.*, 2017). An exception to this trend was observed for blood Pb of which the levels dropped from above 20 µg/L to 8.8 µg/L. Also the levels of the three phthalates were decreasing over a last 4 to 5-years' period for ∑DEHP (from 13.3 to 7.6 µg/g CRT), MnBP (from 16.6 to 11.8 µg/g CRT) and MBzP (from 3.7 to 1.5 µg/g CRT), of which the parent compounds were all restricted of use in toys and childcare articles since 2015 (European Commission, 2015). Similar decreasing trends over a 20-years' time period were seen for the serum concentrations of the persistent pollutants HCB, *p,p'*-DDE, PCBs. TT-MA showed a more fluctuating trend (Supplementary Table 3). Since time trends in exposure parameters may have been lost by using study mean-centering of the individual studies in the mixed model, also models without mean centering of the exposure markers were run. This sensitivity analysis resulted in the same significant relationships.

Pooled analysis of exposure-effect relationships

Of the three genotoxicity markers tested in the pooled analyses, only 8-oxodG appeared to show a consistent positive association with three exposure markers (urinary 1-OHP, Cu, and ∑DEHP). The magnitude of estimates of the associations were confirmed in a sex-stratified analysis. Urinary 8-oxodG is a primary product of DNA repair from oxidative damage to DNA. It is a marker of both: (i) oxidized guanine in the DNA structure repaired by nucleotide excision repair and/or (ii) repair of the damaged DNA building block 8-oxoGTP (by the MTH1 / NUDT1 enzyme), which is in this way prevented of being built into the DNA. This results in water-soluble 8-oxodG molecules that are removed from the body via the urine (Poulsen *et al.*, 2014; Rossner *et al.*, 2016). 8-oxodG, rather than the comet assay or MN was linked with the exposure markers. This may be due to the fact that 8-oxodG

specifically assesses oxidative stress, which is an early event of systemic (and therefore also DNA) stress after several chemical exposures (Klaunig *et al.*, 2011). It was pointed out, that assessment of urinary 8-oxodG, has mainly value in case one can assume that many bodily cells have increased oxidative stress. This is the case in diabetes, obesity and following some drug treatments or possibly exposure (Roszkowski *et al.*, 2011; Guo *et al.*, 2017; Poulsen *et al.*, 2019). This means that one will most probably only detect 8-oxodG if the damage is not limited to a specific organ, but rather systemically. The quantitative interpretation in terms of health risk is not possible (Guo *et al.*, 2017). It is however known that overproduction of reactive oxygen species is linked to cellular senescence (apoptosis) and development of chronic diseases. Guo *et al.* (Guo *et al.*, 2017) recently reviewed the potential use of 8-oxodG as disease marker, and reported a 1.3-5-fold increase in patients with colorectal, gastric, breast cancer, neurodegenerative disorders, diabetes and cardiovascular disease.
In the current pooled analysis we observed: an increase of respectively 5 and 3% in 8-oxodG for a doubling of the urinary 1-OHP level (estimate (95% CI) = 1.05 (1.02-1.08), N=1705 individuals) and the ∑DEHP (1.03(1.005-1.06), N=803) (Figure 2a&c), and an increase of 26% (1.26 (1.16-1.37), N=520) (Figure 2b) for a doubling of urinary Cu. These are minor increases, compared to the above mentioned 1.3-5-fold increase in patients (Guo *et al.*, 2017). However, since this increase applies to the population level, individuals themselves might experience a higher or lower impact. Furthermore, as this association was observed in teenagers, exposed to common environmental concentrations of PAHs, it points to the importance of PAHs, Cu and some phthalates as pollutants with potential health risk.
For PAHs the mechanism of inducing oxidative stress is plausible as they undergo metabolic activation to reactive diol-epoxide metabolites, that form DNA adducts or induce oxidative stress causing mutations (Munoz and Albores, 2011). The urinary levels of 1-OHP measured in the current study were in the range of 40.8-89.3 ng/g creatinine (Table 2), and in the lower range of what was measured in youngsters of USA (12-19y, 101.5 ng/L, Scinicariello and Buser, 2014), Germany (130 ng/L, Schulz *et al.*, 2009) or Canada (110 ng/L, Khoury *et al.*, 2018). 1-OHP can be seen as indicator for exposure to the mixture of PAHs. As earlier reported in Franken *et al.* (Franken, Lambrechts, *et al.*, 2017), the association of 1-OHP with 8-oxodG is in line with findings of several other occupational and environmental studies in adults and children (Chao *et al.*, 2008; Al-Saleh *et al.*, 2013; Kuang *et al.*, 2013; Lai *et al.*, 2013; Nguyen *et al.*, 2014).
Cu is an essential element, which is a crucial constituent of the respiratory enzyme complex cytochrome c oxidase, of the anti-oxidant enzyme superoxide oxidase (SOD) and of the copper transport protein ceruloplasmin. The urinary interquartile concentration range of Cu measured in the adolescents was 6.6-9.0 µg/g CRT, and lower than the levels reported in adult athletes. Cu in urine indicates the proportion excreted to maintain Cu homeostasis in the body (Maynar *et al.*, 2019). However, there is some evidence for a relationship between mainly occupational Cu exposure and adverse health effects, such as genotoxicity (De Olivera *et al.*, 2012), damaged lung function (Skoczyńska *et al.*, 2016), impaired nervous system (Lilis *et al.*, 1985; Kicinski *et al.*, 2015), and renal function (Lilis, Valciukas and Weber, 1983). Interestingly, in a very recent study in pregnant women, a (comparable to our findings) 15.3% increase in 8-oxodG (95% CI: 5.09-26.5) was found in association with an IQR increase in urinary Cu (Kim *et al.*, 2019). Very recently Husain and Mahmood (Husain and Mahmood, 2019) reported cytotoxic and genotoxic effects in *ex-vivo* Cu(II) exposed erythrocytes and lymphocytes. The concentrations applied were above those observed in normal exposure conditions (1h at 0.01-2.5 mM $CuCl_2$). The working principle, being generation of free radicals (Fenton-like reaction), and reactive oxygen and nitrogen species was confirmed in that study. Reduced anti-oxidant levels, damaged proteins and DNA, perturbated energy metabolism in the cells and increased cell damage were reported.
Interestingly, in our study population, the urinary Cu levels were statistically correlated (in the range of Pearson r=0.26-0.40) with urinary Cd, Cr, Ni and Tl and blood total arsenic (but not with urinary total arsenic). It may be speculated that the association of Cu with 8-oxodG observed in our pooled analysis, may therefore, partly serve as a proxy for other metals present in the body. Dutton *et al.* (Dutton *et al.*, 2019) reported the metals Ni, Cu, Co and As were highly present in historically

contaminated Ni-refinery soils. One of the three individual studies (FLEHS-2 GZ) included in the pooled analysis is situated in an area with increased ambient air levels of Ni and Cr, at the time of the human biomonitoring. The urinary levels of Cr, Ni and Tl, that were also measured in the adolescents of the included FLEHS studies, showed a non-significant positive association with 8-oxodG.

The relation of the sum of DEHP metabolites with 8-oxodG was earlier reported in the pooled analysis of FLEHS-2 and FLEHS-3 study (Franken, Lambrechts, *et al.*, 2017). Others reported a similar significant association for several phthalates in Brazilian children aged between 6-14y (Rocha *et al.*, 2017), in Saudi Arabian children of 3-9y (Lee *et al.*, 2019). The same trend, but not significant was seen in American children of 10-13y old (Kataria *et al.*, 2017). As reported by van 't Erve *et al.* (Van'T Erve *et al.*, 2019) - showing the association between oxidative stress (8-isoPGF$_{2\alpha}$) and inflammation (PGF$_{2\alpha}$) and phthalate exposure in pregnant women - phthalates have been shown to cause oxidative stress by free radicals generation from liver macrophages (Kupffer cells). Ferguson (Ferguson *et al.*, 2014) showed a non-significant trend of IL-6 serum levels increased in pregnant women, mainly in relation with the secondary oxidated and carboxylated metabolites of DEHP, aside from a significant increase in urinary 8-oxodG and 8-isoPGF$_{2\alpha}$.

For the association of 8-oxodG and urinary 1-OHP, Cu or phthalates, pooling of the data increased the power of observing the association. Sensitivity analysis using either a fixed effect model or mixed model and either, study-centered or non-study-centered exposure, or by leaving one study out of the pooled analysis, confirmed those associations. However, pooling also showed, that associations of exposure markers vs. genotoxicity outcomes, observed in one or more of the individual surveys, was not confirmed in the pooled analysis (see Supplementary Table 4). This was mainly apparent for the association between ambient PM vs. the comet assay and urinary Ni vs. 8-oxodG. The latter was observed in the hot spot industrial area (sampled in two (FLEHS-2 GZ and GZ) of the three surveys) included in the pooled data. In this area with a diversity of industrial activities, also the urinary levels of total arsenic, Cr and Cu measured in the adolescents living near the plant, were associated with the oxidative stress marker 8-oxodG (Supplementary Table 4). Except for Cu, these associations were also not significant in the overall pooled dataset. This means, that the exposure marker may represent different pollutant mixtures in different studies. This may be especially true for the included surveys that were spread over a 20-years-time period and over different hot spot areas (industry, port). This points to the fact that pooling of data may dilute results. In other words, hot spot biomonitoring has the advantage of studying a specific and local environmental problem and pollutant cocktail.

Covariates used in pooled analysis

In the population examined, outdoor temperature averaged over 7 days before blood collection caused the levels of DNA migration in the comet assay and the number of MN to increase. This was earlier described and recently reviewed in Azqueta *et al*. (submitted). It was stated that the impact of meteorology/seasonal variations on the level of DNA damage measured via the comet assay, may be related to seasonal changes including variation in temperature, solar radiation (strength and duration), air pollution [ozone, particles, (semi-)volatile compounds], diet (antioxidants, cooking processes), allergy (pollen), physical exercise, time spent outdoors/in the sun, and wearing less skin-covering clothes. Boys had compared to girls, significantly higher levels of DNA migration in the comet assay ($p=0.02$). This was similar to the effect of gender (higher in male compared to female) on the levels of 8-oxodG (Nakano *et al.*, 2003; Di Minno *et al.*, 2016). However, Møller (Møller, 2019) reviewed recently the impact of age and gender on the comet assay data, and concluded that the influence of both covariates on DNA damage in the comet assay, may be mediated by lifestyle factors or external exposures, rather than direct effects of age and sex.
Our results confirmed lower 8-oxodG levels with increasing BMI within normal non-obese BMI ranges (Kasai *et al.*, 2001; Irie *et al.*, 2005), also in the SEM model. In this association there might be a

contribution of an indirect effect via educational level. This was also observed from the SEM analysis; higher educational levels were inversely associated with smoking as well as with BMI. From the SEM of urinary Cu vs. 8-oxodG, it appeared that BMI was inversely associated with urinary Cu. This was earlier reported in children (6-17 year) (p= 0.075) (Błażewicz *et al.*, 2013). On the other hand, a study in pregnant women found higher urinary Cu levels for women with very high BMI ≥ 25kg/m^2 compared to women with BMI < 25kg/m^2 (Kim *et al.*, 2018).
Nine % of the adolescents were smokers. The impact of smoking on the genotoxicity markers was not observed, and for the comet assay, we even observed an inverse association. The latter may be driven by the fact that the majority of smokers was observed in the pilot study of 1999, in which overall the absolute comet assay levels were lower, compared to the later surveys. The latter lower comet levels may have been due to changes in the comet assay methodology in that study. Nonetheless, in general, quite some studies show discrepancies considering the effect of smoking on the comet assay results. This may be mediated by several factors such as the number of cigarettes smoked, the period over which they are smoked, and the type of cigarettes (reviewed in Azqueta *et al*., submitted). In the SEM analysis, we observed an indirect effect of smoking on 8-oxodG via 1-OHP.

For the significant associations observed in the pooled analysis (1-OHP, urinary Cu and ∑DEHP vs. 8-oxodG), a causal DAG was adopted as a visual aid to check for relevant influencing factors and for the completeness of the model used in the pooled analysis. Creatinine had the major influence on 8-oxodG and 1-OHP. It confirmed the need to additionally include it as covariate in the regression model, as previously proposed (Barr *et al.*, 2005; O'Brien *et al.*, 2016) and also done in our analysis. Gender was of influence on urinary Cu. Girls had higher urinary levels, which confirmed a previous finding in female adults (Schuhmacher, Domingo and Corbella, 1994). A possible link between ∑DEHP metabolite concentrations and BMI could be explained by higher energy intake in persons with a higher BMI, causing higher DEHP intake, as demonstrated previously (Campbell Jr. *et al.*, 2018). The exposure to DEHP is greater at higher temperature, as the rate of DEHP leaching from PVC into lipid solutions (e.g. foodstuffs) is temperature-dependent (Rose *et al.*, 2012). The urinary DEHP metabolite MEHP was observed to be higher in the general public during winter (Pilka *et al.*, 2015), probably due to indoor heating and leaching from e.g. wall paper and carpets.

Strengths and limitations of the study

The pooled analysis included 2,283 adolescents of a narrow age range, and of which analyses were over the different surveys performed in the same labs. This was a real strength of the current work. The uniform study protocols facilitated pooling of the results, which may be more challenging when pooling completely different studies. In addition, the associations were in line with their known mode of action. A general pitfall of pooling studies may be the lack of specificity of the exposure-response associations. Meaning that entangling the effect of single exposures may be less clear, as the variability in exposure mixtures people were exposed to, may vary greatly over time and over the different populations included.
By combining the studies and correction for multiple testing, the association between PAH exposure and 8-oxodG, and Cu vs. 8-oxodG levels observed in individual studies were confirmed. These urinary exposure biomarkers reflect exposure over days/hours. Even within individuals, large variability in exposure may exist, if urine is collected at different points in time, unless exposure is more continuous over time. Also, the effect biomarker 8-oxodG reflected short-term oxidative stress or DNA damage, which may vary. By pooling of the results, that effect was smoothened i.e. more randomized over a larger study population.

5. Conclusions

Pooling of the data improved the power of some of the associations observed in the individual surveys. However, pooling of studies ran over a long timeframe and in different hot spot areas also diluted effects. Indeed, the exposure profile (mixture of compounds) and the intensity of exposure - and thus, the impact on oxidative stress and genotoxicity outcome - may possibly change over different surveys. Despite this possible drawback, pooled statistical analysis of nine surveys run over a period of 20 years, showed that the urinary PAH metabolite 1-OH, Cu and the sum of the metabolites of the phthalate DEHP, were associated with 8-oxodG adducts in urine. 8-oxodG is a systemic marker of oxidative stress, a mechanism induced by these chemicals, and which was visible in the teenagers. This indicated that the marker is an appropriate tool for assessment of environmental oxidative stress already observed at this young age.

Acknowledgements

This FLEHS- studies were commissioned, financed and steered by the Ministry of the Flemish Community (Department of Economics, Science and Innovation; Flemish Agency for Care and Health; and Department of Environment). The work on the pooled analysis was financed by the Flemish Agency for Care and Health.

Bibliography

Al-Saleh, I. *et al.* (2013) 'Polycyclic aromatic hydrocarbons (PAHs) as determinants of various anthropometric measures of birth outcome', *Science of the Total Environment*. doi: 10.1016/j.scitotenv.2012.12.021.

Aylward, L. L. *et al.* (2009) 'Derivation of Biomonitoring Equivalents for di-n-butyl phthalate (DBP), benzylbutyl phthalate (BzBP), and diethyl phthalate (DEP)', *Regulatory Toxicology and Pharmacology*. doi: 10.1016/j.yrtph.2009.09.003.

Aylward, L. L. *et al.* (2010) 'Biomonitoring equivalents for hexachlorobenzene', *Regulatory Toxicology and Pharmacology*. doi: 10.1016/j.yrtph.2010.06.003.

Aylward, L. L. *et al.* (2013) 'Evaluation of biomonitoring data from the CDC national exposure report in a risk assessment context: Perspectives across chemicals', *Environmental Health Perspectives*. doi: 10.1289/ehp.1205740.

Azqueta, A. *et al.* (2014) 'Comet assay to measure DNA repair: Approach and applications', *Frontiers in Genetics*. doi: 10.3389/fgene.2014.00288.

Baeyens, W. *et al.* (2014) 'Trace metals in blood and urine of newborn/mother pairs, adolescents and adults of the Flemish population (2007-2011)', *International Journal of Hygiene and Environmental Health*. doi: 10.1016/j.ijheh.2014.06.007.

Barr, D. B. *et al.* (2005) 'Urinary creatinine concentrations in the U.S. population: implications for urinary biologic monitoring measurements', *Environ Health Perspect*. 2005/02/03, 113(2), pp. 192–200. doi: 10.1289/ehp.7337.

Błażewicz, A. *et al.* (2013) 'Differences in Trace Metal Concentrations (Co, Cu, Fe, Mn, Zn, Cd, and Ni) in Whole Blood, Plasma, and Urine of Obese and Nonobese Children', *Biological Trace Element Research*, 155(2), pp. 190–200. doi: 10.1007/s12011-013-9783-8.

Bolognesi, C. *et al.* (1997) 'Age-related increase of baseline frequencies of sister chromatid exchanges, chromosome aberrations, and micronuclei in human lymphocytes', *Cancer Epidemiology Biomarkers and Prevention*.

Browne, M. W. and Cudeck, R. (1992) 'Alternative Ways of Assessing Model Fit', *Sociological Methods & Research*. doi: 10.1177/0049124192021002005.

Byrne, B. M. (1998) 'Structural equation modeling with LISREL, PRELIS, and SIMPLIS: Basic concepts, applications, and programming', *Structural equation modeling with LISREL, PRELIS, and SIMPLIS: Basic concepts, applications, and programming.* Lawrence Erlbaum Associates Publishers, pp. 412, xiii, 412–xiii.

Campbell Jr., J. L. *et al.* (2018) 'Excretion of Di-2-ethylhexyl phthalate (DEHP) metabolites in urine is related to body mass index because of higher energy intake in the overweight and obese', *Environ Int*. 2018/02/09, 113, pp. 91–99. doi: 10.1016/j.envint.2018.01.023.

Carmines, E. and McIver, J. (1981) 'Analyzing models with unobserved variables: Analysis of covariance structures', in *Social measurement: Current issues*. doi: 10.1177/004057368303900411.

Chao, M. R. *et al.* (2008) 'Repeated measurements of urinary methylated/oxidative DNA lesions, acute toxicity, and mutagenicity in coke oven workers', *Cancer Epidemiology Biomarkers and Prevention*. doi: 10.1158/1055-9965.EPI-08-0721.

Collins, A. (2004) 'The comet assay for DNA damage and repair', *Molecular Biotechnology*.

Collins, A. *et al.* (2014) 'The comet assay as a tool for human biomonitoring studies: The ComNet Project', *Mutation Research - Reviews in Mutation Research*. doi: 10.1016/j.mrrev.2013.10.001.

Cooke, M. S., Olinski, R. and Loft, S. (2008) 'Measurement and meaning of oxidatively modified DNA lesions in urine', *Cancer Epidemiology Biomarkers and Prevention*. doi: 10.1158/1055-9965.EPI-07-0751.

Costa, S. *et al.* (2011) 'Occupational exposure to formaldehyde: Genotoxic risk evaluation by comet assay and micronucleus test using human peripheral lymphocytes', *Journal of Toxicology and Environmental Health - Part A: Current Issues*. doi: 10.1080/15287394.2011.582293.

De Coster, S. *et al.* (2008) 'Pollutant effects on genotoxic parameters and tumor-associated protein levels in adults: A cross sectional study', *Environmental Health: A Global Access Science Source*. doi: 10.1186/1476-069X-7-26.

De Craemer, S. *et al.* (2016) 'Investigating unmetabolized polycyclic aromatic hydrocarbons in adolescents' urine as biomarkers of environmental exposure', *Chemosphere*. doi: 10.1016/j.chemosphere.2016.04.017.

De Craemer, S. *et al.* (2017) 'Metals, hormones and sexual maturation in Flemish adolescents in three cross-sectional studies (2002–2015)', *Environment International*. doi: 10.1016/j.envint.2017.02.014.

Croes, K. *et al.* (2014) 'Monitoring chlorinated persistent organic pollutants in adolescents in Flanders (Belgium): Concentrations, trends and dose-effect relationships (FLEHS II)', *Environment International*. doi: 10.1016/j.envint.2014.05.022.

Dutton, M. D. *et al.* (2019) 'Towards an exposure narrative for metals and arsenic in historically contaminated Ni refinery soils: Relationships between speciation, bioavailability, and bioaccessibility', *Science of the Total Environment*. doi: 10.1016/j.scitotenv.2019.05.164.

Efsa (2010) 'Scientific Opinion on Lead in Food', *EFSA Journal*.

European Commission (2015) *Directive (EU) 2015/863 Amends EU RoHS to Restrict 4 New Phthalates: DEHP, BBP, DBP and DIBP*. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32015L0863&from=EN.

Fenech, M. *et al.* (1999) 'The HUman MicroNucleus Project - An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans', in *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*. doi: 10.1016/S1383-5742(99)00053-8.

Fenech, M. (2007) 'Cytokinesis-block micronucleus cytome assay', *Nature Protocols*. doi: 10.1038/nprot.2007.77.

Ferguson, K. *et al.* (2014) 'Urinary Phthalate Metabolite Associations with Biomarkers of Inflammation and Oxidative Stress Across Pregnancy in Puerto Rico', *Environmental science & technology*, 48. doi: 10.1021/es502076j.

Franken, C., Koppen, G., *et al.* (2017) 'Environmental exposure to human carcinogens in teenagers and the association with DNA damage', *Environmental Research*. doi: 10.1016/j.envres.2016.10.012.

Franken, C., Lambrechts, N., *et al.* (2017) 'Phthalate-induced oxidative stress and association with asthma-related airway inflammation in adolescents', *International Journal of Hygiene and*

Environmental Health. doi: 10.1016/j.ijheh.2017.01.006.

Geens, T. *et al.* (2014) 'Determinants of bisphenol A and phthalate metabolites in urine of Flemish adolescents', *Environmental Research*. doi: 10.1016/j.envres.2014.07.020.

Guo, C. *et al.* (2017) 'Potential application of the oxidative nucleic acid damage biomarkers in detection of diseases', *Oncotarget*. doi: 10.18632/oncotarget.20801.

Hays, S. M. *et al.* (2008) 'Biomonitoring Equivalents (BE) dossier for cadmium (Cd) (CAS No. 7440-43-9)', *Regulatory Toxicology and Pharmacology*. doi: 10.1016/j.yrtph.2008.05.008.

Den Hond, E. *et al.* (2009) 'Determinants of polychlorinated aromatic hydrocarbons in serum in three age classes-Methodological implications for human biomonitoring', *Environmental Research*. doi: 10.1016/j.envres.2009.02.007.

Den Hond, E. *et al.* (2013) 'Biomarkers of human exposure to personal care products: Results from the Flemish Environment and Health Study (FLEHS 2007-2011)', *Science of the Total Environment*. doi: 10.1016/j.scitotenv.2013.05.087.

Husain, N. and Mahmood, R. (2019) 'Copper(II) generates ROS and RNS, impairs antioxidant system and damages membrane and DNA in human blood cells', *Environmental Science and Pollution Research*. doi: 10.1007/s11356-019-05345-1.

Irie, M. *et al.* (2005) 'Occupational and lifestyle factors and urinary 8-hydroxydeoxyguanosine', *Cancer Sci*. 2005/09/01, 96(9), pp. 600–606. doi: 10.1111/j.1349-7006.2005.00083.x.

Janssen, S. *et al.* (2008) 'Spatial interpolation of air pollution measurements using CORINE land cover data', *Atmospheric Environment*. doi: 10.1016/j.atmosenv.2008.02.043.

Kasai, H. *et al.* (2001) 'Life style and urinary 8-hydroxydeoxyguanosine, a marker of oxidative dna damage: effects of exercise, working conditions, meat intake, body mass index, and smoking', *Jpn J Cancer Res*. 2001/02/15, 92(1), pp. 9–15. doi: 10.1111/j.1349-7006.2001.tb01041.x.

Kataria, A. *et al.* (2017) 'Exposure to bisphenols and phthalates and association with oxidant stress, insulin resistance, and endothelial dysfunction in children', *Pediatric Research*. doi: 10.1038/pr.2017.16.

Khoury, C. *et al.* (2018) 'Human biomonitoring reference values for some non-persistent chemicals in blood and urine derived from the Canadian Health Measures Survey 2009–2013', *International Journal of Hygiene and Environmental Health*. doi: 10.1016/j.ijheh.2018.03.003.

Kicinski, M. *et al.* (2015) 'Neurobehavioral function and low-level metal exposure in adolescents', *International Journal of Hygiene and Environmental Health*. doi: 10.1016/j.ijheh.2014.09.002.

Kim, S. S. *et al.* (2018) 'Urinary trace metals individually and in mixtures in association with preterm birth', *Environ Int*, 121, pp. 582–590. doi: https://doi.org/10.1016/j.envint.2018.09.052.

Kim, S. S. *et al.* (2019) 'Exposure to 17 trace metals in pregnancy and associations with urinary oxidative stress biomarkers', *Environ Res*. 2019/11/05, 179(Pt B), p. 108854. doi: 10.1016/j.envres.2019.108854.

Kirman, C. R. *et al.* (2011) 'Biomonitoring Equivalents for DDT/DDE', *Regulatory Toxicology and Pharmacology*. doi: 10.1016/j.yrtph.2011.03.012.

Klaunig, J. E. *et al.* (2011) 'Oxidative stress and oxidative damage in chemical carcinogenesis', *Toxicology and Applied Pharmacology*. doi: 10.1016/j.taap.2009.11.028.

Koppen, G. *et al.* (2002) 'Persistent organochlorine pollutants in human serum of 50-65 years old women in the Flanders Environmental and Health Study (FLEHS). Part 1: Concentrations and regional differences', *Chemosphere*. doi: 10.1016/S0045-6535(02)00111-X.

Koppen, G. *et al.* (2007) 'A battery of DNA effect biomarkers to evaluate environmental exposure of Flemish adolescents', *Journal of Applied Toxicology*. doi: 10.1002/jat.1174.

Koppen, G. *et al.* (2018) 'The comet assay in human biomonitoring: Cryopreservation of whole blood and comparison with isolated mononuclear cells', *Mutagenesis*. doi: 10.1093/mutage/gex034.

Kuang, D. *et al.* (2013) 'Dose-response relationships of polycyclic aromatic hydrocarbons exposure and oxidative damage to DNA and lipid in coke oven workers', *Environmental Science and Technology*. doi: 10.1021/es401639x.

Lai, C. H. *et al.* (2013) 'Exposure to cooking oil fumes and oxidative damages: A longitudinal study in Chinese military cooks', *Journal of Exposure Science and Environmental Epidemiology*. doi: 10.1038/jes.2012.87.

Lee, I. *et al.* (2019) 'Urinary phthalate metabolites among children in Saudi Arabia: Occurrences, risks, and their association with oxidative stress markers', *Science of the Total Environment*. doi: 10.1016/j.scitotenv.2018.11.025.

Lilis, R. *et al.* (1985) 'Effects of low-level lead and arsenic exposure on copper smelter workers', *Archives of Environmental Health*. doi: 10.1080/00039896.1985.10545887.

Lilis, R., Valciukas, J. A. and Weber, J. P. (1983) 'Epidemiologic study of renal function in copper smelter workers', *Environmental Health Perspectives*. doi: 10.2307/3429806.

Maynar, M. *et al.* (2019) 'Influence of a 6-month physical training program on serum and urinary concentrations of trace metals in middle distance elite runners', *Journal of the International Society of Sports Nutrition*. doi: 10.1186/s12970-019-0322-7.

Di Minno, A. *et al.* (2016) '8-Hydroxy-2-Deoxyguanosine Levels and Cardiovascular Disease: A Systematic Review and Meta-Analysis of the Literature', *Antioxidants & Redox Signaling*. Mary Ann Liebert, Inc., 24(10), pp. 548–555. doi: 10.1089/ars.2015.6508.

Møller, P. (2019) 'Effect of age and sex on the level of DNA strand breaks and oxidatively damaged DNA in human blood cells', *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*. doi: 10.1016/j.mrgentox.2018.11.010.

Munoz, B. and Albores, A. (2011) 'DNA Damage Caused by Polycyclic Aromatic Hydrocarbons: Mechanisms and Markers', in *Selected Topics in DNA Repair*. doi: 10.5772/22527.

Nakano, M. *et al.* (2003) 'Oxidative DNA damage (8-hydroxydeoxyguanosine) and body iron status: a study on 2507 healthy people', *Free Radic Biol Med*. 2003/10/30, 35(7), pp. 826–832. doi: 10.1016/s0891-5849(03)00432-5.

Nguyen, T. T. U. *et al.* (2014) 'Urinary 1-hydroxypyrene and 8-hydroxydeoxyguanosine Levels among coke-oven workers for 2 consecutive days', *Journal of Occupational Health*. doi: 10.1539/joh.13-0222-OA.

O'Brien, K. M. *et al.* (2016) 'Environmental Chemicals in Urine and Blood: Improving Methods for Creatinine and Lipid Adjustment', *Environ Health Perspect*. 2015/07/24. National Institute of Environmental Health Sciences, 124(2), pp. 220–227. doi: 10.1289/ehp.1509693.

De Olivera, J. V. D. *et al.* (2012) 'Occupational genotoxicity among copper smelters', *Toxicology and*

Industrial Health. doi: 10.1177/0748233711422735.

Ostling, O. and Johanson, K. J. (1984) 'Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells', *Biochemical and Biophysical Research Communications*. doi: 10.1016/0006-291X(84)90411-X.

Pilka, T. *et al.* (2015) 'Relationship between variation of seasonal temperature and extent of occupational exposure to phthalates', *Environ Sci Pollut Res Int*. 2014/08/02, 22(1), pp. 434–440. doi: 10.1007/s11356-014-3385-7.

Poulsen, H. E. *et al.* (2014) 'Detection and interpretation of 8-oxodG and 8-oxoGua in urine, plasma and cerebrospinal fluid', *Biochimica et Biophysica Acta - General Subjects*. doi: 10.1016/j.bbagen.2013.06.009.

Poulsen, H. E. *et al.* (2019) 'Oxidatively generated modifications to nucleic acids in vivo: Measurement in urine and plasma', *Free Radical Biology and Medicine*. doi: 10.1016/j.freeradbiomed.2019.10.001.

Rocha, B. A. *et al.* (2017) 'Urinary concentrations of 25 phthalate metabolites in Brazilian children and their association with oxidative DNA damage', *Science of the Total Environment*. doi: 10.1016/j.scitotenv.2017.01.193.

Rose, R. J. *et al.* (2012) 'The effect of temperature on di(2-ethylhexyl) phthalate leaching from PVC infusion sets exposed to lipid emulsions', *Anaesthesia*. John Wiley & Sons, Ltd, 67(5), pp. 514–520. doi: 10.1111/j.1365-2044.2011.07006.x.

Rosseel, Y. (2012) 'Lavaan: An R package for structural equation modeling', *Journal of Statistical Software*. doi: 10.18637/jss.v048.i02.

Rossner, P. *et al.* (2016) 'Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine analysis by an improved ELISA: An inter-laboratory comparison study', *Free Radical Biology and Medicine*. doi: 10.1016/j.freeradbiomed.2016.03.016.

Roszkowski, K. *et al.* (2011) 'Oxidative damage DNA: 8-oxogua and 8-oxodG as molecular markers of cancer', *Medical Science Monitor*. doi: 10.12659/MSM.881805.

Schermelleh-Engel, K., Moosbrugger, H. and Müller, H. (2003) 'Evaluating the fit of structural equation models: Tests of significance and descriptive goodness-of-fit measures', *MPR-online*.

Schoeters, G. *et al.* (2012) 'Concept of the Flemish human biomonitoring programme', *International Journal of Hygiene and Environmental Health*. doi: 10.1016/j.ijheh.2011.11.006.

Schoeters, G. *et al.* (2017) 'Three cycles of human biomonitoring in Flanders − Time trends observed in the Flemish Environment and Health Study', *International Journal of Hygiene and Environmental Health*. doi: 10.1016/j.ijheh.2016.11.006.

Schuhmacher, M., Domingo, J. L. and Corbella, J. (1994) 'Zinc and copper levels in serum and urine: relationship to biological, habitual and environmental factors', *Sci Total Environ*. 1994/05/30, 148(1), pp. 67–72. doi: 10.1016/0048-9697(94)90376-x.

Schulz, C. *et al.* (2009) 'Revised and new reference values for environmental pollutants in urine or blood of children in Germany derived from the German Environmental Survey on Children 2003-2006 (GerES IV)', *International Journal of Hygiene and Environmental Health*. doi: 10.1016/j.ijheh.2009.05.003.

Schulz, C. *et al.* (2012) 'Reprint of " Update of the reference and HBM values derived by the German

Human Biomonitoring Commission"', *International Journal of Hygiene and Environmental Health*. doi: 10.1016/j.ijheh.2012.01.003.

Scinicariello, F. and Buser, M. C. (2014) 'Urinary polycyclic aromatic hydrocarbons and childhood obesity: NHANES (2001-2006)', *Environmental Health Perspectives*. doi: 10.1289/ehp.1307234.

Singh, N. P. *et al.* (1988) 'A simple technique for quantitation of low levels of DNA damage in individual cells', *Experimental Cell Research*. doi: 10.1016/0014-4827(88)90265-0.

Skoczyńska, A. *et al.* (2016) 'Association between the Type of Workplace and Lung Function in Copper Miners', *BioMed Research International*. doi: 10.1155/2016/5928572.

Staessen, J. A. *et al.* (2001) 'Renal function, cytogenetic measurements, and sexual development in adolescents in relation to environmental pollutants: A feasibility study of biomarkers', *Lancet*. doi: 10.1016/S0140-6736(00)04822-4.

Sughis, M. *et al.* (2012) 'Adverse health effects of child labor: High exposure to chromium and oxidative DNA damage in children manufacturing surgical instruments', *Environmental Health Perspectives*. doi: 10.1289/ehp.1104678.

Textor, J. *et al.* (2016) 'Robust causal inference using directed acyclic graphs: The R package "dagitty"', *International Journal of Epidemiology*. doi: 10.1093/ije/dyw341.

Tice, R. R. *et al.* (2000) 'Single cell gel/comet assay: Guidelines for in vitro and in vivo genetic toxicology testing', in *Environmental and Molecular Mutagenesis*. doi: 10.1002/(SICI)1098-2280(2000)35:3<206::AID-EM8>3.0.CO;2-J.

Toyokuni, S. *et al.* (1997) 'Quantitative immunohistochemical determination of 8-hydroxy-2'-deoxyguanosine by a monoclonal antibody N45.1: Its application to ferric nitrilotriacetate-induced renal carcinogenesis model', *Laboratory Investigation*.

Umweltbundesamt (2017) *Human-Biomonitoring (HBM) values, derived by the Human Biomonitoring Commission of the German Environment Agency, date February 2017*. Available at: https://www.umweltbundesamt.de/sites/default/files/medien/355/bilder/dateien/hbm-werte_engl_stand_2017_02_06_2.pdf (Accessed: 30 January 2020).

Van'T Erve, T. J. *et al.* (2019) 'Phthalates and Phthalate Alternatives Have Diverse Associations with Oxidative Stress and Inflammation in Pregnant Women', *Environmental Science and Technology*. doi: 10.1021/acs.est.8b05729.

Vrijens, J. *et al.* (2014) 'Trace metal concentrations measured in blood and urine of adolescents in Flanders, Belgium: Reference population and case studies Genk-Zuid and Menen', *International Journal of Hygiene and Environmental Health*. doi: 10.1016/j.ijheh.2013.10.001.