Multiplex Genotyping as a Biomarker for Susceptibility to Carcinogenic Exposure in the FLEHS Biomonitoring Study

Hans B. Ketelslegers,1 Ralph W.H. Gottschalk,1 Gudrun Koppen,2 Greet Schoeters,2 Willy F. Baeyens,3 Nicolas A. van Larebeke,4 Joost H.M. van Delft,1 and Jos C.S. Kleinjans1

1Department of Health Risk Analysis and Toxicology, Maastricht University, Maastricht, The Netherlands; 2Flemish Institute for Technological Research, Mol, Belgium; 3Analytical and Environmental Chemistry Department, ANCH, Vrije Universiteit Brussel, Brussels, Belgium; and 4Department of Radiotherapy, Nuclear Medicine and Experimental Cancerology, Ghent University, Ghent, Belgium

Abstract

Cancer has been suggested to result from interactions between genetic and environmental factors, and certain subgroups in the general population may be at increased risk because of their relatively higher susceptibility to environmental carcinogens. The current study, part of a large biomonitoring study conducted in Flanders from 2002 to 2006 (The Flanders Environment and Health Survey), aims to determine these susceptible subpopulations based on multiple genotypic differences between individuals. A random selection of 429 adolescents and 361 adults was genotyped for 36 polymorphisms in 23 genes selected because of their known role in carcinogen metabolism, DNA repair, and oxidative stress. In both age groups, relationships between endogenous exposure to organochloride substances (polychlorinated biphenyl, hexachlorobenzene, dichlorodiphenyl dichloroethane), metals (cadmium, lead), and urinary metabolites (1-hydroxypyrene, trans-trans muconic acid) versus genotoxic effects (Comet assay and micronuclei in lymphocytes, and urinary 8-hydroxydeoxyguanosine) were investigated. In addition, in the study among adults, the relationship of these exposures with several tumor markers (prostate-specific antigen, carcinoembryonic antigen, and p53) was tested. The impact of the genotype on established exposure-effect relationships was evaluated. Eight exposure-effect relationships were found, including three novel associations, with an impact of various genotypes, predominantly affecting biotransformation and oxidative stress response. This study shows that at least part of the interindividual differences in relationships between carcinogen exposure and genotoxic effect can be explained by genotypic differences, enabling the identification of more susceptible subgroups for environmental cancer risks. This may be of relevance for environmental health policy setting. (Cancer Epidemiol Biomarkers Prev 2008;17(8):1902–12)

Introduction

It has been estimated that the incidence of cancer can be reduced by as much as 85% upon preventing exposure to carcinogenic environmental components (1). Therefore, continued efforts are needed to investigate the effect of hazardous environmental exposures on environmental cancer risk and eventually translate this knowledge into preventive policy measures. Molecular epidemiologic approaches have the advantage of applying methodologies that enable investigating the preclinical effects of exposure by using biological markers (biomarkers) present in human cells, tissues, and body fluids (2). These biomarkers comprise measurements of the so-called internal dose of an environmental carcinogen (e.g., urinary metabolites resulting from carcinogen metabolism), biologically effective dose (e.g., DNA damage reflecting certain genotoxic exposures), and early biological effects (e.g., deleterious mutations). The EU Environmental Health Action plan stimulates biomarker studies among relevant human populations (http://www.eu-humanbiomonitoring.org/sub/back/ehap.htm).

The Flanders Environment and Health Survey (FLEHS) is a large-scale biomonitoring study conducted in Flanders from 2002 to 2006 and assigned by the Flemish Government (www.milieu-en-gezondheid.be). Its aim is to obtain a better perspective on health risks associated with exposure to harmful environmental compounds among inhabitants of different regions of Flanders to eventually underpin new policy measures.

Conventional risk assessment methods may underestimate the individual's risk for exposures to environmental carcinogens because of the default assumption that all individuals within a certain population possess equal susceptibility to a specific carcinogen dose (1). Although the established risk assessment procedure for setting exposure limits to environmental carcinogens is considered sufficiently safe because of the application of the relatively rigid linear, nonthreshold, extrapolation model and the inclusion additional safety factors, environmental health policy making may benefit from more information on the degree of interindividual variations in environmental cancer risk. Investigating
Materials and Methods

Study Population. Volunteers were all recruited upon advertisement by the FLESH Biomonitoring and Surveillance team. The campaign was approved by the ethical committee of the University of Antwerp, and every individual signed an informed consent before collecting blood. Blood samples were donated by all individuals for analysis of exposure (internal dose) and effect biomarkers. Information on age, sex, and smoking habits was obtained by questionnaire. Two age groups were defined, coming from across Flanders and consisting of 429 adolescents and 361 adults; this represented a selection from a grand total of 4,800 subjects who participated in the FLESH biomonitoring study, which has run from 2002 to 2006.

The adolescents were sampled between October 2003 and July 2004 and consisted of 256 males and 173 females, with an average age of 15 ± 0.5 years. Fifty-four individuals reported smoking 7 ± 7 (range, 1-41) cigarettes per day. These 429 adolescents are a representative subset of the larger source population, enrolled by the FLEHS via 50 schools located in Flanders, and sampled between October 2003 and July 2004. Inclusion criteria were the following: being born in 1988 or 1989, studying in the third year of secondary school, living for at least 5 years in the same area, and giving informed consent (both adolescent and parents).

Subsequently, 361 adults aged 58 ± 4 years at the time of inclusion were sampled between September 2004 and June 2005. This study population was composed of 185 males and 176 females, of whom 65 individuals reported to smoke 17 ± 9 (range, 1-40) cigarettes per day. These 361 adults are also a representative selection of the larger source population, randomly sampled by the FLEHS via communal address databases between September 2004 and June 2005. Inclusion criteria were age between 50 and 65 years, living in Flanders for more than 5 years, and being able to complete questionnaires in Dutch.

Sample Collection. Approximately 200 mL of urine and 40 mL of blood were sampled from each participant to carry out various analyses. The creatinine content in urine was determined spectrophotometrically by Algemeen Medisch Laboratorium in Antwerp, Na2EDTA (10% volume for volume) was added to whole blood, whereas serum was prepared by immediate centrifugation of the coagulated blood. Samples were stored at −20°C until analysis.

WBC and DNA Isolation. WBCs were isolated by destroying RBCs using lysis buffer containing 155 mMmol/L NH4Cl, 10 mMmol/L KHCO3, and 10 mMmol/L EDTA of 0°C (blood-buffer ratio is 1:2). After two rounds of (a) incubation with lysis buffer on ice for 30 minutes and (b) centrifugation (10 minutes at 1,000 rpm at 4°C), the cells were washed with 1 mL. 150 mMmol/L NaCl and stored at −80°C before DNA isolation.

DNA isolation was done in a 96-well format using the Invisorb Blood Midi HTS 96 Kit/C (Invitrogen) and stored in 96-well plates at −4°C until genotyping.

Selection of Polymorphisms. Oxidative stress, 36 single-nucleotide polymorphisms in 23 genes involved in biotransformation, oxidative stress, and DNA repair, with the aim to investigate the impact of these genotypes plus gender on various exposure-effect relationships.

Genotyping. Genotyping was done by primer extension using the Snapshot methodology (Applied Biosystems). Four different multiplex single-base extension primers were designed as previously described. PCR was carried out on four different multiplex PCRs (Table 1) on a Tgradient 96-well thermal cycler (Biometra), each in a 10-μL volume using 96-well plates (described previously in ref. 8). Values for Tm were optimized for every multiplex PCR individually: 56°C for multiplex 1, 60°C for multiplex 2, 57°C for multiplex 3, and 60°C for multiplex 4 (Table 1). PCR products were subsequently incubated (37°C for 45 minutes) with 4 μL Exo-SAP-IT (Amersham) to digest contaminating deoxyribonucleotide triphosphates and PCR primers. Enzymes were deactivated at 75°C (15 minutes).

Genotyping was done by primer extension using the Snapshot methodology (Applied Biosystems). Four different multiplex single-base extension reactions were conducted on the corresponding multiplex PCR products using 96-well plates. The protocol used for single-base extension was described previously (8). After genotyping, single-base extension products were mixed with 13 μL deionized formamide containing 0.4 μL Genescan 120 LIZ size standard, denatured at 95°C for 5 minutes, and thereafter analyzed on an ABI Prism 3100 genetic analyzer using Genscan Analysis software (version 3.7).

growth genetic polymorphisms predisposing for increased susceptibility in monitored populations may thus be of relevance.

In the FLEHS study, individuals were monitored for exposure to organochlorides, heavy metals, polycyclic aromatic hydrocarbons (PAH), and benzene. PAHs and benzene are established environmental genotoxins, whereas organochlorides and heavy metals are known to produce free radicals and thereby lead to oxidative DNA damage, and thus represent a cancer risk (3-7). To elucidate the genotoxic effects of these exposures among the Flemish populations, DNA damage levels as measured by the Comet assay and as micronuclei in lymphocytes, urinary 8-hydroxydeoxyguanosine and several serum tumor markers were analyzed.

In the current study, for the purpose of determining more susceptible subpopulations, a random selection of 790 individuals from the FLESH study population was genotyped for 36 polymorphisms in 23 critical genes involved in biotransformation, oxidative stress, and DNA repair, with the aim to investigate the impact of these genotypes plus gender on various exposure-effect relationships.
Chemical Analysis of Biomarkers of Exposure. Lead (Pb) and cadmium (Cd) concentrations in whole blood were determined after an acid digestion pretreatment destroying the organic matrix, followed by high-resolution inductively coupled plasma–mass spectrometry detection.

Analysis of serum polychlorinated biphenyls and chlorinated pesticides (hexachlorobenzene and dichlorodiphenyl dichloroethane, a metabolite of dichlorodiphenyltrichloroethane) was based on methods as previously described by Gomara et al. (9) and Covaci and Schepens (10). Shortly, blood serum was mixed with formic acid, internal standards were added, and the mixture was equilibrated in an ultrasonic bath. Subsequent elution was done using a solid-phase extraction cartridge, which was washed, dried, and placed on top of a multilayer column filled with anhydrous sodium sulfate and silica with sulfuric acid. Polychlorinated biphenyls and chlorinated pesticides were eluted and concentrated, and subsequently analyzed using gas chromatography combined with an electron capture detector. The detection limit of all chlorinated compounds in serum was 0.02 μg/L.

Determination of 1-hydroxypyrene (a metabolite of pyrene) and tt-muconic acid (a metabolite of benzene) in urine are based on those of Angerer and Schaller (11, 12). The determination of 1-hydroxypyrene was done with high-performance liquid chromatography with fluorescence detector. The detection limit was 0.060 μg/L. tt-Muconic acid was determined in urine by means of ion chromatography using solid-phase extraction–SAX columns. High-performance liquid chromatography was used to separate the extract from other compounds, and quantification was done by a UV detector. The detection limit was 8.6 μg/L.

Measurement of the Early Biological Effect Markers. Loss of DNA integrity was evaluated by the alkaline Comet assay as described by Singh et al (13). Median percentages of DNA migration in the tail areas were determined and used as a measure of DNA damage. Micronuclei frequencies were investigated using the cytokinesis block micronucleus assay on whole blood.

### Table 1. Overview of the included single-nucleotide polymorphisms and their phenotypic characteristics

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>db SNP ID</th>
<th>Effect on enzymatic function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2 A&gt;C-154</td>
<td>rs762551</td>
<td>Higher inducibility</td>
</tr>
<tr>
<td>CYP1A1 T&gt;C-3801</td>
<td>rs9030838</td>
<td>Higher enzyme activity</td>
</tr>
<tr>
<td></td>
<td>rs1048943</td>
<td></td>
</tr>
<tr>
<td>CYP1B1 V432L</td>
<td>rs1056836</td>
<td>Higher enzyme activity</td>
</tr>
<tr>
<td></td>
<td>rs1800440</td>
<td></td>
</tr>
<tr>
<td>CYP2E1 G&gt;T-70</td>
<td>rs6413420</td>
<td>Higher enzyme activity</td>
</tr>
<tr>
<td>CYP3A4 A&gt;G-391</td>
<td>rs2740374</td>
<td>Higher enzyme activity</td>
</tr>
<tr>
<td>GSTM1 Del</td>
<td>rs947894</td>
<td>Decreased enzyme activity</td>
</tr>
<tr>
<td>GSTP1 I105V</td>
<td>rs179981</td>
<td></td>
</tr>
<tr>
<td>GSTT1 Del</td>
<td>rs1801280</td>
<td></td>
</tr>
<tr>
<td>NAT2 I114T</td>
<td>rs1799930</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs179931</td>
<td></td>
</tr>
<tr>
<td>mEH Y113H</td>
<td>rs1051740</td>
<td>Decreased enzyme activity</td>
</tr>
<tr>
<td></td>
<td>rs2234922</td>
<td>Increased enzyme activity</td>
</tr>
<tr>
<td>MTHFR A429E</td>
<td>rs1801133</td>
<td>Decreased enzyme activity</td>
</tr>
<tr>
<td></td>
<td>rs1801133</td>
<td></td>
</tr>
<tr>
<td>COMT V108M</td>
<td>rs4680</td>
<td></td>
</tr>
<tr>
<td>DNA repair</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRCC1 R194W</td>
<td>rs1799782</td>
<td>Increased enzyme activity</td>
</tr>
<tr>
<td></td>
<td>rs25487</td>
<td>Decreased enzyme activity</td>
</tr>
<tr>
<td>XRCC3 T241M</td>
<td>rs1052559</td>
<td>Decreased enzyme activity</td>
</tr>
<tr>
<td>XPD K751Q</td>
<td>rs28046</td>
<td>Decreased enzyme activity</td>
</tr>
<tr>
<td>BRCA2 G&gt;A-26 (5`UTR)</td>
<td>rs1799943</td>
<td>Decreased enzyme activity</td>
</tr>
<tr>
<td>APE1 D148G</td>
<td>rs3136820</td>
<td>Decreased enzyme activity</td>
</tr>
<tr>
<td>OGGl S326C</td>
<td>rs1052133</td>
<td>Decreased enzyme activity</td>
</tr>
<tr>
<td>General stress</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mSOD V16A</td>
<td>rs1799725</td>
<td>Decreased enzyme activity</td>
</tr>
<tr>
<td>CAT C&gt;T-262</td>
<td>rs1001179</td>
<td>Lower catalase activity</td>
</tr>
<tr>
<td>MPO G&gt;A-463</td>
<td>rs233227</td>
<td>Higher enzyme activity</td>
</tr>
<tr>
<td>NQO1 P187S</td>
<td>rs1800566</td>
<td>Reduced enzyme activity</td>
</tr>
<tr>
<td>GPX1 P198L</td>
<td>rs1050450</td>
<td>Lower enzyme activity</td>
</tr>
</tbody>
</table>
| NOTE: (1) is 10-plex PCR; (2), 9-plex PCR; (3), 8-plex PCR; and (4), 9-plex PCR (see Materials and Methods). Sets of single-nucleotide polymorphisms were analyzed together. Abbreviations: SNP, single-nucleotide polymorphism; ID, identification.
cultures, based on standard procedures (14). For each individual, 1,000 to 2,200 cells were evaluated for the presence of micronuclei using the metafer automatic program from Metasystems.

The competitive immunosorbent assay (Gentaur) based on ELISA was used to measure urinary 8-hydroxydeoxyguanosine levels according to the manufacturer instructions.

The serum protein level of the tumor marker p53 was analyzed using the enzyme immunometric assay and titerzyme enzyme immunometric assay p53 (Assay Designs). Prostate-specific antigen and carcinoembryonic antigen levels were measured using solid-phase chemiluminescent immunometric assay and Immulite 2000 (DPC, Los Angeles, CA). Samples were analyzed for prostate-specific antigen within 24 hours after collection.

Statistical Analysis. First, the putative effect of smoking on exposure was tested by comparing levels of the exposure markers between smokers and non-smokers using a Mann-Whitney U test. In case that smoking significantly affected biomarker levels, subsequent analyses were conducted among nonsmokers because primary focus was on the exposure to environmental compounds. In case that no effect of smoking was observed, further statistical analyses were done on the total population, cigarette smoking being regarded as an independent variable.

The subsequent analysis has been adapted from our statistical methods described earlier (15) and summarized in Fig. 1. Shortly, after elimination of smokers, if necessary, relationships between markers for endogenous exposure and genotoxic effect were investigated by linear regression analysis. For each individual, the deviation from this relationship was determined by dividing the level of response that was actually observed (O) by the level that was expected (E) according to the regression line. Using this approach, the population was divided into two subgroups: (a) so-called low responders, thus having an O/E<1, and (b) high responders, that is, the more sensitive subjects, showing an O/E>1 (an O/E = 1 was not observed). Subsequently, to identify the most important factors for discrimination of the different subgroups, binary logistic regression analysis has been conducted to classify each individual into one of the two subgroups based on the putative variables cigarettes per day (only if smokers were included), gender, and all single-nucleotide polymorphisms. The investigated genotypes were coded 2 in case of double variants, 0 for wild type, and 1 for heterozygous individuals (15). Results are presented as mean ± SE.

Results

A total of 28,440 genotypes was generated in the overall study population. Statistical analyses assessing the impact of these genotypes on various exposure-effect relationships were conducted in the adolescent and adult population separately, and obtained results will therefore be described for each subpopulation. If the distribution of the biological markers was not normal, a log transformation has been applied to the data (Table 2).

Adolescent Population. Because blood Cd ($P = 0.000$) and urinary 1-hydroxypyrene ($P = 0.013$) were found to be significantly elevated in smokers, subsequent analyses of these markers were conducted solely in nonsmokers. Of all possible exposure-effect relationships, only chlorobenzene ($P = 0.046$) and ethylbenzene ($P = 0.022$) exposures were significantly related to levels of DNA damage levels as determined by Comet assay (Table 2A). Dosimeter data for the different benzene exposure markers were only available in 54 individuals.

In case of the exposure-effect relationship between chlorobenzene exposure and Comet DNA damage, no significant predictors for the observed interindividual variation were found among evaluated single-nucleotide polymorphisms (Table 3). In contrast, $CAT$ C-262T ($P = 0.027$) and $GSTT1*0$ ($P = 0.035$) polymorphisms seemed the most important genetic variations affecting Comet DNA damage levels in association with exposure to ethylbenzene (Table 3). For the $CAT$ C-262T polymorphism, individuals carrying the double-variant allele had 1.4 times higher DNA damage levels than $CAT$ C-262T.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Overview of the statistical analysis. If smoking significantly affected the exposure marker level (Mann-Whitney U test), the analysis was conducted in nonsmokers only. Exposure-effect relationships were analyzed by linear regression, and two subgroups were made based on the regression line. Subsequent classification of the individuals into one of these subgroups (low or high responders) was conducted using binary logistic regression analysis.

*Only in total population*
Table 2. Results of the correlation analyses between exposure and effect markers, presented as \( R^2 \) or standardized \( R^2 \), in the adolescent (A) and adult (B) population [exposure markers are shown in rows, early effect markers are shown in columns (with the exception of the first)]

<table>
<thead>
<tr>
<th></th>
<th>Smoking*</th>
<th>Comet</th>
<th>8-OHdG</th>
<th>PSA</th>
<th>CEA</th>
<th>p53</th>
<th>MN</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p,p’DDE</td>
<td>0.544</td>
<td>–0.079/–0.026</td>
<td>–0.250/–0.017</td>
<td>–0.030/0.015</td>
<td>0.067/0.038</td>
<td>0.452/0.068</td>
<td>0.103/0.056</td>
</tr>
<tr>
<td>HCB</td>
<td>0.517</td>
<td>–0.157/–0.073</td>
<td>1.807/0.158</td>
<td>–0.059/0.032</td>
<td>–0.045/0.035</td>
<td>0.485/0.098</td>
<td>0.401/0.037</td>
</tr>
<tr>
<td>Cd_blood</td>
<td>0.562</td>
<td>–0.032/–0.028</td>
<td>0.146/0.221</td>
<td>–0.100/0.012</td>
<td>0.008/0.011</td>
<td>0.414/0.041</td>
<td>0.930/0.017</td>
</tr>
<tr>
<td>Cd_urine</td>
<td>0.000</td>
<td>0.254/0.029</td>
<td>–0.014/0.012</td>
<td>–0.148/0.124</td>
<td>0.041/0.034</td>
<td>0.303/0.007</td>
<td>0.282/0.024</td>
</tr>
<tr>
<td>Pb</td>
<td>0.510</td>
<td>0.127/0.048</td>
<td>–1.146/–0.090</td>
<td>–0.055/0.032</td>
<td>–0.257/0.168</td>
<td>0.290/0.052</td>
<td>–0.228/–0.078</td>
</tr>
<tr>
<td>1-OHP</td>
<td>0.000</td>
<td>0.023/0.020</td>
<td>0.749/0.129</td>
<td>–0.104/–0.148</td>
<td>0.033/0.059</td>
<td>–0.067/–0.026</td>
<td>0.069/0.106</td>
</tr>
<tr>
<td>tt-MA</td>
<td>0.074</td>
<td>–0.087/–0.077</td>
<td>1.032/0.173</td>
<td>–0.019/0.023</td>
<td>0.070/0.110</td>
<td>–0.011/–0.004</td>
<td>0.049/0.067</td>
</tr>
</tbody>
</table>

B wild-type individuals (\( P = 0.018; \) Fig. 2A). Individuals lacking the GSTT1 gene had 1.2 times higher DNA damage levels as compared with subjects carrying the wild-type GSTT1 gene variant in response to ethylbenzene exposure (Fig. 2B), although not statistically significant (\( P = 0.159 \)). No significant role for gender was observed.

**Adult Population.** Investigated exposure biomarkers in adults, associations of smoking with these biomarkers, and exposure-effect relationships are shown in Table 2B. Cd, in blood and in urine, and urinary 1-hydroxypyrene levels were significantly elevated by smoking (\( P = 0.000 \) for each biomarker; Table 2B). Therefore, all subsequent statistical analyses of these biomarkers were conducted in nonsmokers. Significant exposure-effect relationships were established between urinary levels of the early effect marker 8-hydroxydeoxyguanosine and four of the investigated exposure markers, namely, blood hexachlorobenzene (\( P = 0.003 \)), urinary Cd (\( P = 0.007 \) in nonsmokers), urinary 1-hydroxypyrene (\( P = 0.029 \) in nonsmokers), and urinary trans-trans muconic acid (\( P = 0.001 \); Table 2B). Moreover, two tumor markers in blood, namely, carcinoembryonic antigen and p53, were significantly associated with Pb in blood (\( P = 0.004 \)) and in urinary Cd (\( P = 0.003 \), in nonsmokers), respectively (Table 2B). Finally, a positive correlation between hexachlorobenzene exposure and micronuclei levels was observed (\( P = 0.000 \); Table 2B).

The observed associations will be detailed below; it is noted that because of limited space, error bars corresponding to these results are not shown, and data are presented as numbers.

**Hexachlorobenzene versus 8-Hydroxydeoxyguanosine.** A positive exposure-effect relationship was found between hexachlorobenzene exposure and 8-hydroxydeoxyguanosine DNA damage levels. Females have 1.1 times higher 8-hydroxydeoxyguanosine DNA damage levels upon hexachlorobenzene exposure than males (\( P = 0.01 \)). In addition, interindividual variations in developing DNA damage after exposure to hexachlorobenzene significantly depend on the mEH genotype (Table 3). Hexachlorobenzene-related 8-hydroxydeoxyguanosine levels were 0.9 times lower in carriers of one or more mEH H199R variant alleles compared with wild-type individuals; however, this difference was not statistically significant (\( P = 0.055 \)).

**Hexachlorobenzene versus Micronuclei.** Hexachlorobenzene exposure was found to have a positive effect on micronuclei levels. Within this relationship, 1.3 times higher micronuclei levels were observed in females compared with males (\( P < 0.001 \)). Moreover, 0.9 times

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**NOTE:** All exposure markers, the tumor markers, and micronuclei data were log-transformed to meet normality. If smoking significantly affected the exposure marker level (Mann-Whitney U; \( P \) values shown in the first column), the correlation analysis was conducted on nonsmokers. Significant correlations are marked (\( P < 0.05 \)).

**Abbreviations:** DDE, dichlorodiphenyl dichloroethane; HCB, hexachlorobenzene; PCB, polychlorinated biphenyl; 1-OHP, 1-hydroxypyrene; tt-MA, trans-trans muconic acid; 8-OHdG, 8-hydroxydeoxyguanosine; PSA, prostate-specific antigen; CEA, carcinoembryonic antigen; MN, micronuclei.

*Smoking indicates effect of smoking on exposure marker; \( P \) values were obtained by Mann-Whitney U test.

0.05 \( > P \geq 0.01 \).

0.01 \( > P \geq 0.001 \).

0 \( P \geq 0.001 \).
lower micronuclei levels were found in individuals with two variants for the mnsOD V472A genotype (P = 0.032) and for individuals carrying at least one variant for the CYP1A1 T3801C genotype (0.86 times for heterozygous individuals and 0.65 times for double variants; P = 0.001) compared with the respective wild-type genotypes.

Urinary Cd versus 8-Hydroxydeoxyguanosine. Exposures to Cd as measured in urine was found to be associated with levels of 8-hydroxydeoxyguanosine in nonsmokers. The deletion of the GSTT1 gene was found to be a significant predictor of interindividual differences within this relationship (Table 3). For individuals carrying the wild-type allele for GSTT1, 0.9 times lower DNA damage levels were observed compared with individuals lacking the GSTT1 gene (P = 0.039). No role of gender was found.

Urinary Cd versus p53. A positive exposure-effect relationship between urinary Cd and serum p53 was observed in nonsmokers. GSTM1*0 (P = 0.004) and BRCA2 N372H (P = 0.024) were found to contribute significantly to interindividual variations within this relationship (Table 3). Wild-type GSTM1 individuals had 1.1 times higher p53 levels in association with urinary Cd excretion than individuals lacking the GSTM1 gene, although not statistically significant (P = 0.106). Moreover, serum p53 levels in individuals carrying the double-variant allele for the BRCA2 N372H polymorphism were observed to be 1.3 and 1.4 times higher as compared with wild-type and heterozygous individuals (P = 0.024), respectively. No role of gender was observed.

Table 3. Results of genotype, gender, and smoking habits on the different exposure-effect relationships in the adolescent and adult populations

<table>
<thead>
<tr>
<th>Relationship</th>
<th>Significantly differently distributed variables between high and low responders</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adolescents</strong></td>
<td><strong>Response</strong></td>
<td><strong>Effect</strong></td>
</tr>
<tr>
<td>Chlorine-benzene * Comet</td>
<td>No significant variables</td>
<td><strong>Variant allele associated with increased Comet levels</strong></td>
</tr>
<tr>
<td>Ethylbenzene * Comet</td>
<td>CAT C-262T (0.027)</td>
<td><strong>Increased Comet levels in null individuals</strong></td>
</tr>
<tr>
<td>HCB * 8-OHdG</td>
<td>Sex (0.008)</td>
<td>Higher 8-OHdG levels in females</td>
</tr>
<tr>
<td></td>
<td>mEH H133R (0.033)</td>
<td>Lower 8-OHdG levels in individuals with at least one variant allele</td>
</tr>
<tr>
<td>HCB * MN</td>
<td>Sex (0.000)</td>
<td>Higher MN levels in females</td>
</tr>
<tr>
<td></td>
<td>mnsOD (0.019)</td>
<td>Lower MN levels in dv</td>
</tr>
<tr>
<td></td>
<td>GSTM1*0 (0.000)</td>
<td>Lower MN levels in individuals with at least one variant allele</td>
</tr>
<tr>
<td>Cd_urate * 8-OHdG</td>
<td>GSTT1*0 (0.041)</td>
<td>Increased 8-OHdG levels in null individuals</td>
</tr>
<tr>
<td>1-OHP * 8-OHdG</td>
<td>Sex (0.012)</td>
<td>Females have higher 8-OHdG levels</td>
</tr>
<tr>
<td></td>
<td>mEH H133R (0.023)</td>
<td>Lower 8-OHdG levels in individuals with at least one variant allele</td>
</tr>
<tr>
<td></td>
<td>GSTM1*0 (0.004)</td>
<td><strong>Variant allele associated with increased 8-OHdG levels</strong></td>
</tr>
<tr>
<td>tt-MA * 8-OHdG</td>
<td>Sex (0.002)</td>
<td>Higher 8-OHdG levels in females</td>
</tr>
<tr>
<td>Pb * CEA</td>
<td>Smoking (0.000)</td>
<td>Smokers have higher CEA levels</td>
</tr>
<tr>
<td>CEA level</td>
<td>BRCA2 N372H (0.046)</td>
<td>Higher CEA levels in dv</td>
</tr>
<tr>
<td>Cd_urate * p53</td>
<td>GSTM1*0 (0.004)</td>
<td>Lower p53 levels in null individuals</td>
</tr>
<tr>
<td></td>
<td>BRCA2 N372H (0.024)</td>
<td>Higher p53 levels in individuals with dv</td>
</tr>
</tbody>
</table>

NOTE: When smoking significantly affected the level of the exposure marker, the relationship was investigated in nonsmokers. Corresponding P value is given between brackets.

Abbreviations: hz, heterozygous individuals; dv, double variants.

1 Analysis in nonsmokers.
levels (Table 3). Although smoking was not found to elevate the level of Pb exposure ($P = 0.510$), smokers had 2.7 times higher carinoembryonic antigen levels as compared with nonsmokers ($P < 0.001$) in response to Pb exposure. In addition, variations around the exposure-effect relationship were also found to be predicted by the $BRC2 A N372H$ polymorphism. Observed Pb-associated carinoembryonic antigen levels were 1.9 and 1.6 times higher in individuals carrying the double-variant allele as in heterozygous and wild-type individuals, respectively ($P = 0.003$). No effect of gender has been observed.

**Discussion**

In this study, a random selection taken from the FLEHS study population, comprising 429 adolescents and 361 adults, was genotyped for 36 single-nucleotide polymorphisms in 23 genes involved in relevant pathways with respect to environmental cancer risk assessment. In both subpopulations, several important and, in some cases, novel exposure-effect relationships between environmental toxicants and effect markers were established. These will be described separately below. With one exception, more susceptible subgroups could be defined within all significant relationships by analysis of genetic variants in relevant genes. Furthermore, gender seemed to influence susceptibility to various environmental carcinogens. This stresses the significant role that genetic-based interindividual differences play in biomonitoring projects studying environmental health effects.

**Adolescents.** DNA damage levels measured by Comet assay were used as a biomarker for genotoxic effects in association with several environmental exposures in the adolescent subpopulation. Of the investigated toxicants, only chlorobenzene and ethylbenzene were significantly associated with this biomarker; both associations have not yet been described in the literature. The main pathway of benzene biotransformation involves formation of metabolites that are known to generate reactive oxygen species through redox cycling (16, 17). These reactive oxygen species eventually induce oxidative DNA damage; exposure to benzene in mice has been shown to induce oxidative DNA damage measured by the Comet assay (18, 19). Interindividual variations around the regression line were observed. In case of chlorobenzene exposure, however, no genetic susceptible subgroups could be defined. However, regarding ethylbenzene exposure, catalase and $GSTT1$ genotypes significantly affected the relationship between ethylbenzene exposure and DNA damage. Increased levels compared with the expected response based on the regression line were observed in carriers of the $CAT C^{262}T$ polymorphism and in individuals lacking $GSTT1$ activity. Catalase and $GSTT1$ are involved in the primary defense against (environmentally induced) oxidative stress. Both polymorphisms have been associated with reduced enzyme activity, which leads to higher levels of reactive metabolites and increased levels of reactive oxygen species and, thus, higher DNA damage levels (20, 21). Therefore, $GSTT1$-null individuals and/or individuals carrying the $CAT C^{262}T$ polymorphism may be more susceptible for developing health effects induced by reactive oxygen species resulting from environmental exposure to ethylbenzene, which is in agreement with our observations; $GSTT1$-null individuals were found to have 1.2 times higher DNA damage levels compared with $GSTT1$ wild-types, whereas the $CAT C^{262}T$ polymorphism was observed to be associated with a 1.4 times higher DNA damage levels compared with wild-type individuals.

**Adults.** Urinary excretion of the DNA repair product 8-hydroxydeoxyguanosine is the most commonly used biomarker for assessing oxidative DNA damage as induced by (environmental) genotoxicants in combination with the efficiency of repair of such damage (22). Several studies have shown an effect of environmental exposures to various agents on urinary 8-hydroxydeoxyguanosine levels (23, 24). In the current study, in adults, an association of hexachlorobenzene in blood, urinary Cd, urinary 1-hydroxypyrene, and urinary trans-trans muconic acid with 8-hydroxydeoxyguanosine levels was observed.

Within the relationships between 1-hydroxypyrene and 8-hydroxydeoxyguanosine, and also between trans-trans muconic acid and 8-hydroxydeoxyguanosine, an effect of gender was found. Higher exposure-related DNA damage levels were observed in females than in males. Gender also affected the relationship between hexachlorobenzene exposure and micronuclei levels; again, higher micronuclei levels upon hexachlorobenzene exposure were found in females compared with males. Although these results are difficult to interpret because little is known about the mechanisms relating gender differences and carcinogen susceptibility, they are consistent with earlier reports that women have a higher susceptibility toward various carcinogens than men (25, 26).

In addition to this effect of gender, an effect of CYP1A1 $T^{461}N$ and $mEH H^{139}R$ on the exposure-effect relationship between 1-hydroxypyrene and urinary 8-hydroxydeoxyguanosine was found. CYP1A1 is a phase I biotransformation enzyme that activates certain carcinogenic compounds such as several PAHs into highly reactive metabolites. A key role for CYP1A1 in pyrene metabolism has been described (27), and variants of the CYP1A1 gene have been associated with increased enzyme activity (28-31). The CYP1A1 $T^{461}N$ polymorphism described here results in a greater capacity of metabolizing PAHs, and, thus, higher levels of reactive metabolites are formed. These reactive metabolites are known to induce oxidative stress and, eventually, oxidative DNA damage (32). Therefore, within the described exposure-effect relationship between 1-hydroxypyrene and 8-hydroxydeoxyguanosine levels, it may be expected that individuals carrying the CYP1A1 $T^{461}N$ polymorphism are more susceptible in developing oxidative DNA damage upon PAH exposure compared with wild-type individuals. Indeed, the observed 1-hydroxypyrene-associated urinary 8-hydroxydeoxyguanosine levels were 1.2 times higher in individuals carrying the CYP1A1 $T^{461}N$ polymorphism than in CYP1A1 $T^{461}N$ wild-type individuals. The role of $mEH$ is more complex because it may catalyze phases I and II biotransformation reactions (33). In general, $mEH$ has been described as a very important phase II enzyme (33). At least two functional polymorphisms have been determined in $mEH$, of which a high activity variant of the enzyme is coded by the $mEH H^{139}R$ polymorphism in exon 4 (34). In the current study, individuals carrying...
the mEH $H^{139R}$ polymorphism had 0.9 times lower 8-hydroxydeoxyguanosine levels as compared with mEH $H^{139R}$ wild types. Although the level of this effect is very low, the observed pattern is as expected because the high activity variant of the enzyme ($mEH H^{139R}$, $mEH R^{139R}$) leads to more detoxification and, thus, to lower DNA damage levels.

A similar effect of mEH on 8-hydroxydeoxyguanosine levels was found in association with hexachlorobenzene exposure. Hexachlorobenzene is a persistent organochloric pesticide that accumulates in humans and is widespread in the environment because it is not well degraded (35). The oxidative metabolites of hexachlorobenzene are able to bind to macromolecules such as DNA (36). Urinary levels of 8-hydroxydeoxyguanosine were observed to increase in association with hexachlorobenzene exposure, and $mEH H^{139R}$ wild-type individuals were defined as the more susceptible subgroup. Similar to the effect observed in response to PAH exposure, the level of the effect was marginal (0.9 times lower levels in heterozygous and homozygous carriers of the $mEH H^{139R}$ polymorphism compared with wild

**Figure 2.** Effect of CAT C-262T (A) and GSTT1 null (B) genotype on the relationship between ethyl-benzene exposure and Comet levels in adolescents, presented as O/E ratio. Results are presented as mean ± SE. Dashed line, the situation where the expected response equals the observed response.
types); however, the observed pattern indicates again a protective effect of the mEH H135R polymorphism, where the low activity of the wild-type enzyme may lead to less detoxification of reactive metabolites and, thus, to higher DNA damage levels.

In addition, exposure to hexachlorobenzene was also found to be associated with increased micronuclei levels. It has been described that hexachlorobenzene is a genotoxic carcinogen and is able to produce a significant increase in micronuclei frequencies (36). Within this exposure-effect relationship, lower hexachlorobenzene-related micronuclei levels were observed for individuals carrying the mnSOD V16A variant and for individuals carrying the CYP1A1 T3801C variant alleles. The variant allele of the CYP1A1 T3801C genotype leads to an increase in CYP1A1 enzyme activity (37). However, the role of this enzyme in biotransformation is rather complex because it can both activate and detoxify certain compounds. In addition, little is known regarding its role in the biotransformation of hexachlorobenzene.

Nevertheless, the observed results for CYP1A1 T3801C might be explained by a higher activity of the CYP1A1 T3801C double variant in the detoxification of hexachlorobenzene, which leads to lower hexachlorobenzene-related micronuclei levels. Moreover, it has been shown both in rats (38) and in human breast tumors (39) that organochlorine pesticides such as hexachlorobenzene are associated with an increased oxidative stress. Regarding mnSOD, the mnSOD V16A variant is associated with a decrease in enzyme activity and, hence, an impaired antioxidant capacity against the oxidative metabolites produced during the metabolism of hexachlorobenzene. This is in contrast to what would be expected because less detoxification would lead to a higher accumulation of oxidative metabolites and, thus, higher micronuclei levels. Therefore, these results regarding mnSOD remain inconclusive.

Furthermore, an important exposure-effect relationship between Cd and urinary 8-hydroxydeoxyguanosine levels was found, which is affected by the GSTT1 genotype. Cd is a highly toxic metal and a well-known occupational (e.g., heavy metal mining, waste incinerators, and farm fertilizers) and environmental pollutant (water, food, and air contaminations; ref. 40). Occupational exposure to Cd has been associated with lung, prostate, pancreas, and kidney cancer, and it has been correspondingly classified as a category 1 carcinogen by IARC (41). Cd is not directly genotoxic, but it may affect genome instability by inhibition of DNA repair, depletion of cellular antioxidants such as glutathione (reduced), and indirect formation of reactive oxygen species (through the replacement of iron, zinc, and copper from various proteins such as ferritin, thereby increasing the level of free metal ions that participate in oxidative stress via fenton reactions; refs. 42-44). This Cd-induced oxidative stress has been described to cause 8-hydroxydeoxyguanosine-related DNA strand breaks (45). In addition, in the current study, a positive exposure-effect relationship between Cd exposure and 8-hydroxydeoxyguanosine levels was found. Within this relationship, individuals lacking GSTT1 activity have 1.1 times higher oxidative DNA damage levels after Cd exposure as compared with GSTT1-positive individuals. GSTT1 is an enzyme involved in the reduced glutathione pathway, one of the most essential antioxidant defense mechanisms. Thus, GSTT1-null individuals have an impaired antioxidant defense mechanism that results in an enhanced effect of oxidative stress, induced indirectly by Cd exposure, and thus, to higher 8-hydroxydeoxyguanosine DNA damage levels. This is confirmed by our observations.

Exposure to Cd was also found to be related to an increase in serum p53 levels, an association described for the first time in this study. It has been suggested by Xie and Shaikh (46) that Cd can activate p53 by the indirect generation of oxidative stress. For example, Cd has been reported to generate hydrogen peroxide (7), which in turn is known to trigger p53 transcriptional activity (47). Moreover, Cd is known to interfere with DNA repair, which in turn may also induce apoptosis (40). Analysis of the p53 tumor suppressor gene is proposed as a biomarker for effect in biomonitoring studies (48, 49). Within the observed exposure-effect relationship between Cd exposure and p53 levels, individuals homozygously carrying the BRCA2 N372H polymorphism were found to have significantly higher serum p53 levels than BRCA2 N372H wild-type and heterozygous individuals (1.3 and 1.4 times higher compared with wild-type and heterozygous individuals, respectively). In addition to the interference of Cd with the DNA repair system, carrying the BRCA2 N372H polymorphism, associated with a decrease in DNA repair, may lead to even higher DNA damage levels and, thus, an increase in Cd-induced serum p53 levels compared with average levels.

Another tumor marker, carcinoembryonic antigen, was also found to be increased upon exposure to a heavy metal, Pb. This is the first report that describes this relationship. Pb is an indirectly acting genotoxic metal, present in the environment as a pollutant of, for example, heavy metal mining, traffic waste by leaded fuel and as a food contaminant (e.g., drinking water by use of Pb pipelines). It is classified as carcinogenic by IARC, and (occupational) exposure to Pb has been reported to cause DNA damage as determined by Comet assay (50-52). In the current study, Pb-induced carcinoembryonic antigen levels were 1.9 and 1.6 times increased in carriers of the BRCA2 N372H polymorphism as compared with heterozygous and wild-type individuals, respectively. We cannot explain the functionality of elevated blood carcinoembryonic antigen levels in this case. However, it is striking that relatively higher levels of serum carcinoembryonic antigen have been reported in smokers compared with nonsmokers (53). In agreement to these findings, also in our study, smokers were observed to have 2.7 times higher carcinoembryonic antigen levels than nonsmokers upon Pb exposure.

Overall. In both subpopulations, the genotype was shown to significantly influence interindividual susceptibilities to environmental carcinogens. However, in most cases, observed impacts of these genotypes were relatively low. This may be the result of the rather low exposure levels of the Flemish population to the investigated compounds, which lead to weak exposure-effect relationships and, as a consequence, a low impact of genetic variations, including gender, predisposing for increased susceptibility. However, all observed relationships and effects of the genotype are biologically plausible and are statistically significant, which reduces the chance of bias.
Regarding the complexity of carcinogen exposure and the number of pathways that is involved in the modification of their effects, it is unlikely that one single-nucleotide polymorphism can be held responsible for these interindividual differences in genotoxic response. Investigating multiple polymorphisms in different pathways simultaneously should therefore be considered. Moreover, because these low-penetration polymorphisms are risk factors for developing health effects in response to (environmental) exposures, future studies assessing genotyping as a biomarker for susceptibility must shift interest from exploring the effect of the genotype on the specific biomarker, toward the investigation of the effect of the genotype on the exposure-effect relationship between markers of interest. It is noted that in these types of study design investigating a large number of variables simultaneously, there is a chance of “multiple comparisons.” However, in accordance with the views formulated by Rothman (54), and because all observed findings are biologically plausible, in this study, P values are presented without adjustment for multiple testing.

In conclusion, this study has provided new insights in exposure-effect relationships in humans exposed to certain environmental contaminants and, secondly, has quantified the role of genetic polymorphisms in these relationships, enabling the identification of more susceptible subgroups. These findings suggest a role of interindividual differences in environmental cancer risk assessment. Although the results of this study should be interpreted carefully because overall effects were relatively modest and they comprise biomarker analysis instead of ultimate cancer incidence, environmental cancer risk assessment should challenge the assumption that all individuals are uniformly vulnerable to environmental pollutants.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Multiplex Genotyping as a Biomarker for Susceptibility to Carcinogenic Exposure in the FLEHS Biomonitoring Study

Hans B. Ketelslegers, Ralph W.H. Gottschalk, Gudrun Koppen, et al.


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