Exposure to polycyclic aromatic hydrocarbons among never smokers in Golestan Province, Iran, an area of high incidence of esophageal cancer – a cross-sectional study with repeated measurement of urinary 1-OHPG in two seasons

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are by-products of incomplete combustion of organic matter and occur as mixtures of complex and variable composition. The major sources of PAHs are tobacco smoking, some occupational exposures (such as coke oven working), air pollution, and diet, particularly charbroiled or fried foods (Unwin et al., 2006; Perello et al., 2008; Kamangar et al., 2009). The International Agency for Research on Cancer has classified benzo[a]pyrene, a prototype PAH, and exposures in several occupations with heavy exposure to PAHs as carcinogenic to humans (Baan et al., 2009). Consistent associations between those occupational exposures and cancers of the lung and skin have been shown in epidemiological studies, and strong mechanistic evidence and carcinogenicity in several animal species has been reported for benzo[a]pyrene (Baan et al., 2009). An association between PAHs and urinary bladder has also been reported in...
several studies (Bosetti et al., 2007). Factors such as the route of exposure, the chemical composition of the mixture, the presence of co-exposures, and genetic susceptibility are likely to explain inter-organ differences in the carcinogenicity of PAH (IARC Working Group, 2010).

Cross-sectional studies in high-incidence areas of esophageal squamous cell carcinoma (ESCC) in Linxian (China) and Golestan Province (Iran) indicate that the inhabitants of these areas are highly exposed to PAHs (Roth et al., 1998a,b, 2001; Wornat et al., 2001; Kamangar et al., 2005; Islami et al., 2009b). The hypothesis of a carcinogenic role of PAH on the esophageal mucosa is supported by experimental and epidemiological studies, including those showing an increased risk of ESCC among chimney sweeps (Hogstedt et al., 1982; Evanoff et al., 1993) and tobacco smokers (Hecht, 2003). We have recently reported in a case–control study in Golestan Province higher levels of antibody against benzo[a]pyrene diol epoxide-1-modified guanosine in non-tumoral esophageal biopsies from patients with biopsy-proven ESCC than in biopsies from control subjects (Abedi-Ardekani et al., 2010).

In Golestan Province, where both ESCC rates and exposure to PAHs are high, the sources of exposure to PAHs are not well characterized. High PAH exposure levels (compared to several other populations; see Discussion) in Golestan have been observed among non-smokers as well as smokers, and only 15% of the variance in PAH levels in urine was accounted for by known factors, such as age, sex, place of residence (rural versus urban), and tobacco use (Kamangar et al., 2005). We conducted an exploratory molecular epidemiological study on healthy, never-smoking female inhabitants of Golestan Province to investigate the patterns of exposure to PAHs, sources of PAHs other than tobacco smoking, and the association of several lifestyle and genetic factors with 1-hydroxypyrene glucuronide (1-OHPG) levels in urine. 1-OHPG is a stable PAH metabolite that reflects recent (within the past 24 h) exposure to mixed PAHs (Roth et al., 2001). The half-life for urinary excretion of 1-OHPG ranges from 6 to 35 h and peak urine concentration occurs a few hours following exposure (Strickland et al., 1996).

MATERIALS AND METHODS

STUDY SUBJECTS

For this study, 111 participants in the Golestan Cohort Study were randomly selected. The Golestan Cohort Study is a prospective study that recruited 50,045 participants, 40–75 years of age, from eastern parts of Golestan Province between January 2004 and June 2008, with 40,013 participants from 326 villages and 10,032 from urban areas (Pourshams et al., 2010). In order to investigate sources of PAHs other than tobacco smoking, specifically potential exposure from cooking methods, only never-smoking women were enrolled in this study because preparation of food in the area is usually done by women, so they might provide more precise information about food consumption and cooking methods. Furthermore, smoking among women in Golestan is uncommon; therefore, erroneous assignment of tobacco use is less likely among women. Also, if exposure to PAHs is to be a major cause of ESCC in Golestan, this should be true in both men and women, as incidence of ESCC is high in both sexes. The age adjusted incidence rates (ASR) for ESCC in men and women in Golestan are 43 and 36 per 10^5 person-years, respectively (Semnani et al., 2006), with higher rates in eastern than in western parts of the province (Mahboubi et al., 1973). The overall ASR for esophageal cancer among men and women in the more developed areas of the world is 6.5 and 1.2 per 10^5 person-years, respectively; the respective rates in the less developed areas are 11.8 and 5.7 (Iemal et al., 2011). An earlier study did not show any difference by sex in 1-OHPG levels in urine samples in Golestan (Kamangar et al., 2005).

DATA AND BIOLOGICAL SAMPLE COLLECTION

Data and biological sample collection from all participants took place in two rounds: one from December 2006 to January 2007 (hereafter referred to as the winter round) and the other from August to early September 2007 (the summer round). In the winter round, information on demographic characteristics and duration of using a heating system (in hours) during the previous 24 h and the fuel used for heating was collected from all participants. All food and beverage items consumed the previous day were recorded on an open questionnaire. Height and weight of participants were also measured by trained research staff. Questionnaire data on exposure to second-hand smoke and cooking practice were collected in both rounds. We asked questions about exposure to second-hand smoke in general and over the previous 24 h, and in case of any exposure, the numbers of cigarettes of second-hand smoke per day was recorded. We asked study participant whether they did cooking in general and over the previous 24 h, and when the reply was positive, cooking frequency was recorded. We also collected data on cooking methods in the household, including frying, boiling, baking, and barbecuing in general (and not over the previous 24 h), as well as frying intensity, which was assessed by questioning the change in foods’ color caused by frying. For this, we proposed four options: no frying, little change in color, becoming golden, and becoming brown or darkening. For our analyses, the first two categories were considered as no/little frying and the third and fourth categories were combined and considered as high-temperature frying.

In order to obtain repeated urine samples in two seasons, in both winter and summer rounds a single spot urine sample was collected from each participant and stored at −20°C. In the winter round, 10 ml of peripheral venous blood was collected in EDTA containing tubes. Buffy coat containing white blood cells were obtained and stored at −70°C until genotyping. All biological samples were collected in mornings or early afternoons during weekdays. In both seasons, questionnaire data and biological samples were collected from each participant on the same day. The study was reviewed and approved by the Institutional Review Board of the Digestive Disease Research Center of Tehran University of Medical Sciences. Informed written consent was obtained from all participants.

LABORATORY ASSESSMENTS

Details of the laboratory assessments are available in the Section “Appendix.” Briefly, 1-OHPG concentration was measured in 4.5-ml spot urine specimens at the Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, United States, using the assay developed by
Strickland et al. (1994). This was done with synchronous fluorescence spectroscopy (Perkin Elmer LS50B Luminescence spectrometer, Norwalk, CT, USA) using a wavelength difference of 34 nm between excitation and emission. The limit of detection was 0.01 ng 1-OHPG/ml urine and the recovery of the assay was 95–100%. Fourteen control samples were used to assess the reliability of 1-OHPG measurements. With the mean of 5.74 pmol/ml (SD: 0.64), the coefficient of variation was 11.1%.

Genotyping was performed at the Department of Health Risk Analysis and Toxicology, Maastricht University, Maastricht, the Netherlands. Genetic variants including single nucleotide polymorphisms (SNPs) or deletions in the following genes were determined: genes for phase I metabolizing enzymes myeloperoxidase (MPO) and cytochromes P450s (CYP1A1, CYP1A2, CYP1B1, CYP2E1, and CYP3A4) and for phase II metabolizing enzymes glutathione S-transferases (GSTM1, GSTP1, GSTT1), and N-acetyltransferase 2 (NAT2). Details of the primer development are available elsewhere (Knaapen et al., 2004; Ketelslegers et al., 2006). Genotyping was performed by the single base extension (SBE) method using SnaPShot (Applied Biosystems, Nieuwerkerk, a.d. Ijssel, the Netherlands), and primer 3 and Netprimer software were used to design SBE primers (Knaapen et al., 2004; Ketelslegers et al., 2006; Langie et al., 2010). Finally, the samples were analyzed on an ABI Prism 3100 genetic analyser using Genescan Analysis software. Polymerase chain reaction (PCR) primers were designed using Primer 3 and Netprimer software. More detailed information about the studied genetic variants and genotyping is presented in the Section “Appendix.”

STATISTICAL ANALYSIS

Food recall data over the previous 24 h were transformed to specific food group intake values. Intake of food groups was categorized into two groups, as below median and equal to or above median. When medians included zero, the intakes were categorized as no intake and any intake. Intake of red meat (≥ median versus < median) showed an association with 1-OHPG level in preliminary analyses, so it was examined in further analyses as a continuous variable (log-transformed), as well as a categorical variable with three groups. Nearly half of the participants did not consume red meat over the previous 24 h, so they were included in one group and those who ate red meat were categorized in two equal-sized groups according to their intake. For the continuous variable, a constant value of 0.1 was added to red meat intake values before log-transformation.

The normality of the 1-OHPG variable distributions was assessed by histograms and the Shapiro–Wilk W test, and the distributions were found to be severely skewed. With log-transformation, the distribution became normal, so we used log-transformed 1-OHPG values in all further analyses. Creatinine (Cr)-adjusted 1-OHPG levels in winter and summer samples, from participants with paired samples only (n = 106), were compared using paired t-tests. Linear regression models were used to investigate the association between Cr-adjusted 1-OHPG level and variables of interest. Categorical results from genotyping were treated as follows: the wild-type homozygote group was categorized as the reference genotype and coded as 0 (zero), the heterozygote group was coded as 1, and the homozygote variant group was coded as 2. Results from the summer round were adjusted for age (continuous), place of residence (urban; rural), ethnicity (non-Turkmen; Turkmen), body mass index (BMI, kg/m², log-transformed), and frequency of second-hand smoke exposure over the previous 24 h (none; 1–2; ≥3 cigarettes/day). Results from the winter round were additionally adjusted for red meat (none; 1–24; ≥25) and processed meat (no use, some use) consumption over the previous 24 h. For all linear regression models, we report regression coefficients and P-values (two-sided) to illustrate the associations. For categorical variables with more than two categories, P-values for trend were obtained from adjusted regression models by assigning consecutive numbers to categories within each variable.

RESULTS

1-OHPG LEVELS, OVERALL AND IN ASSOCIATION WITH DEMOGRAPHIC FACTORS AND BODY MASS INDEX

Table 1 shows median levels of 1-OHPG and creatinine, and median and 25th and 75th percentiles of Cr-adjusted 1-OHPG in urine samples by demographic characteristics. 1-OHPG levels in urine were obtained for 108 participants in winter and 109 participants in summer. Overall, the median 1-OHPG level in summer was 2.5-fold higher than the median level in winter. On the other hand, the creatinine levels in summer were approximately twice as high as in winter, indicating that urine samples collected in winter were more diluted than the summer urine samples. Therefore, the Cr-adjusted 1-OHPG level did not show a seasonal difference (P = 0.40); levels in both winter and summer samples were approximately 110 μmol/molCr. For each of the categories of demographic variables, Cr-adjusted 1-OHPG levels in summer and winter were not significantly different. Cr-adjusted 1-OHPG levels in the urban area seemed to be the same in summer and winter (0.110 and 0.111 μmol/molCr, respectively; P = 0.68). The median level in rural areas changed from 0.105 μmol/molCr in winter to 0.136 μmol/molCr in summer (P = 0.46). Approximately 68% of participants used natural gas for heating in winter; 32% used kerosene. There was no association between 1-OHPG levels in urine and fuels used for heating or duration of using heating systems over the previous 24 h (data not shown).

There was no major variation in Cr-adjusted 1-OHPG levels by different categories of age, place of residence, ethnicity, and education (Table 2). There was an association between Cr-adjusted 1-OHPG in winter and BMI (P for trend = 0.03), but the association was disappeared after adjustments for other factors. Cr-adjusted 1-OHPG level in summer was not associated with BMI.

1-OHPG LEVELS AND FOOD INTAKE

Food intake data were available only for the winter round of the study. Table 3 shows the relation between Cr-adjusted 1-OHPG levels in urine and intake of selected food groups over the previous 24 h. Intake of red meat and processed meat were associated with 1-OHPG levels. Regression coefficient was 0.208 (P = 0.03) for red meat and 0.218 (P = 0.02) for processed meat consumption; however, only 2.9% of participants consumed processed meat over the previous 24 h. Regression coefficient for intake of fat was −0.191 (P = 0.06). The coefficients for other food groups were smaller than the above values.
Table 1 | Creatinine and 1-OHPG levels in urine in relation to demographic characteristics.

| Demographic characteristics | Winter | Summer | P for seasonal diff. |
|-----------------------------|--------|--------|---------------------|
| No. (%) Median 1-OHPG Cr Median Cr-adjusted 1-OHPG (p25, p75) | No. (%) Median 1-OHPG Cr Median Cr-adjusted 1-OHPG (p25, p75) | seasonal diff. |
| All participants 111 (100) 1.03 121.2 0.107 (0.057, 0.166) 2.54 243.7 0.113 (0.047, 0.242) | 0.40 |
| AGE | | | |
| < Median (48 years) 54 (48.6) 1.19 127.9 0.109 (0.062, 0.151) 2.73 241.1 0.125 (0.049, 0.256) | 0.33 |
| ≥ Median 57 (51.4) 0.90 96.9 0.099 (0.054, 0.205) 2.16 243.7 0.092 (0.046, 0.219) | 0.80 |
| PLACE OF RESIDENCE | | | |
| Urban 44 (39.6) 1.24 128.1 0.110 (0.058, 0.171) 2.16 234.8 0.111 (0.047, 0.228) | 0.68 |
| Rural 67 (60.4) 0.98 97.0 0.105 (0.056, 0.161) 2.70 243.7 0.136 (0.046, 0.254) | 0.46 |
| ETHNICITY | | | |
| Non-Turkmen 24 (21.6) 0.91 158.4 0.080 (0.045, 0.136) 1.63 223.7 0.096 (0.043, 0.179) | 0.67 |
| Turkmen 87 (78.4) 1.1 1102.7 0.118 (0.062, 0.182) 2.79 255.6 0.133 (0.049, 0.243) | 0.47 |
| EDUCATION | | | |
| No school 78 (70.3) 1.03 102.1 0.114 (0.059, 0.195) 2.70 246.8 0.128 (0.047, 0.248) | 0.43 |
| Some school 33 (29.7) 1.11 162.9 0.087 (0.054, 0.149) 2.16 233.6 0.086 (0.050, 0.200) | 0.75 |

1-OHPG levels in urine were obtained from 108 participants in winter and 109 participants in summer. The biomarker levels are presented in following units: 1-OHPG, pmol/ml; creatinine, mg/dl; creatinine-adjusted 1-OHPG, μmol/molCr.

*P*-value for difference in Cr-adjusted 1-OHPG levels in winter and summer (in logarithmic scale), calculated using paired t-tests. The number of participant with paired results was 106.

1-OHPG, 1-hydroxypyrene glucuronide; Cr, creatinine.

Table 2 | Crude and adjusted beta-coefficients and *P*-values for the association of demographic characteristics and body mass index with creatinine-adjusted 1-hydroxypyrene glucuronide levels in urine.

| Characteristics | Winter | Summer |
|-----------------|--------|--------|
| Age, years* | | |
| Crude β | P | Adjusted β 1 | P | Adjusted β 2 | P | Crude β | P | Adjusted β 1 | P |
| −0.040 | 0.68 | −0.048 | 0.63 | −0.112 | 0.26 | −0.055 | 0.57 | −0.062 | 0.52 |
| PLACE OF RESIDENCE | | | |
| Urban Reference | −0.035 | 0.72 | −0.121 | 0.29 | −0.111 | 0.32 | Reference | 0.034 | 0.73 | −0.037 | 0.74 |
| Rural Reference | 0.084 | 0.39 | 0.137 | 0.25 | 0.115 | 0.32 | Reference | 0.077 | 0.43 | 0.098 | 0.38 |
| ETHNICITY | | | |
| Non-Turkmen Reference | −0.021 | 0.83 | −0.029 | 0.82 | 0.032 | 0.79 | Reference | −0.060 | 0.53 | −0.089 | 0.45 |
| Turkmen Reference | 0.213 | 0.03 | 0.132 | 0.20 | 0.130 | 0.19 | Reference | −0.037 | 0.70 | −0.006 | 0.96 |

*Beta-coefficients and *P*-values come from linear regression models, in which Cr-adjusted 1-OHPG level was in logarithmic scale. Adjusted β 1: results were adjusted for other variables listed in the table and frequency of second-hand smoke exposure over the previous 24 h (none; 1–2; ≥3 cigarettes/day) in the respective season. Adjusted β 2: results from winter were additionally adjusted for red meat (none; 1–24; ≥25g) and processed meat (no use, any use) consumption over the previous 24 h.

*Mean (SD).*

1-OHPG Levels and Second-hand Smoking

Exposure to second-hand tobacco smoke from three or more cigarettes over the previous 24 h compared to those who were not exposed to tobacco smoke (Table 4) was associated with increased levels of 1-OHPG; this was stronger in summer (β = 0.254; *P* = 0.008) than in winter (β = 0.156; *P* = 0.12). The coefficient for the correlation between 1-OHPG levels and exposure to second-hand smoke from one or two cigarettes was −0.019 in winter (*P* = 0.86) and −0.152 in summer (*P* = 0.13).
Table 3 | Crude and adjusted beta-coefficients and P-values for the association between intake of selected food groups (over the previous 24 h) and creatinine-adjusted 1-OHPG levels in urine in winter.

| Food group intake | No. (%) | Median (p25, p75) | Crude β | P-value | Adjusted β | P-value |
|--------------------|---------|-------------------|----------|----------|------------|----------|
| RED MEAT           |         |                   |          |          |            |          |
| Categorical        |         |                   |          |          |            |          |
| None               | 51 (48.6) | 0.082 (0.046, 0.150) | Reference | – | Reference |          |
| 1–24 g             | 27 (25.7) | 0.082 (0.058, 0.152) | 0.069 | 0.50 | 0.116 | 0.26 |
| ≥25 g              | 27 (25.7) | 0.148 (0.099, 0.310) | 0.257 | 0.01 | 0.249 | 0.02 |
| P for trend        |         |                   | 0.02 | 0.02 |            |          |
| Continuous         | –       | –                 | 0.206 | 0.04 | 0.208 | 0.03 |
| PROCESSED MEAT     |         |                   |          |          |            |          |
| No intake          | 102 (97.1) | 0.105 (0.058, 0.152) | Reference | – | Reference |          |
| Any intake         | 3 (2.9) | 0.326 (0.311, 0.550) | 0.222 | 0.02 | 0.218 | 0.02 |
| CHICKEN            |         |                   |          |          |            |          |
| No intake          | 62 (59.1) | 0.098 (0.064, 0.161) | Reference | – | Reference |          |
| Any intake         | 43 (40.9) | 0.118 (0.048, 0.205) | 0.009 | 0.92 | 0.103 | 0.31 |
| FISH               |         |                   |          |          |            |          |
| No intake          | 100 (95.2) | 0.110 (0.062, 0.166) | Reference | – | Reference |          |
| Any intake         | 5 (4.8) | 0.073 (0.046, 0.099) | −0.080 | 0.42 | −0.144 | 0.14 |
| MEAT (ANY KIND)b  |         |                   |          |          |            |          |
| 0–12 g (1st quartile) | 28 (26.7) | 0.082 (0.060, 0.152) | Reference | – | Reference |          |
| 13–28 g            | 27 (25.7) | 0.080 (0.039, 0.135) | 0.000 | 1.00 | −0.015 | 0.90 |
| 29–49 g            | 25 (23.8) | 0.135 (0.105, 0.207) | 0.237 | 0.05 | 0.149 | 0.23 |
| ≥50 g              | 25 (23.8) | 0.109 (0.062, 0.188) | 0.104 | 0.21 | −0.019 | 0.88 |
| P for trend        |         |                   | 0.14 | 0.85 |            |          |
| EGG                |         |                   |          |          |            |          |
| No intake          | 72 (68.6) | 0.109 (0.057, 0.174) | Reference | – | Reference |          |
| Any intake         | 33 (31.4) | 0.105 (0.064, 0.151) | 0.000 | 1.00 | −0.016 | 0.87 |
| DAIRY PRODUCTS     |         |                   |          |          |            |          |
| < Median (120 g)   | 52 (49.5) | 0.110 (0.063, 0.151) | Reference | – | Reference |          |
| ≥ Median           | 53 (50.5) | 0.105 (0.051, 0.210) | 0.021 | 0.84 | 0.020 | 0.84 |
| FAT, OIL           |         |                   |          |          |            |          |
| < Median (32 g)    | 52 (49.5) | 0.123 (0.065, 0.206) | Reference | – | Reference |          |
| ≥ Median           | 53 (50.5) | 0.097 (0.046, 0.144) | −0.137 | 0.16 | −0.191 | 0.06 |
| BREAD              |         |                   |          |          |            |          |
| < Median (240 g)   | 50 (47.8) | 0.129 (0.056, 0.210) | Reference | – | Reference |          |
| ≥ Median           | 55 (52.4) | 0.097 (0.064, 0.150) | −0.055 | 0.58 | −0.006 | 0.95 |
| RICE               |         |                   |          |          |            |          |
| < Median (110 g)   | 52 (49.5) | 0.109 (0.067, 0.150) | Reference | – | Reference |          |
| ≥ Median           | 53 (50.5) | 0.099 (0.054, 0.188) | −0.028 | 0.76 | −0.071 | 0.48 |
| PASTA              |         |                   |          |          |            |          |
| No intake          | 90 (85.7) | 0.110 (0.063, 0.154) | Reference | – | Reference |          |
| Any intake         | 15 (14.3) | 0.080 (0.036, 0.210) | −0.064 | 0.52 | −0.065 | 0.50 |
| OTHER CEREALS      |         |                   |          |          |            |          |
| No intake          | 89 (84.8) | 0.109 (0.063, 0.171) | Reference | – | Reference |          |
| Any intake         | 16 (15.2) | 0.095 (0.054, 0.147) | −0.040 | 0.68 | −0.020 | 0.84 |
| CEREALS (ANY KIND) |         |                   |          |          |            |          |
| < Median (396 g)   | 49 (46.7) | 0.105 (0.063, 0.188) | Reference | – | Reference |          |
| ≥ Median           | 56 (53.3) | 0.109 (0.058, 0.152) | −0.053 | 0.59 | −0.036 | 0.72 |
| LEGUMES            |         |                   |          |          |            |          |
| < Median (50 g)    | 60 (57.1) | 0.114 (0.086, 0.165) | Reference | – | Reference |          |
| ≥ Median           | 45 (42.9) | 0.094 (0.044, 0.161) | −0.104 | 0.29 | −0.098 | 0.32 |

(continued)
Table 4 also shows the relation between Cr-adjusted 1-OHPG levels in urine, making bread at home and the intensity of food frying. Making bread at home more than once a week in summer was associated with increased 1-OHPG (β = 0.203; P = 0.04); P for trend was 0.04. Such an association was not observed in winter. With regard to frying intensity, the strongest positive correlations were observed with high-temperature frying of red meat (β = 0.144; P = 0.15) and onion (β = 0.126; P = 0.20) in winter. There was an inverse association between high-temperature frying of vegetables and 1-OHPG levels in summer (β = -0.223; P = 0.03). There was no association between 1-OHPG level in urine and several other variables related to food preparing (data not shown), including cooking over the previous day or regular cooking in general, cooking rice, boiling the food groups presented in Table 4, and fuels used for baking bread or preparing foods. Nearly all of the participants used natural gas for baking or cooking. Whereas high-temperature frying was a common cooking practice (Table 4), barbecuing was uncommon and had no association with 1-OHPG levels (data not shown).

1-OHPG LEVELS AND GENOTYPES

Table 5 shows the distribution of the studied genotypes, P-value for Hardy–Weinberg equilibrium for each gene, and the relation between Cr-adjusted 1-OHPG levels in winter and the genotypes. A few genes were not in Hardy–Weinberg equilibrium; none of them had an association with 1-OHPG levels. The pattern of association were generally different in the winter and summer rounds. However, relatively consistent results were found for the GT + TT (versus GG) genotypes of CYP2E1-05, which showed fairly strong inverse association with 1-OHPG levels in winter (β = -0.176; P = 0.07) and summer (β = -0.184; P = 0.07). In winter, the “null” genotype of GSTT1-02 was associated with increased 1-OHPG levels (β = 0.228; P = 0.02), while there were inverse associations for GG genotype (versus AA + GA genotypes) of CYP1B1-07 (β = -0.256; P = 0.008). In summer, the CT + TT genotype of GSTP1-02 (β = -0.218; P = 0.03) showed an inverse and the “null” genotype of GSTM1-02 (β = 0.198; P = 0.04) showed a positive association with 1-OHPG levels.

DISCUSSION

This study showed similar levels of a urinary biomarker of PAH exposure in summer and winter. Red and processed meat intake and GSTT1-02 polymorphism showed correlations with 1-OHPG levels in winter, while CYP1B1-07 polymorphism had an inverse correlation. In summer, making bread at home, second-hand smoke, and GSTM1-02 polymorphism were correlated with 1-OHPG levels, but GSTP1-02 polymorphism showed an inverse association; food intake data were not available for this season.

Participants in the current study, who were all female non-smokers, had higher 1-OHPG levels compared to non-smokers without occupational exposure to PAHs in several other studies that used the same methodology as this study for assessing 1-OHPG; in fact, the levels in this study were comparable to those of smokers in the other studies (Table 6). The higher SD for Cr-adjusted 1-OHPG in the current study suggests that inter-individual variability is larger in our study participants than in other populations. Almost all of the participants in the current study used natural gas or kerosene for heating in winter. The similarity of the Cr-adjusted 1-OHPG levels in summer and winter implies that exposure to PAHs through heating in winter does not have a major effect on total exposure in Golestan. As we used...
Table 4 | Crude and adjusted Beta-coefficients and P-values for the association of second-hand smoke exposure (over the previous 24 h), frying intensity, and several genetic factors with creatinine-adjusted 1-hydroxypyrene glucuronide levels in urine*.

| Characteristics                  | Winter                        | Summer                       |
|----------------------------------|-------------------------------|------------------------------|
|                                  | No. (%)                       | Crude β                      | Adjusted β 1 | Adjusted β 2 | No. (%)                       | Crude β                      | Adjusted β 1 | Adjusted β 2 |
| **SECOND-HAND SMOKE**            |                               |                              |              |              |                               |                              |              |              |
| None                             | 80 (72.1)                     | Reference –                  | Reference –  | Reference – | 84 (75.7)                     | Reference –                  | Reference – | Reference –  |
| 1–2 cigarettes/day               | 18 (16.2)                     | −0.034                       | 0.73         | −0.019      | 0.86 −0.029                   | 0.78                        |
| ≥ 3 cigarettes/day              | 13 (11.7)                     | 0.115                        | 0.13         | 0.132       | 0.19 0.156                   | 0.12                        |
| *P for trend*                    | 0.24                          | 0.28                         | 0.21         |              | 0.182 0.06                     | 0.178 0.07                  |
| **MAKING BREAD AT HOME**         |                               |                              |              |              |                               |                              |              |              |
| No                               | 63 (59.4)                     | Reference –                  | Reference –  | Reference – | 79 (72.5)                     | Reference –                  | Reference – | Reference –  |
| Yes                              | 43 (40.6)                     | 0.121                        | 0.22         | 0.084       | 0.41 0.053                   | 0.59                        |
| < Weekly                         | 19 (17.9)                     | 0.107                        | 0.29         | 0.024       | 0.82 0.063                   | 0.55                        |
| Once/week                        | 11 (10.4)                     | 0.014                        | 0.89         | −0.004      | 0.97 −0.054                  | 0.59                        |
| > Once/week                      | 13 (12.3)                     | 0.121                        | 0.23         | 0.125       | 0.59 0.080                   | 0.44                        |
| *P for trend*                    | 0.25                          | 0.21                         | 0.58         |              | 9 (8.3) 0.203                 | 0.04 0.203                  |
| **FRYING INTENSITY**             |                               |                              |              |              |                               |                              |              |              |
| Red meat                         |                               |                              |              |              |                               |                              |              |              |
| No/little frying                 | 33 (31.1)                     | Reference –                  | Reference –  | Reference – | 46 (42.2)                     | 0.108 0.27                   | 0.087 0.39   |
| High-temperature frying          | 73 (68.8)                     | 0.149                        | 0.13         | 0.187       | 0.07 0.144                   | 0.15                        |
| Chicken                          | 19 (17.9)                     | Reference –                  | Reference –  | Reference – | 21 (19.3)                     | Reference –                  | Reference – | Reference –  |
| No/little frying                 | 87 (82.1)                     | −0.095                       | 0.33         | −0.107      | 0.30 −0.081                   | 0.42                        |
| High-temperature frying          | 15 (14.1)                     | Reference –                  | Reference –  | Reference – | 21 (19.3)                     | Reference –                  | Reference – | Reference –  |
| Onion                            | 91 (85.9)                     | 0.101                        | 0.30         | 0.126       | 0.21 0.126                   | 0.20                        |
| High-temperature frying          | 76 (71.7)                     | Reference –                  | Reference –  | Reference – | 61 (56.0)                     | Reference –                  | Reference – | Reference –  |
| Vegetables                       | 30 (28.3)                     | −0.018                       | 0.86         | 0.023       | 0.81 0.023                    | 0.81                        |
| Fish                             | 22 (20.7)                     | Reference –                  | Reference –  | Reference – | 55 (50.5)                     | Reference –                  | Reference – | Reference –  |
| High-temperature frying          | 84 (79.3)                     | 0.152                        | 0.12         | 0.006       | 0.95 0.097                   | 0.33                        |
| Potato                           | 31 (29.2)                     | Reference –                  | Reference –  | Reference – | 57 (52.4)                     | Reference –                  | Reference – | Reference –  |
| No/little frying                 | 75 (70.8)                     | −0.024                       | 0.81         | −0.041      | 0.69 −0.055                   | 0.57                        |
| High-temperature frying          | 14 (13.2)                     | Reference –                  | Reference –  | Reference – | 17 (15.9)                     | Reference –                  | Reference – | Reference –  |
| Eggplant                         | 92 (86.8)                     | −0.027                       | 0.78         | −0.066      | 0.52 −0.051                   | 0.61                        |

*Beta-coefficients and P-values come from linear regression models, in which creatinine (Cr)-adjusted 1-OHPG level in logarithmic scale was the dependent variable. Adjusted β 1: results were adjusted for age (continuous), place of residence (urban, rural), ethnicity (non-Turkmen, Turkmen), body mass index (kg/m², logarithmic scale), and frequency of exposure to second-hand smoke over the previous 24 h (none; 1–2; ≥3 cigarettes/day) in the respective season. Adjusted β 2: results from winter were additionally adjusted for red meat (none; 1–24; ≥25 g) and processed meat (no use, any use) consumption over the previous 24 h.

Paired t-tests, any substantial overall intra-individual variability in the two seasons is unlikely. In concordance with this finding, the fuels used for heating and the duration of their use did not show any association with 1-OHPG levels.

Diet seems to be the main source of PAHs among non-smokers who do not have occupational exposure to those compounds in studies from other regions of the world (Straif et al., 2005). However, information on the role of diet in exposure to PAHs in Golestan is limited. Only two earlier studies in Golestan have investigated such association. These studies assessed the PAH levels in commonly eaten foods and in bread and rice (the two major staple foods in the region), but they did not find high levels on average (Joint Iran-IARC Study Group, 1977; Hakami et al., 2008). In one of those studies, however, when the daily intake of benzo[a]pyrene was estimated from its levels in cooked rice, bread, and drinking water, the daily intake of the compound in Golestan was higher than in a low-risk area in Iran (Hakami et al., 2008). In the current study, participants were questioned about the frying intensity of the consumed food in general rather than over the previous 24 h. 1-OHPG levels in urine reflect recent exposure (within hours) to...
Table 5 | P-values for Hardy–Weinberg equilibrium and crude and adjusted Beta-coefficients and P-values for the association between polymorphisms in several phase I and phase II metabolizing enzyme genes and creatinine-adjusted 1-hydroxypyrene glucuronide levels in urine.

| Polymorphism | Genotype | No. (%) | P | Winter, beta-coefficients | Summer, beta-coefficients |
|--------------|----------|---------|---|---------------------------|---------------------------|
|              |          |         |   | Crude β | P-value | Adjusted β | 1 | P-value | Adjusted β 1 | P-value |           | Crude β | P-value | Adjusted β 1 | P-value |
|              |          |         |   |          |          |            |   |          |            |          |           |          |          |            |          |
| PHASE I ENZYMES |          |         |   |          |          |            |   |          |            |          |           |          |          |            |          |
| CYP1A1-01    | AA       | 90 (84.1) | 0.37 | Reference | – | Reference | – | Reference | – | Reference | – | –0.039 | 0.7 | –0.044 | 0.65 |          |
|              | rs1048943| GA      | 17 (15.9) | 0.07 | 0.141 | 0.16 | 0.06 | 0.55 | Reference | – | Reference | – | 0.079 | 0.43 | 0.091 | 0.35 |          |
| CYP1A1-03    | CC       | 97 (91.5) | 0.65 | Reference | – | Reference | – | Reference | – | Reference | – | –0.062 | 0.63 | –0.001 | 0.99 |          |
|              | rs1799814| CA      | 9 (8.5) | –0.16 | 0.11 | –0.176 | 0.08 | –0.177 | 0.07 | Reference | – | Reference | – | 0.078 | 0.54 | 0.121 | 0.33 |          |
| CYP1A2-03    | CC       | 24 (22.2) | <0.001 | Reference | – | Reference | – | Reference | – | Reference | – | 0.074 | 0.46 | 0.11 | 0.28 |          |
|              | rs762551 | CA      | 41 (38.0) | 0.082 | 0.52 | 0.074 | 0.56 | 0.019 | 0.88 | Reference | – | Reference | – | –0.168 | 0.1 | –0.129 | 0.2 |          |
|              | GA       | 43 (39.8) | 0.084 | 0.51 | 0.111 | 0.38 | 0.065 | 0.6 | Reference | – | Reference | – | 0.074 | 0.46 | 0.11 | 0.28 |          |
| CYP1B1-05    | CC       | 58 (54.2) | 0.01 | Reference | – | Reference | – | Reference | – | Reference | – | –0.143 | 0.16 | 0.132 | 0.2 |          |
|              | rs1056836| GC      | 41 (38.0) | –0.005 | 0.96 | 0.025 | 0.96 | 0.025 | 0.81 | Reference | – | Reference | – | 0.01 | 0.92 | 0.034 | 0.74 |          |
| CYP1B1-07    | AA       | 70 (66.0) | 0.07 | Reference | – | Reference | – | Reference | – | Reference | – | –0.011 | 0.91 | 0.014 | 0.89 |          |
|              | rs1800440| GA      | 31 (29.3) | –0.129 | 0.18 | –0.102 | 0.31 | –0.04 | 0.68 | Reference | – | Reference | – | 0.02 | 0.14 | 0.031 | 0.75 |          |
|              | GG       | 5 (4.7) | –0.298 | 0.002 | –0.265 | 0.01 | –0.261 | 0.008 | Reference | – | Reference | – | –0.246 | 0.01 | –0.203 | 0.05 |          |
| CYP2E1-05    | AA       | 102 (95.3) | 0.8 | Reference | – | Reference | – | Reference | – | Reference | – | –0.011 | 0.91 | 0.031 | 0.75 |          |
|              | GA       | 5 (4.7) | 0.15 | 0.13 | 0.138 | 0.18 | 0.166 | 0.09 | Reference | – | Reference | – | 0.131 | 0.18 | 0.154 | 0.12 |          |
|              | GG       | 77 (72.0) | 0.18 | Reference | – | Reference | – | Reference | – | Reference | – | –0.072 | 0.47 | –0.098 | 0.33 |          |
| MPO-02       | GA       | 24 (22.4) | 0.006 | 0.95 | 0.015 | 0.88 | 0.073 | 0.47 | Reference | – | Reference | – | –0.015 | 0.88 | –0.005 | 0.96 |          |
|              | AA       | 6 (5.6) | 0.007 | 0.95 | 0.01 | 0.93 | 0.033 | 0.74 | Reference | – | Reference | – | –0.015 | 0.88 | –0.005 | 0.96 |          |

(Continued)
### PHASE II ENZYMES

| Polymorphismb | Genotype | No. [%] | Winter, beta-coefficients | Summer, beta-coefficients | P-value | Adjusted β | 1 | P-value | Adjusted β | 2 | P-value | Adjusted β |
|---------------|----------|---------|--------------------------|---------------------------|---------|------------|---|---------|------------|---|---------|------------|
| **GSTP1-01** |          |         |                          |                            |         |            |   |         |            |   |         |            |
| AA            | 64 (69.3) | 0.02    | Reference                |                            |         |            |   | Reference|                            |   |         |            |
| GA            | 37 (34.2) | 0.092   | Reference                |                            | 0.069   | 0.23       |   | Reference|                            |   |         |            |
| GT            | 1 (0.9)   | 0.052   | Reference                |                            | 0.07    | 0.21       |   | Reference|                            |   |         |            |
| **GSTP1-02** |          |         |                          |                            |         |            |   |         |            |   |         |            |
| CC            | 89 (82.4) | 0.34    | Reference                |                            | 0.003   | 0.23       |   | Reference|                            |   |         |            |
| CT            | 18 (16.7) | 0.19    | Reference                |                            | 0.034   | 0.27       |   | Reference|                            |   |         |            |
| TT            | 1 (0.9)   | 0.057   | Reference                |                            | 0.052   | 0.25       |   | Reference|                            |   |         |            |
| **GSTM1-02** |          |         |                          |                            |         |            |   |         |            |   |         |            |
| +             | 56 (51.9) | –       | Reference                |                            | 0.148   | 0.23       |   | Reference|                            |   |         |            |
| –             | 52 (48.1) | –       | Reference                |                            | 0.119   | 0.13       |   | Reference|                            |   |         |            |
| **GSTT1-02** |          |         |                          |                            |         |            |   |         |            |   |         |            |
| +             | 19 (17.6) | –       | Reference                |                            | 0.268   | 0.2        |   | Reference|                            |   |         |            |
| –             | 69 (82.4) | –       | Reference                |                            | 0.107   | 0.25       |   | Reference|                            |   |         |            |
| **NAT2-02**  |          |         |                          |                            |         |            |   |         |            |   |         |            |
| AA            | 1 (0.9)   | 0.042   | Reference                |                            | 0.045   | 0.72       |   | Reference|                            |   |         |            |
| GA            | 18 (16.7) | 0.107   | Reference                |                            | 0.195   | 0.22       |   | Reference|                            |   |         |            |
| TT            | 1 (0.9)   | 0.057   | Reference                |                            | 0.116   | 0.24       |   | Reference|                            |   |         |            |
| **NAT2-06**  |          |         |                          |                            |         |            |   |         |            |   |         |            |
| AA            | 50 (46.3) | 0.094   | Reference                |                            | 0.003   | 0.15       |   | Reference|                            |   |         |            |
| GA            | 50 (46.3) | 0.097   | Reference                |                            | 0.001   | 0.12       |   | Reference|                            |   |         |            |
| **NAT2-08**  |          |         |                          |                            |         |            |   |         |            |   |         |            |
| AA            | 8 (7.4)   | 0.097   | Reference                |                            | 0.002   | 0.16       |   | Reference|                            |   |         |            |

*Beta-coefficients and P-values come from linear regression models, in which creatinine-adjusted 1-OHPG level in logarithmic scale was the dependent variable. Adjusted β 1: results were adjusted for age (continuous), place of residence (urban; rural), ethnicity (non-Turkmen; Turkmen), body mass index (kg/m², logarithmic scale), and frequency of exposure to second-hand smoke over the previous 24 h (none; 1–2; ≥3 cigarettes/day) in the respective season. Adjusted β 2: results from winter were additionally adjusted for red meat (none; 1–24; ≥25 g) and processed meat (no use, any use) consumption over the previous 24 h. For the full names of genes, see Section “Materials and Methods.”*
Table 6 | Examples of 1-OHPG levels assessed with the same methodology in different studiesa.

| Study; country | Smoking status | No. | Mean (SD) | Study participants |
|----------------|----------------|-----|-----------|--------------------|
| CRUDE 1-OHPG   |                |     |           |                    |
| Current study; Golestan Province, Iran | Never-smoker (winter) | 108 | 1.87 (2.70) | Healthy women |
|                 | Never-smoker (summer) | 109 | 3.70 (3.98) | |
| Kamangar et al. (2005); Golestan Province, Iran | Neversmoker | 49 | 3.7 (NA)b | Healthy individuals |
|                 | Former-smoker | 20 | 2.7 (NA)b | |
|                 | Current-smoker | 30 | 6.9 (NA)b | |
| Fagundes et al. (2006); Brazil | Non-smoker | 103 | 1.14 (NA)b | Healthy individuals |
|                 | Smoker | 96 | 3.76 (NA)b | |
| Vineis et al. (1996); Italy | Non-smoker | 50 | 0.55 (0.05) | Healthy men |
|                 | Smoker | 49 | ~1.05 (0.20)c | |
| Kang et al. (1995); USA | Non-smoker | 10 | 0.23 (0.11) | Healthy men, after 2 weeks free of charbroiled or smoked foods |
|                 | Smoker | 10 | 6.5 (1.5) | Healthy men, the day after eating 225 g charbroiled meat |
| Gunier et al. (2008); USA | Did not smoke in last 24 h | 299 | 0.16 (NA)b | Healthy female school teachers or administrators |
|                 | Smoked in last 24 h | 5 | 1.61 (NA)b | |
|                 | Did not smoke but ate grilled meat in last 24 h | 134 | 0.25 (NA)b | |
|                 | Neither smoked nor ate grilled meat in last 24 h | 165 | 0.06 (NA)b | |
| CR-ADJUSTED 1-OHPG |                |     |           |                    |
| Current study; Golestan Province, Iran | Never-smoker (winter) | 108 | 0.15 (0.17) | Healthy women |
|                 | Never-smoker (summer) | 109 | 0.21 (0.31) | |
| Lai et al. (2005); Taiwan | Non-smoker | 16 | 0.07 (0.04) | Healthy office worker women |
|                 | Smoker | 8 | 0.18 (0.07) | |
|                 | Non-smoker | 42 | 0.16 (0.10) | Healthy female highway toll station workers |
| Lee et al. (2003); South Korea | Non-smoker | 53 | 1.14 (NR) | Healthy painters in a shipyard |
|                 | Smoker | 122 | 1.96 (NR) | |
| Lee et al. (2002); South Korea | Non-smoker | 8 | 0.12 (0.03) | Healthy administrative employees in an industrial waste incinerating site |
|                 | Smoker | 10 | 0.21 (0.07) | |
|                 | Non-smoker | 5 | 0.09 (0.04) | Healthy waste incineration workers |
|                 | Smoker | 20 | 0.28 (0.09) | |

aCrude 1-OHPG and Cr-adjusted 1-OHPG levels are presented in pmol/ml urine and mol/mol Cr, respectively.
bMedians were presented.
cSmokers were divided to two groups; 1-OHPG level was 1.0 (SD 0.15) in flue-cured tobacco users (n=31) and 1.1 (SD 0.23) in air-cured tobacco smokers (n=16).

PAHs, so the level may not be an optimal indicator of exposure to PAHs from fried food in general rather than in the previous few hours. This fact and also the association between red meat intake and 1-OHPG level suggests that fried red meat may be an important source of PAH among those who use this food in Golestan, although the association for fried red meat did not reach statistical significance in our study. Frying has been shown to increase the PAH content of foods; for example, in a study in Spain, the total PAH content of raw lamb increased from 5.5 μg/kg fresh weight to 16.9 μg/kg after frying (Perello et al., 2008). In our study, the association between BMI and 1-OHPG level in winter may be related to food intake, because the association disappeared following adjustments for other factors, including red meat intake. Making bread at home in summer showed an association with 1-OHPG levels. On the other hand, there was no association between 1-OHPG and making bread at home in winter. The fuel used for making bread in both seasons was natural gas, so it is not clear why such a practice was associated with increased exposure to PAHs in only one round of our study. Some possible explanations may be that the association is due to chance or that making bread at home is a proxy for another habit or practice which is associated with higher exposure to PAHs in summer than in winter. Only a limited number of studies have investigated the association between second-hand smoke and PAHs or their metabolites in urine (Scherer et al., 1992; Suwan-Ampai et al., 2009). A recent large study reported that elevated urinary concentrations of most...
PAH metabolites are associated with exposure to second-hand smoke (Suwan-Ampai et al., 2009). Our findings also suggest a similar association in both seasons, although the association in winter was weaker than in summer. Our small sample size may be one of the reasons for not observing a statistically significant association in winter. There was no increase in 1-OHPG levels with exposure to tobacco smoke from one or two cigarettes; this amount of exposure might not be sufficient to increase 1-OHPG levels in urine.

Phase I enzymes in the metabolic pathways of PAHs, including CYPs and MPO, usually catalyze PAHs to more reactive metabolites. Phase II enzymes, such as GSTs, catalyze the conjugation of PAHs or their reactive metabolites to compounds that are more water-soluble, so that they are more readily excreted (Chen et al., 2007). Several studies have investigated the association between polymorphisms in genes encoding the above enzymes and levels of PAH metabolites in urine, but the results have not been very consistent (Alexandrie et al., 2000; Schoket et al., 2001; Apostoli et al., 2003; Abnet et al., 2007; Chen et al., 2007; Chuang and Chang, 2007; Petchpoung et al., 2011). In our study, polymorphisms in some CYP genes were associated with lower levels of 1-OHPG, while deletions in GSTM1 and GSTT1 were associated with elevated levels. However, except for CYP2E1-05, the other polymorphisms did not show a consistent pattern in winter and summer. The significance and repeatability of these findings need to be examined in further studies.

While the Caspian Littoral region in general is a humid area, the areas with highest incidence of ESCC in Golestan have a relatively dry climate (Mahboubi, 1971). The urine samples collected in winter were more diluted than summer urine, which may be related to greater dehydration of inhabitants in Golestan in summer than in winter. From a methodological point of view, this suggests the importance of adjustment for creatinine levels when a single spot urine sample is analyzed for PAH metabolites. Different patterns of association in the winter and summer rounds in our study, e.g., with regard to BMI, suggest exposure to different sources of PAH in different seasons in Golestan. These potential variations should also be considered in future investigations.

Our study has strengths and limitations. We collected extensive questionnaire data, which allowed us to examine the associations of interest while considering the influence of several other lifestyle-related factors. We collected samples in two seasons to reduce the effect of inter-individual and seasonal variations. However, not having 24-h food intake data in the second round (summer) did not allow us to compare the pattern of exposure to PAHs in the two seasons with regard to diet and to adjust the analyses in the summer for diet. Furthermore, multiple comparisons were done in our analyses. Therefore, some of the statistically significant results might have arisen by chance. However, at least regarding exposure to red meat intake, the results seem to be robust.

In summary, the study confirms high exposure to PAHs of the general population in Golestan, which does not seem to be related to heating in winter, and for which certain foods may be important factors among individuals who consume those foods. With regard to esophageal cancer, it is possible that drinking hot tea, a habit common in Golestan, exposes esophageal cells to higher amounts of ingested PAHs than in individuals who drink their tea at moderate temperatures (Islami et al., 2009a,c). Exposure to second-hand tobacco smoke also showed an association with PAHs, but the prevalence of the exposure was low. Although high-temperature frying is a common cooking practice in Golestan, approximately 50% of participants in our study did not consume red meat in the previous 24 h. We were not able to identify the factors explaining the high levels of PAH in all participants, suggesting that there is no single factor responsible for this pattern in our study population and, very likely, in Golestan. The potential differences in pattern of exposure in summer and winter also points to variability of major sources of PAHs. Results of this pilot study may be helpful in determining the issues to be focused on in future studies, e.g., PAH content in fried foods and potential seasonal variation in sources of PAHs. Further studies on biomarkers of internal exposure (e.g., PAH-related DNA adducts) and on the potential association of exposure to PAHs and risk of esophageal cancer are also warranted. Due to possible associations of exposure to ingested PAHs with several other health outcomes, including cancer of the urinary bladder and cardiovascular diseases (Curfs et al., 2005; Ramos and Moorthy, 2005; Baan et al., 2009), the benefits of identifying and avoiding the preventable exposures to PAHs in the general population may go beyond esophageal cancer.

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APPENDIX

DETAILS OF LABORATORY ASSESSMENTS

Assessment of 1-hydroxypyrene glucuronide (1-OHPG)

In order to hydrolyze acid-labile conjugated metabolites, each urine sample was treated with 1 N HCl at 90°C for 1 h. Under these hydrolysis conditions, 1-OHPG is stable. Then, 1-OHPG was loaded on methanol/water primed Sep-Pak C18 cartridges (Waters, Milford, MA, USA). After being washed with water and 30% methanol in water, the samples were eluted by the same volume of 80% methanol in water. With mild heating under vacuum, the eluate was concentrated to approximately one-eighth of the initial volume. Adding 7.5 mM phosphate-buffered saline (PBS), with pH 7.4, the volume was increased to 4 ml. The samples were loaded on immunoaffinity columns containing CNBr-activated Sepharose 4B conjugated with monoclonal antibody 8E11 which recognizes 1-OHPG. Then the samples were washed with 7.5 mM PBS and 25% methanol in 7.5 mM PBS, and 1-OHPG was eluted with 70% methanol in 7.5 mM PBS. The samples were dried and re-dissolved in 2 ml of water. The 1-OHPG content of the samples was measured with synchronous fluorescence spectroscopy (Perkin Elmer LS50B Luminescence spectrometer, Norwalk, CT, USA) using a wavelength difference of 34 nm between excitation and emission. The limit of detection was 0.01 ng 1-OHPG/ml urine and the recovery of the assay was 95–100%. The adjustment of 1-OHPG levels for creatinine was done as following:

\[
\text{Creatinine (mg/dl)} \times 0.0884 = \text{creatinine (μmol/ml)}
\]

\[
\text{Cr - adjusted 1-OHPG (mol/molCr)} = 1 - \text{OHPG (pmol/ml)/creatinine (μmol/ml)}
\]

Genotyping

DNA was isolated by standard phenol extraction procedures and PCR was done as reported previously (Knaapen et al., 2004) in two multiplex PCR reactions. The multiplexes included the following polymorphisms; series A: CYP1A2-03 (rs762551), NAT2-02 (rs1799931), NAT2-06 (rs1801280), NAT2-08 (rs1799930), GSTM1-02 (deletion), GSTP1-01 (rs1695), GSTP1-02 (rs1138272), and GSTT1-02 (deletion); series B: CYP1A1-01 (rs1048943), CYP1A1-03 (rs1799814), CYP1B1-05 (rs1056836), CYP1B1-07 (rs1800440), CYP2E1-05 (rs6413420), CYP3A4-02 (rs2740574), and MPO-02 (rs2333227). PCR was carried out in a Tgradient 96-well Thermal cycler (Biometra, Goettingen, Germany) in a 10-μl volume, containing PCR buffer (Invitrogen, Breda, the Netherlands), 0.2 mmol/l deoxynucleotide triphosphates (Invitrogen), 1.75 mmol/l MgCl2 (Invitrogen), 0.25 unit Platinum Taq-Polymerase (Invitrogen), and 40 ng template DNA. The final concentration of the primers was 0.2 μmol/l. PCR conditions were: denaturation at 94°C for 3 min; 30 cycles of 94°C for 30 s, 56°C (series A)/60°C (series B) for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. The samples were incubated at 37°C (45 min) with 4 μl Exo-SAP-IT (Amersham, Roosendaal, the Netherlands) to degrade deoxynucleotide triphosphates and PCR primers. The enzymes were deactivated at 75°C (15 min).

Genotyping was performed by single base extension (SBE) method using SnaPshot (Applied Biosystems, Nieuwerkerk, a.d. IJssel, the Netherlands), and primer 3 and Netprimer software were used to design SBE primers. Following SBE, the samples were incubated at 37°C (1 h) with 1 unit shrimp alkaline phosphatase (Amersham) to degrade the unincorporated deoxynucleotide triphosphates. Two multiplex genotyping experiments (as described above) were used for SBE reactions. SBE products were diluted and mixed with deionized formamide containing Genescan 120 LIZ size standard. The mixture was denatured at 95°C for 5 min. Finally, the samples were analyzed on an ABI Prism 3100 genetic analyser using Genescan Analysis software.