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Evaluation of urinary 1-hydroxypyrene, *S*-phenylmercapturic acid, *trans*,*trans*-muconic acid, 3-methyladenine, 3-ethyladenine, 8-hydroxy-2′-deoxyguanosine and thioethers as biomarkers of exposure to cigarette smoke

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Abstract

The objective was to evaluate the utility of urinary 1-hydroxypyrene (1-OHP), *S*-phenylmercapturic acid (*S*-PMA), *trans*,*trans*-muconic acid (*t*,*t*-MA), 3-methyladenine (3-MeAd), 3-ethyladenine (3-EtAd), 8-hydroxy-2′-deoxyguanosine (8-OHdG) and thioethers as biomarkers for assessing the exposure in adult smokers who switched from smoking conventional cigarettes to candidate potential reduced exposure products (PREP) or who stopped smoking. Two electrically heated smoking systems (EHCSS) were used as prototype cigarettes that have significant reductions in a number of mainstream smoke constituents as measured by smoking machines relative to those from conventional cigarettes. Urine samples were collected from a randomized, controlled, forced-switching study in which 110 adult smokers of a conventional cigarette brand (CC1) were randomly assigned to five study groups. The groups included the CC1 smoking group, a lower-tar conventional cigarette (CC2) smoking group, EHCSS1 group, EHCSS2 group and a no smoking group that were monitored for 8 days. Biomarkers were measured at baseline and day 8. The daily excretion levels of these biomarkers were compared among the groups before and after switching, and the relationships between the daily excretion levels of these biomarkers and cigarette smoking-related exposure were investigated using Pearson product-moment correlation and multiple regression analyses. It was concluded that under controlled study conditions: (1) 1-OHP, *S*-PMA and *t*,*t*-MA are useful biomarkers that could differentiate exposure between smoking conventional and EHCSS cigarettes or between smoking conventional cigarettes and no smoking; between *S*-PMA and *t*,*t*-MA, the former appeared to be more sensitive; (2) 3-MeAd could only differentiate between smoking conventional cigarettes and no smoking; the results for 3-EtAd were not conclusive because contradictory results were observed; (3) 8-OHdG had a questionable association with smoking and therefore the utility of this biomarker for smoking-related exposure could not be established; and (4) urinary excretion of thioethers as a biomarker lacked sensitivity to demonstrate a clear dose–response relationship in conventional cigarette smokers, although it could differentiate the excretion levels between those subjects who smoked a conventional cigarette and those who stopped smoking.

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Introduction

Cigarette smoke is a complex mixture containing more than 4000 chemical constituents (Dube & Green 1982). Evaluation of exposure to cigarette smoke in humans based on measurement of levels of biomarkers has been reported in several studies (Benowitz 1983, Benowitz et al. 1994, Byrd et al. 1998, Hecht 1999, 2002, 2003, Phillips 2002). Recently, the US Institute of Medicine proposed that an assessment of a potential reduced exposure products (PREP) cigarette should include quantification in smokers of biomarkers of those constituents thought to be present at lower levels in smoke (Stratton et al. 2001). However, any biomarker used to estimate exposure should be evaluated for its sensitivity and selectivity.

In a previous study (Roethig et al. 2005), the present authors evaluated smokers' exposure from four different cigarettes by use of the following biomarkers: urinary nicotine and five of its metabolites, mutagenic substances excreted in urine, carbon monoxide (CO) in exhalate and carboxyhaemoglobin (COHb). Differences in the levels of these biomarkers were observed in smokers smoking cigarettes of different designs (conventional cigarettes and the first-generation electrically heated cigarette smoking systems (EHCSS); Patskan & Reininghaus 2003) and in smokers who had been assigned to a no smoking group.

The present study evaluated the utility of several other urinary biomarkers including 1-hydroxypyrene (1-OHP), *S*-phenylmercapturic acid (*S*-PMA), *trans*,*trans*-muconic acid (*t*,*t*-MA), 3-methyladenine (3-MeAd), 3-ethyladenine (3-EtAd), 8-hydroxy-2′-deoxyguanosine (8-OHdG) and thioethers. Two EHCSS cigarettes were used as prototype cigarettes whose mainstream deliveries of a number of smoke constituents were significantly lower compared with conventional cigarettes. This allowed us to examine whether the excretion levels of these biomarkers responded to the change of external exposure after switching from conventional cigarettes. The changes of biomarker excretion levels in the no smoking group were also investigated. Several other aspects of these biomarkers were also examined (World Health Organization 2000): the reliability of the analytical methods and stability of these biomarkers, the dose–response relationships, the biological variability, and potential confounding factors. Demonstration of dose–response was evaluated using the number of cigarettes smoked per day as a dose estimate. Additionally, toxicokinetics of these biomarkers were also taken into consideration; available information regarding elimination half-lives of the various biomarkers was used in designing the experiments. The reasons for selecting these biomarkers for evaluation are described below.

1-OHP

The measurement of 1-OHP as a surrogate biomarker for overall PAH exposure has been reported in numerous environmental and occupational studies (Jongeneelen et al. 1985, Strickland et al. 1996, Jongeneelen 1997, Dor et al. 1999, Castano-Vinyals et al. 2004). There are also many reports on the relationships between cigarette smoking-related exposure or dietary intake of PAHs and the 1-OHP excretion levels (Van Rooij et al. 1994, Scherer et al. 2000, Heudorf & Angerer 2001, Viau et al.

2002). It has been shown that the 1-OHP excretion levels in smokers are about twice as high as those in non-smokers, although the difference could be as high as 50-fold (Hecht 2002, IARC 2004).

S-*PMA and* t,t-*MA*

Both *S*-PMA and *t*,*t*-MA are minor metabolites of benzene (Melikian et al. 2002, Qu et al. 2003) and have been validated as suitable biomarkers for monitoring benzene exposure in occupational and environmental exposure as well as in tobacco smoke exposure studies (Qu et al. 2000, 2003, Hecht 2002, Melikian et al. 2002, Cocco et al. 2003, IARC 2004). Unmetabolized benzene in urine has also been used as a biomarker for environmental and occupational exposure to benzene (Ong & Lee 1994). *S*-PMA is formed by degradation of the glutathione conjugate of benzene epoxide and *t*,*t*-MA is formed via benzene ring oxidation and ring cleavage to *trans*,*trans*-muconaldehyde, which is further oxidized to *t*,*t*-MA. *S*-PMA is believed to be a more specific biomarker than *t*,*t*-MA because there is a dietary confounding factor for *t*,*t*-MA. Sorbic acid, a common food preservative (Boogaard & Van Sittert 1996, Scherer et al. 1998, Pezzagno et al. 1999, Weaver et al. 2000), is also metabolized to *t*,*t*-MA. Significantly higher *t*,*t*-MA levels were found in smokers (range 0.06–0.30 mg g^{-1} creatinine) than in non-smokers (0.04–0.09 mg g^{-1} creatinine). Boogaard and Van Sittert (1996) reported significantly higher levels of *S*-PMA in smokers (1.71 μmol mol^{-1} creatinine) than in non-smokers (0.94 μmol mol^{-1} creatinine).

3-MeAd and 3-EtAd

Alkylating agents can react with DNA and the alkylated bases are readily removed from the nucleotides by DNA repair mechanisms and then excreted in urine (Shuker & Farmer 1992). Higher levels of 3-MeAd and 3-EtAd were found in the urine of smokers than in non-smokers as reported in several controlled studies and a population-based study (Hecht 1999, IARC 2004). Although several smoke constituents have methylating potential (e.g. *N*-nitrosodimethylamine, *N*-nitrosomethylethylamine, NNK and methyl halides), it is unclear which smoke constituents are responsible for the increased excretion of 3-MeAd in smokers. It is also unknown which smoke constituent causes increased excretion of 3-EtAd in smokers (Prevost & Shuker 1996).

8-OHdG

8-OHdG is one of the most predominant free radical-induced products of oxidative DNA damage with miscoding potential (Cheng et al. 1992). DNA repair results in the excretion of 8-OHdG in urine, and 8-OHdG can be measured as a non-invasive biomarker to reflect whole-body oxidative damage (Kasai 1997). Cigarette smoking has been shown to elevate the urinary 8-OHdG excretion by 16–50%, although negative results have also been reported (Nia et al. 2001). It was reported that 4 weeks of smoking cessation resulted in a decrease in 8-OHdG excretion by 21% (Prieme et al. 1998). However, Pilger et al. (2001) reported that in a longitudinal study the intra-individual variability (17–106%) in urinary 8-OHdG was greater than the increase in

excretion levels due to smoking, suggesting that the 8-OHdG excretion may be influenced by complex factors.

Thioethers

Urinary thioethers (measured as the total amount of various thioethers) have been frequently measured as a biomarker of exposure to electrophiles (Van Doorn et al. 1981, Henderson et al. 1984). However, such measurement does not provide any structural information about the electrophiles that eventually result in the excretion of thioethers. In several studies (Heinonen et al. 1983, Aringer & Lidums 1988, Bos et al. 1992, Scherer et al. 1996), increased urinary thioether levels have been reported for smokers (on average ranging from 60 to 135 μmol/24 h) compared with non-smokers (on average 40–110 μmol/24 h). In one study (Van Doorn et al. 1979), much lower urinary thioethers levels were reported for smokers (20–34 μmol/24 h) and non-smokers (5 μmol/24 h).

Materials and methods

Test products

The four cigarette products used were evaluated for tar, nicotine and CO deliveries in mainstream smoke using standardized (FTC) methods. Tar, nicotine and CO yields of the cigarettes used in this study are shown in Table I.

EHCSS1 (ACCORD First Generation EHCSS Series E4) delivered approximately 3 mg tar. EHCSS2 (OASIS First Generation EHCSS Series E4) included a charcoal filter and delivered approximately 2 mg tar per cigarette. *In vitro* investigations showed that the lower pyrolysis/combustion temperature from EHCSS results in a significant reduction (25–90%) in 44 machine measured mainstream smoke constituents relative to mainstream smoke from a University of Kentucky standard cigarette (1R4F) (Stabbert et al. 2003). These reduced mainstream constituents included, but not limited to, polycyclic aromatic hydrocarbons (PAHs), *N*-nitrosamines, monocyclic aromatic hydrocarbons such as benzene, and several α,β-unsaturated aldehydes. A detailed description of the technical design and chemical composition of the mainstream smoke of the electrically heated cigarettes (EHCSS1) has already been published (Patskan & Reininghaus 2003, Stabbert et al. 2003). In the current study the two types of EHCSS were compared with two conventional cigarette (CC) brands: the CC1 (Marlboro LIGHTS) delivered 11 mg tar and the CC2 (Marlboro ULTRA) delivered 3 mg tar per cigarette.

Table I. FTC tar, nicotine and carbon monoxide (CO) yields of the test products.

Test product	Tar (mg/cigarette)	Nicotine (mg/cigarette)	CO (mg/cigarette)
CC1	11	0.8	12
CC2	3	0.3	4
EHCSS1	3	0.2	0.7
EHCSS2	2	0.2	0.7

Subjects and urine collection

The study that yielded the urine samples for the current investigation was a single-centre, open-label, randomized, forced-switching, controlled-smoking and parallel-group study. Subjects were confined to the clinical research facility during the course of the study. The detailed study design and subject characteristics have been reported elsewhere (Roethig et al. 2005). Briefly, after baseline investigation, 110 CC1 smokers (who had smoked this product for at least 1 year before the start of the study) were randomized into one of the five following groups: CC1, CC2, EHCSS1, EHCSS2 and no smoking. Twenty subjects were randomized into each of the first four smoking groups, while 30 subjects were randomized into the no smoking group. In the four smoking groups, each subject was limited to his/her maximum daily cigarette consumption as determined from acclimation on the day before the baseline during the 10-day study and smoked the designated type of cigarette evenly throughout each study day. The actual number of cigarettes smoked on each study day was based on a collection of the cigarette butts by the clinical staff. Meals served during the study were planned by a dietician to avoid high mutagen content, particularly PAHs. The menus at baseline and day 8 were identical. Subjects had no access to other sources of food during the course of this study. Urine samples (24-h collection) were collected as described previously and the reserved volumes were used for the present study. However, for some subjects there were inadequate volumes for biomarker measurements described here. These samples were mainly distributed in the EHCSS1 and no smoking groups at day 8. All the available samples were stored at -20^{o}C for about 6 months before shipment on dry ice to the Analytisch-biologisches Forschungslabor (ABF) GmbH, Munich, Germany, where the analyses were performed within 2 months.

Analytical methods

The development and evaluation of analytical methods were performed at ABF Munich. The limit of detection (LOD) and lower limit of quantification (LLOQ) for each method, except for thioethers, were determined by the signal-to-noise (S/N) ratio using S/N ratios of 3 and 10, respectively. Those for thioethers were determined from the variation of a blank sample. The upper limit of quantification (ULOQ) for each method was determined as the highest analyte concentration for which linearity could be demonstrated. For the 1-OHP, *S*-PMA, 3-MeAd, 3-EtAd and 8-OHdG methods, accuracy (relative recovery) and precision were determined by using urine samples from non-smokers spiked with different levels of analytes; for the *t*,*t*-MA method, they were determined by using certified reference urine samples from Receipe, Munich, Germany. Stability of analytes in urine when stored at -20°C for 12 months or longer was verified before performing this study. The performance of each method is summarized in Table II.

1-OHP in urine

1-OHP in urine was determined by an HPLC fluorescence method after enzymatic deconjugation with β-glucuronidase/arylsulfatase and solid-phase extraction (SPE) according to Jongeneelen and Anzion (1990). Briefly, 10 ml urine were adjusted to pH 5 with 4 N HCl and hydrolysed with 0.06 U β-glucuronidase/arylsulfatase (Roche,

Mannheim, Germany) for 16 h at 37°C. The hydrolysed sample was subjected to SPE on C18 cartridges (Bond Elut® C18, 500 mg, Varian, Darmstadt, Germany). After washing with 10 ml water, the analyte was eluted with 4 ml methanol. The eluate was evaporated and redissolved in 4 ml 20 mM phosphate buffer/methanol (50/50). A total of 10 µl of the extract was injected into an HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a C18 column (Symmetry Shield RP18, Waters GmbH, Eschborn, Germany). Gradient chromatography was applied using methanol/20 mM phosphate buffer (50/50) and methanol (increasing from 25 to 90% over 20 min). The analyte was detected by using a fluorescence detector (Model HP 1056A, Agilent Technologies) at 238 nm (excitation wavelength) and 394 nm (emission). The retention time for 1-OHP was 20.4 min.

S-PMA in urine

S-PMA in urine was determined by an unpublished LC-MS/MS method using an SPE procedure and d_5-*S*-PMA as an internal standard. Briefly, after adding 20 ng d_5-*S*-PMA to 5 ml urine, the sample was subjected to SPE on an anion-exchange cartridge (Oasis MAX®, Waters). The cartridge was washed with 3 ml water containing 5% ammonia and 3 ml methanol and then eluted with 4 ml ethyl acetate containing 5% formic acid. The eluate was evaporated to dryness and redissolved in 100 µl methanol/water (50/50). LC-MS/MS was performed with an API 2000 triple quadrupole mass spectrometer (Applied Biosystems, Darmstadt, Germany) coupled to an HPLC system Model 1100 (Agilent Technologies) by injecting 10 µl extract onto a silica bonded reversed-phase column (Luna C18(2), Phenomenex, Aschaffenburg, Germany) and eluting with a mobile phase of 10 mM ammonium acetate, pH 4.3 and methanol using a gradient of 80 to 5% ammonium acetate buffer and 20 to 95% methanol. Retention times were 4.54 and 4.56 min for d_5-*S*-PMA and *S*-PMA, respectively. The MS/MS was operated in the atmospheric pressure negative chemical ionization (APCI) mode. The following ion transitions were monitored: d_5-*S*-PMA: m/z 243 →114, *S*-PMA: m/z 238 →109.

t,t-*MA in urine*

t,*t*-*MA* in urine was determined by gas chromatography with mass-selective detection (GC-MS) according to Ruppert et al. (1995), with modifications. Briefly, after adding 2 µg internal standard (d_4-*t*,*t*-MA) to 1 ml urine acidified with acetic acid, the sample was subjected to SPE (anion exchange cartridge, Baker SPE, J. T. Baker, Griesheim, Germany). The SPE cartridge was washed with 3 ml 1% acetic acid and eluted with 3 ml 10% acetic acid. The eluate was dried under vacuum and the residue derivatized with borotrifluoride/methanol. The reaction mixture was extracted three times with heptane (1 ml each). The combined extracts were evaporated under a stream of nitrogen to 50–100 µl. A total of 1 µl extract was injected into the GC-MS system (MD 800, Thermo Finnigan, Egelsbach, Germany), equipped with a fused silica column (Zebron ZB 50, Phenomenex). Retention times for *t*,*t*-*MA* and d_4-*t*,*t*-MA were 6.18 and 6.17 min, respectively. The MS detector was operated in the electron-impaction (EI) mode. The following ions were monitored in the selected ion-monitoring mode: *t*,*t*-MA-dimethylester: m/z 139 (quantifier) and 170 (qualifier), d_4-*t*,*t*-MA-dimethylester: m/z 143 (quantifier) and 174 (qualifier). The recovery of the

SPE step was determined using a pooled urine sample from non-smokers spiked with 0.01 to 1.00 μg ml^{-1} *t,t*-MA.

3-MeAd and 3-EtAd in urine

3-MeAd and 3-EtAd in urine were determined by liquid chromatography with tandem mass spectrometry (LC-MS/MS) after SPE using d_5-3-EtAd as an internal standard. Briefly, after adding 10 ng internal standard to 50 ml urine, the sample was subjected to SPE using MCX cartridges (Oasis®, Waters). The cartridge was washed sequentially with 6 ml 0.1 M phosphate buffer, pH 7.0, 6 ml 0.1 M HCl, 6 ml methanol, 10 ml toluene and 6 ml ethyl acetate. After elution with 6 ml 5% methanol in ethyl acetate saturated with ammonia, the eluate was evaporated to dryness at 55°C in a stream of nitrogen. The residue was redissolved in 20 μl methanol and diluted by adding 20 μl water. LC-MS/MS was performed with an API 2000 triple quadrupole mass spectrometer (Applied Biosystems) coupled to an HPLC system Model 1100 (Agilent Technologies). A total of 10 μl extract was introduced to a silica-bonded reversed-phase column (Synergi MAX RP 80, Phenomenex). The mobile phase was 10 mM ammonium acetate buffer and methanol, with a gradient of 95 to 20% ammonium acetate and 5 to 80% methanol. Retention times were 5.07, 5.09 and 4.65 min for d_5-3-EtAd, 3-EtAd and 3-MeAd, respectively. The MS/MS was operated in the atmospheric pressure positive chemical ionization (APCI) mode. The following ion transitions were monitored for quantification: d_5-3-EtAd: m/z 169 →137, 3-EtAd: m/z 164 →136, and 3-MeAd: m/z 150 →123.

8-OHdG in urine

8-OHdG in urine was determined by an LC-MS/MS method after SPE according to Renner et al. (2000). Briefly, 3 ml urine were subjected to SPE on a LiChrolut EN® cartridge (Merck, Darmstadt, Germany). After washing with 3 ml 25 mM potassium dihydrogen phosphate, pH 5.5, 2 ml water and 2 ml acetonitrile, the cartridge was eluted with 2 ml methanol. The extract was evaporated to dryness in a stream of nitrogen and redissolved in 300 μl 10 mM ammonium acetate containing 2% methanol, pH 4.3. LC-MS/MS (API 2000 triple quadrupole mass spectrometer, Applied Biosystems coupled to an HPLC system Model 1100, Agilent Technologies) was performed by injecting 10 μl extract into the system operated in the positive electro-spray ionization (ESI) mode. A silica gel reversed-phase column (Supersphere 100 RP18 endcapped, Merck) was used as a stationary phase and a gradient of 10 mM ammonium acetate, pH 4.3 (99 to 20%) and methanol (1 to 80%). The retention time was 7.2 min. The following mass transfers were monitored: 284 →168 (quantifier), 284 →140 (qualifier) and 284 →112 (qualifier).

Thioethers in urine

Thioethers in urine were determined photometrically according to Aringer and Lidums (1988), which was modified to incorporate a microplate reader. Briefly, 5 ml urine were extracted three times with ethyl acetate (8 ml each). The combined extracts were evaporated to dryness at 40°C under reduced pressure and the residue was redissolved in 2 ml 2 μM ascorbic acid. One half of the extract was measured for compounds containing free sulfhydryl groups by reaction with 5,5-dithio-*bis*-(2-

Table II. Analytical method performance characteristics.

	LOD	LLOQ	ULOQ	Accuracy (relative recovery rate, %)	Precision (CV%)
1-OHP	5 pg ml^{-1}	9 pg ml^{-1}	1000 pg ml^{-1}	88.6–91.7	4.6 ($n=14$)
S-PMA	10 pg ml^{-1}	20 pg ml^{-1}	10000 pg ml^{-1}	97.6–100.4	3.7–5.3 ($n=5$)
t,*t*-MA	0.003 μg ml^{-1}	0.01 μg ml^{-1}	1000 μg ml^{-1}	92[a]; 83–92[b]	3.0 ($n=4$)
3-MeAd	0.002 ng ml^{-1}	0.006 ng ml^{-1}	160 ng ml^{-1}	83–104	6.2–7.6 ($n=10$)
3-EtAd	0.002 ng ml^{-1}	0.006 ng ml^{-1}	800 ng ml^{-1}	100–112	4.7–7.7 ($n=10$)
8-OHdG	0.1 ng ml^{-1}	0.2 ng ml^{-1}	10 ng ml^{-1}	95.4–115.4	0.9–3.6 ($n=3$)
Thioethers	4.7 nmol ml^{-1}	14.1 nmol ml^{-1}	160 nmol ml^{-1}	n.a.[c]	15.6 ($n=10$)

[a]Accuracy as determined by certified standard.
[b]Recovery of the SPE step.
[c]n.a., Not available.

nitrobenzoic acid) at 405 nm. The other half was subjected to alkaline hydrolysis under a stream of nitrogen in order to cleave the thioethers, resulting in the corresponding sulfhydryl compounds, which were also measured after reaction with 5,5-dithio-*bis*-(2-nitrobenzoic acid). The concentration of thioethers in a sample was the difference between the two measurements. The method was calibrated by using *N*-acetyl-cysteine as an external standard.

Statistical methods and data analysis

A linear mixed model for repeated measures analysis of variance was used to test for differences in daily biomarker excretion levels between the study groups (CC1, CC2, EHCSS1, EHCSS2 and no smoking) and time points. The model included terms for study group, time point and study group/time point interaction. Multiple comparisons were performed using the pairwise *t*-test. SAS Proc Mixed (SAS Institute, Inc, Cary, NC, USA) was used to conduct the analysis.

The relationships between the daily biomarker excretion levels and number of cigarettes smoked per day were examined using Pearson product-moment correlation analysis. The analysis was also used to examine the correlations in biomarker excretion levels between different biomarkers or between baseline and day 8 for the CC1 group.

The effect of age, gender, daily cigarette consumption and body mass index (BMI), i.e. body weight (kg)/height^2 (m), on daily biomarker excretion levels was examined by stepwise regression analysis with a maximum r^2 procedure for variable selection.

As a remedy for the non-normal distribution of the biomarker variables, the natural logarithm transformation was applied to all biomarker data for statistical analysis.

Results were considered statistically significant at $p < 0.05$.

Results

Subjects

The main demographics of 110 subjects randomized into five study groups are shown in Table III. The numbers of male and female subjects were almost equally distributed in all groups. Other characteristics of the subjects including age, body weight and height, and BMI were also similar. Daily cigarette consumption was similar in the five groups at baseline and day 8 (Table IV).

Table III. Descriptive characteristics of the study subjects.

Trait	Groups					Overall
	CC1	CC2	EHCSS1	EHCSS2	No smoking	
Gender (n):						
Female	10	9	11	11	14	55
Male	10	11	9	9	16	55
Age (years):						
Mean	30.7	30.8	33.6	29.9	32.1	31.4
SD	10.5	10.6	11.7	10.3	9.7	10.4
Range	21–56	21–56	21–58	21–58	21–50	21–58
Race (n):						
Caucasian	16	19	18	18	28	99
Non-Caucasian	4	1	2	2	2	11
Height (m):						
Mean	1.74	1.75	1.73	1.72	1.74	1.74
SD	0.09	0.09	0.06	0.09	0.10	0.09
Weight (kg):						
Mean	72.0	74.9	71.3	73.9	76.5	74.0
SD	11.3	10.8	13.5	15.0	14.8	13.3
BMI (kg m^{-2}):						
Mean	23.8	24.5	23.7	24.7	25.2	24.5
SD	2.8	3.5	3.5	3.4	3.5	3.3

Daily urinary biomarker excretion levels

Table V shows the daily urinary excretion levels at both baseline and day 8 for each biomarker broken down into five study groups with two p values indicating the statistical significance of the difference between the excretion levels at baseline and those at day 8 within the same group and of the difference between the CC1 group and any other group on the same study day. 1-OHP, *S*-PMA, *t*,*t*-MA, 3-MeAd and 3-EtAd were measured in all study groups, and 8-OHdG and thioethers were only measured in the CC1 and no smoking groups at both baseline and day 8.

At baseline when all groups were smoking CC1, the levels for 1-OHP, *t*,*t*-MA, 8-OHdG and thioethers were similar among different study groups, whereas those for *S*-PMA, 3-MeAd and 3-EtAd were not. For *S*-PMA, the baseline level in EHCSS1, and for 3-EtAd, the baseline levels in the EHCSS1 and EHCSS2 groups, were significantly lower than those in the CC1 group. For 3-MeAd, the baseline levels in

Table IV. Cigarette consumption by the study groups at baseline and day 8.

Group	Baseline			Day 8		
	Mean	SD	Range	Mean	SD	Range
CC1	15.9	6.5	6–26	15.9	6.5	7–28
CC2	14.7	6.3	5–27	14.3	7.5	1–27
EHCSS1	16.2	5.4	6–24	15.5	7.2	2–23
EHCSS2	16.6	4.9	9–26	16.1	4.9	7–23
No smoking	16.4	5.9	5–27	0	0	0

Table V. Daily urinary biomarker excretion levels in study groups at baseline and day 8.

	Group	Day	*n*	Mean[a]	SD	Range	p^b	p^c
1-OHP	CC1	−1	20	0.18	0.11	0.06–0.47	reference	reference
(μg/24 h)		8	20	0.14	0.06	0.06–0.28	0.0934	reference
	CC2	−1	20	0.17	0.09	0.05–0.31	reference	0.7079
		8	20	0.13	0.06	0.03–0.25	0.0156	0.2571
	EHCSS1	−1	20	0.15	0.11	0.04–0.50	reference	0.1586
		8	15	0.04	0.01	0.03–0.06	<0.0001	<0.0001
	EHCSS2	−1	19	0.19	0.22	0.09–1.09	reference	0.9922
		8	19	0.05	0.02	0.02–0.09	<0.0001	<0.0001
	no smoking	−1	30	0.16	0.09	0.04–0.41	reference	0.7765
		8	24	0.04	0.01	0.02–0.06	<0.0001	<0.0001
S-PMA	CC1	−1	19	7.66	7.71	0.84–24.90	reference	reference
(μg/24 h)		8	20	4.49	4.29	0.96–18.50	0.0637	reference
	CC2	−1	20	6.08	5.18	0.60–16.12	reference	0.4640
		8	20	3.10	2.25	0.31–7.01	0.0134	0.1235
	EHCSS1	−1	20	4.33	4.53	0.11–18.74	reference	0.0221
		8	15	0.43	0.25	0.06–1.01	<0.0001	<0.0001
	EHCSS2	−1	19	8.27	7.90	1.13–34.12	reference	0.8812
		8	18	0.39	0.30	0.05–1.24	<0.0001	<0.0001
	no smoking	−1	30	6.52	6.44	0.33–25.75	reference	0.5425
		8	21	0.32	0.26	0.06–1.13	<0.0001	<0.0001
t,*t*-MA	CC1	−1	20	0.26	0.15	0.09–0.69	reference	reference
(mg/24 h)		8	20	0.21	0.11	0.10–0.53	0.0711	reference
	CC2	−1	20	0.23	0.13	0.10–0.71	reference	0.4420
		8	20	0.16	0.08	0.06–0.44	0.0009	0.0878
	EHCSS1	−1	20	0.24	0.13	0.08–0.58	reference	0.5151
		8	15	0.11	0.06	0.05–0.23	<0.0001	0.0003
	EHCSS2	−1	19	0.22	0.06	0.12–0.36	reference	0.5342
		8	19	0.09	0.04	0.04–0.21	<0.0001	<0.0001
	no smoking	−1	30	0.25	0.17	0.09–0.96	reference	0.7966
		8	24	0.13	0.13	0.03–0.52	<0.0001	<0.0001
3-MeAd	CC1	−1	20	7.14	4.00	2.59–19.92	reference	reference
(μg/24 h)		8	20	8.44	5.86	1.70–25.83	0.8292	reference
	CC2	−1	20	14.59	7.34	6.30–27.14	reference	<0.0001
		8	20	9.10	6.31	2.03–24.92	0.0057	0.6829
	EHCSS1	−1	20	13.33	8.22	3.50–33.66	reference	0.0012
		8	14	8.65	8.81	2.70–38.19	0.0338	0.9678
	EHCSS2	−1	18	19.68	9.49	8.94–44.63	reference	<0.0001
		8	19	5.78	3.40	0.05–12.46	<0.0001	0.0507
	no smoking	−1	30	9.59	6.01	3.19–34.47	reference	0.0700
		8	23	1.56	0.67	0.38–2.70	<0.0001	<0.0001
3-EtAd	CC1	−1	20	210.53	69.31	119.70–369.26	reference	reference
(ng/24 h)		8	20	200.83	47.14	110.20–266.02	0.8122	reference
	CC2	−1	20	169.15	62.50	72.00–348.92	reference	0.0614
		8	20	123.29	72.29	7.24–262.96	<0.0001	<0.0001
	EHCSS1	−1	20	124.63	55.21	47.9–250.51	reference	<0.0001
		8	14	102.15	39.25	44.55–154.62	0.0439	<0.0001
	EHCSS2	−1	18	149.66	49.57	53.90–262.06	reference	0.0044
		8	19	87.53	36.63	38.70–178.45	<0.0001	<0.0001
	no smoking	−1	30	184.72	64.78	58.69–295.38	reference	0.1626
		8	23	182.06	72.25	103.11–363.63	0.4498	0.1850
8-OHdG	CC1	−1	20	3.75	1.10	1.98–5.93	reference	reference
(μg/24 h)		8	20	2.94	1.17	0.83–6.52	0.0162	reference

Table V (*Continued*)

	Group	Day	*n*	Mean[a]	SD	Range	p^b	p^c
	no smoking	−1	23	3.24	0.99	1.34–4.87	reference	0.1522
		8	24	2.81	0.93	1.36–4.94	0.1620	0.7991
Thioethers	CC1	−1	19	156.99	81.20	50.42–423.70	reference	reference
(μmol/24 h)		8	19	159.92	75.87	54.00–341.68	0.9329	reference
	no smoking	−1	23	160.93	61.42	81.14–316.10	reference	0.7169
		8	24	72.48	34.64	9.70–158.15	<0.0001	<0.0001

[a]Arithmetic means.
[b]p values for comparisons with the baseline (day −1) values (reference) were obtained from ANOVA after natural log-transformation of the biomarker excretion levels.
[c]p values for comparisons with CC1 group at baseline or day 8 (references) were also obtained from ANOVA after natural log-transformation of the biomarker excretion levels.

the CC2, EHCSS1 and EHCSS2 groups were significantly higher than those in the CC1 group.

At day 8, in the CC1 group, with the exception of 8-OHdG, which showed a significant decrease relative to its baseline level, all other urinary biomarkers measured remained similar ($p > 0.05$) to their respective baseline levels. For the other study groups, with the exceptions of 8-OHdG and 3-EtAd, the excretion levels of the biomarkers measured decreased significantly. At day 8, the 1-OHP, *S*-PMA and *t*,*t*-MA levels in the EHCSS1 and EHCSS2 groups were similar to those in the no smoking group, and were significantly lower than those in the CC1 and CC2 groups. At day 8, there was no significant difference between the CC1 and CC2 groups for 1-OHP, *S*-PMA and *t*,*t*-MA, although after switching the levels of these three biomarkers in the CC2 group decreased significantly relative to their respective baseline levels. For 3-MeAd, although there were significant decreases in the CC2, EHCSS1, EHCSS2 and no smoking groups after switching, only the no smoking group showed a significantly lower level than the CC1 group. For 3-EtAd, the levels in the CC2, EHCSS1 and EHCSS2 groups at day 8 were significantly lower than their respective baseline levels and than that of the CC1 group. However, surprisingly, the 3-EtAd level in the no smoking group remained the same as its baseline level. For 8-OHdG, although the excretion level in the CC1 group decreased significantly at day 8, there was no significant difference between the CC1 and no smoking groups either at baseline or at day 8. For thioethers, the excretion level in the no smoking group not only decreased significantly relative to its baseline level, but also was significantly lower than that of the CC1 group.

The Pearson product-moment correlation coefficients between the daily biomarker excretion levels (log-transformed) and the daily number of cigarettes smoked are summarized in Table VI. Although this analysis was not adjusted for any possible confounding variables, there were significant correlations between daily 1-OHP, *S*-PMA, *t*,*t*-MA, 3-MeAd and 3-EtAd excretion levels and the number of cigarettes in all the CC1 smokers at baseline ($r = 0.62$, 0.52, 0.48, 0.35 and 0.28, respectively, $p < 0.05$), whereas the correlations for 8-OHdG or thioethers were not significant. For 1-OHP, *S*-PMA, *t*,*t*-MA and 3-MeAd the correlations in CC1 and CC2 groups remained significant after switching, but generally were not significant in EHCSS1 and EHCSS2 groups, with the 3-MeAd in the EHCSS2 group being the only exception. It was also noted that the correlation coefficients in the CC2 group were

Table VI. Pearson product-moment correlation between the daily urinary biomarker excretion levels and the number of cigarettes.

	Group	Day	r^a	n	p
1-OHP	all CC1 smokers[b]	−1	0.62	108	<0.0001
	CC1	8	0.46	20	0.0436
	CC2	8	0.76	19	0.0002
	EHCSS1	8	−0.09	15	0.7540
	EHCSS2	8	0.17	19	0.4825
S-PMA	all CC1 smokers[b]	−1	0.52	107	<0.0001
	CC1	8	0.47	20	0.0348
	CC2	8	0.57	19	0.0110
	EHCSS1	8	0.22	15	0.4401
	EHCSS2	8	0.23	18	0.3495
t,*t*-MA	all CC1 smokers[b]	−1	0.48	108	<0.0001
	CC1	8	0.54	20	0.0134
	CC2	8	0.73	19	0.0004
	EHCSS1	8	−0.30	15	0.2716
	EHCSS2	8	0.27	19	0.2618
3-MeAd	all CC1 smokers[b]	−1	0.35	107	0.0003
	CC1	8	0.51	20	0.0209
	CC2	8	0.70	19	0.0008
	EHCSS1	8	0.22	14	0.4524
	EHCSS2	8	0.58	19	0.0097
3-EtAd	all CC1 smokers[b]	−1	0.28	107	0.0031
	CC1	8	−0.02	20	0.9409
	CC2	8	0.42	19	0.0697
	EHCSS1	8	0.45	14	0.1037
	EHCSS2	8	−0.16	19	0.5014
8-OH-dG	all CC1 smokers[c]	−1	−0.07	44	0.6407
	CC1	8	−0.37	20	0.1051
Thioethers	all CC1 smokers[c]	−1	0.17	43	0.2703
	CC1	8	0.00	19	0.9846

[a]Correlation coefficients were obtained after natural log-transformation was applied to both biomarker excretion levels and the number of cigarettes.
[b]All CC1 smokers of five groups were pooled for analysis.
[c]All CC1 smokers of only CC1 and no smoking groups were pooled for analysis.

higher than those in the CC1 group. There was no significant correlation for 3-EtAd, 8-OHdG and thioethers in any of the study groups measured at day 8.

The effects of other factors including gender, age and BMI on the daily excretion levels (log-transformed) of these biomarkers at baseline were also examined by using stepwise multiple regression analysis. For 1-OHP, *t*,*t*-MA and 3-EtAd, in addition to the number of cigarettes, gender also showed significant association with the daily excretion levels. For 8-OHdG, gender was the only variable that significantly associated with its daily excretion levels. But for *S*-PMA, 3-MeAd and thioethers (data not shown), none of these additional variables showed any association with the daily excretion levels. The best fit multiple regression models for 1-OHP, *S*-PMA, *t*,*t*-MA, 3-MeAd, 3-EtAd and 8-OHdG are summarized in Table VII. This analysis revealed that 41, 35 and 15% of the variations in 1-OHP, *t*,*t*-MA and 3-EtAd daily excretion levels, respectively, could be explained by the daily number of cigarettes and

Table VII. Multiple regression models[a] of six urinary biomarkers for the CC1 smokers at baseline.

Response variable	Overall R^2	Predictor variable	Coefficient	Standard error	p
1-OHP	0.41	intercept	−4.61	0.32	<0.0001
		number of cigarettes	0.88	0.11	<0.0001
		gender	0.18	0.09	0.0431
S-PMA	0.27	intercept	−2.49	0.63	0.0001
		number of cigarettes	1.43	0.23	<0.0001
t,*t*-MA	0.35	intercept	−3.49	0.27	<0.0001
		number of cigarettes	0.54	0.09	<0.0001
		gender	0.33	0.07	<0.0001
3-MeAd	0.12	intercept	0.89	0.39	0.0226
		number of cigarettes	0.53	0.14	0.0003
3-EtAd	0.15	intercept	3.97	0.27	<0.0001
		number of cigarettes	0.28	0.09	0.0036
		gender	0.22	0.08	0.0046
8-OHdG	0.22	intercept	0.72	0.15	<0.0001
		gender	0.32	0.12	0.0014

[a]Natural log-transformation was applied for all the response variables and predictor variables except gender. Male = 1; female = 0.

gender; 27 and 12% of the variations in *S*-PMA and 3-MeAd, respectively, could be explained by the daily number of cigarettes alone; and 22% of the variations in 8-OHdG could be explained by the gender alone. Among all CC1 smokers at baseline, male subjects had higher mean daily excretion levels than female subjects for 1-OHP (0.19 ± 0.16 μg/24 h, $n = 55$ versus 0.15 ± 0.08 μg/24 h, $n = 54$), *t*,*t*-MA (0.28 ± 0.16 mg/24 h, $n = 55$ versus 0.20 ± 0.09 mg/24 h, $n = 54$), 3-EtAd (187.18 ± 63.96 ng/24 h, $n = 55$ versus 151.45 ± 64.97 ng/24 h, $n = 53$) and 8-OHdG (3.86 ± 1.30 μg/24 h, $n = 24$ versus 2.82 ± 1.42 μg/24 h, $n = 20$), respectively, although the number of cigarettes smoked by male (16.2 ± 5.8) and female subjects (15.7 ± 5.8) at the baseline were similar ($p = 0.6204$ by *t*-test).

Correlation analysis was performed to evaluate the relationships between these biomarkers with all the CC1 smokers at baseline (Table VIII). Previously measured urinary nicotine equivalents (the molar sum of nicotine and its five major metabolites including cotinine, *trans*-3′-hydroxycotinine, nicotine-*N*-glucuronide, cotinine-*N*-glucuronide and *trans*-3′-hydroxycotinine-*O*-glucuronide) in the same subjects (Roethig et al. 2005) were also included in this analysis. Nicotine equivalents, 1-OHP, *S*-PMA, *t*,*t*-MA and 3-MeAd, significantly correlated with each other. The correlation coefficients between these biomarkers varied from 0.24 (between *t*,*t*-MA and 3-MeAd) to 0.72 (between 1-OHP and nicotine equivalents). Surprisingly, 3-EtAd did not correlate with 3-MeAd, but correlated with all other biomarkers. 8-OHdG and thioethers significantly correlated to each other and both of them correlated with 3-EtAd.

Finally, within-subject correlations (Table IX) between baseline and day 8 for the CC1 group for all biomarkers investigated were examined. Except for 3-EtAd and thioethers, all other biomarkers showed significant correlations between baseline and day 8.

Table VIII. Pearson correlation matrix[a] for the urinary biomarkers at baseline.

	1-OHP	*S*-PMA	*t*,*t*-MA	3-MeAd	3-EtAd	8-OHdG	Thioethers
Nicotine equivalents	0.72 ($n=109$, $p<0.0001$)	0.64 ($n=108$, $p<0.0001$)	0.48 ($n=109$, $p<0.0001$)	0.52 ($n=108$, $p<0.0001$)	0.43 ($n=108$, $p<0.0001$)	−0.03 ($n=44$, $p=0.8233$)	0.21 ($n=43$, $p=0.1848$)
1-OHP		0.57 ($n=108$, $p<0.0001$)	0.48 ($n=109$, $p<0.0001$)	0.47 ($n=108$, $p<0.0001$)	0.51 ($n=108$, $p<0.0001$)	0.32 ($n=44$, $p=0.0346$)	0.27 ($n=43$, $p=0.0835$)
S-PMA			0.37 ($n=108$, $p<0.0001$)	0.39 ($n=107$, $p<0.0001$)	0.33 ($n=107$, $p=0.0005$)	0.17 ($n=43$, $p=0.2845$)	0.29 ($n=43$, $p=0.0559$)
t,*t*-MA				0.24 ($n=108$, $p=0.0108$)	0.33 ($n=108$, $p=0.0006$)	0.25 ($n=44$, $p=0.1008$)	0.21 ($n=43$, $p=0.1841$)
3-MeAd					0.09 ($n=108$, $p=0.3725$)	0.12 ($n=44$, $p=0.4466$)	0.17 ($n=43$, $p=0.2753$)
3-EtAd						0.35 ($n=44$, $p=0.0185$)	0.49 ($n=43$, $p=0.0009$)
8-OHdG							0.43 ($n=43$, $p=0.0039$)

[a]Natural log-transformation was applied to all biomarker excretion levels.

Table IX. Within-subject correlation between baseline and day 8 for the CC1 group.

Biomarker	r	p
1-OHP	0.59	0.0068
S-PMA	0.59	0.0076
t,*t*-MA	0.64	0.0025
3-MeAd	0.49	0.0283
3-EtAd	0.30	0.1969
8-OHdG	0.45	0.0447
Thioether	0.05	0.8405

Discussion

Reliability of the analytical methods

All biomarkers were measured using validated methods. Table II shows the performance characteristics (LOD, LLOQ, ULOQ, recovery and precision) of these methods. All analytical methods had sufficient sensitivity to detect these biomarkers except four samples had concentrations below LODs for *S*-PMA. With the exception of thioethers, all the methods were based on chromatography-mass spectrometry. The samples were extracted and purified using SPE before chromatographic analysis, and there was no indication of any interference from other components in the sample matrix. It was concluded that the analytical methods were reliable and did not significantly contribute to the variation of the results.

1-OHP as a biomarker

In this study, there were also detectable levels of 1-OHP in the no smoking group at day 8 and the 1-OHP excretion levels in the conventional smokers at baseline and at day 8 were about three to four times as high as that in the no smoking group at day 8. 1-OHP has a relatively short elimination half-life ($T_{1/2}$) of 9.8 h (Brzeznicki et al. 1997) following inhalation of pyrene, although a longer half-life (18–20 h) has also been reported (Jongeneelen & Bos 1990, Buckley & Lioy 1992, Van Schooten et al. 1995). Judging from its $T_{1/2}$, it is reasonable to assume that the excretion of 1-OHP in the no smoking group at the end of the study is not due to the carryover from baseline, but mainly due to the pyrene intake through other sources such as diet and ambient air with diet being the overwhelming source (Van Rooij et al. 1994, Sithisarankul et al. 1997, Viau et al. 2002). It can also be assumed that the dietary intake of pyrene contributed to 1-OHP excretion in all study groups. In spite of the dietary confounding factor, the 1-OHP excretion level in the no smoking group at day 8 was significantly lower ($p < 0.0001$) than those of the CC1 and CC2 groups. The 1-OHP excretion levels in EHCSS1 and EHCSS2 groups reached levels similar to that in the no smoking group ($p > 0.05$) and were significantly lower than those in CC1 and CC2 groups ($p < 0.0001$). There was no significant difference between the two EHCSS groups ($p > 0.05$). Given that the daily numbers of cigarettes smoked by each group were similar at baseline and day 8, it could be concluded that the lower levels of 1-OHP excretion in EHCSS1 and EHCSS2 groups compared with the CC1 and CC2 groups were due to the lower levels of smoke exposure. This was further confirmed by the correlation analysis which showed that there were significant correlations between the 1-OHP levels and the daily number of cigarettes in all CC1 smokers at baseline as

well as the CC1 and CC2 groups at day 8, but not in EHCSS1 and EHCSS2 groups at day 8. In fact, the PAH intake from smoking EHCSS cigarettes was nearly negligible as compared with that from diet as evidenced by the almost identical 1-OHP excretion levels in the EHCSS1, EHCSS2, and no smoking groups.

There were considerable inter-individual variations in the 1-OHP excretion levels, indicated by the wide ranges of excretion levels in groups. For example, the inter-individual differences of five groups at baseline varied approximately 6–12-fold. Part of this variability was due to the variability in the number of cigarettes, which differed approximately 3–5-fold. Gender was found to be another influencing factor. The number of cigarettes and gender together explained about 41% of the variability in the 1-OHP excretion levels. There are three possible explanations for the gender effect: first, the dietary intake of PAHs in men might be higher than in women; second, men have a higher metabolic rate than women (Meijer et al. 1992); and third, women tend to take smaller and shorter puffs than men (Eissenberg et al. 1999), which might result in lower exposure levels. A shortcoming of this study is that the puffing behaviour of the smoking subjects was not monitored. Despite that, considering the findings in this study on other biomarkers (*S*-PMA and *t*,*t*-MA, see below), it is reasonable to suspect that there might be a gender difference in dietary intake of pyrene, which resulted in the gender difference in 1-OHP excretion levels. In contrast to the published data (Van Rooij et al. 1994), age was not a determinant of urinary 1-OHP excretion in the present study. Genetic polymorphisms in metabolic genes (Dor et al. 1999, Nerurkar et al. 2000, Heudorf & Angerer 2001), the respiratory retention of pyrene, and metabolic efficiency (Brzeznicki et al. 1997) have also been reported to affect the 1-OHP excretion levels. It should be noted that the differences in the 1-OHP excretion levels after switching to EHCSS1, EHCSS2, and no smoking were largely reduced 2, 4.5, and 3-fold, respectively, while those in the CC1 and CC2 groups were in the same range as at baseline.

Urinary 1-OHP levels in CC1 smokers correlated significantly ($r = 0.72$, $p < 0.0001$) with nicotine equivalents. This is similar to the previously reported correlation between urinary 1-OHP and cotinine ($r = 0.76$, $p < 0.01$) in smokers (Scherer et al. 2000). 1-OHP also showed significant correlations with the other urinary biomarkers measured in CC1 smokers except thioethers. This is the first time that significant correlations between urinary 1-OHP and these biomarkers in conventional cigarette smokers have been reported.

Overall, the findings reported here support the use of urinary 1-OHP excretion as a useful biomarker to differentiate the exposure levels in subjects who smoked the two conventional cigarettes from those who smoked the two EHCSS cigarettes and from those who stopped smoking.

S-*PMA and* t,t-*MA as biomarkers*

In the present study, there were clear and similar patterns in the changes of excretion levels of both biomarkers after switching. Similar to 1-OHP, at day 8, the mean excretion levels within all groups except the CC1 group decreased significantly ($p < 0.05$) for both *S*-PMA and *t*,*t*-MA. Both *S*-PMA and *t*,*t*-MA had significant correlations with the daily number of cigarettes in both CC1 and CC2 smoking subjects, but there was no correlation in the two EHCSS groups, indicating that

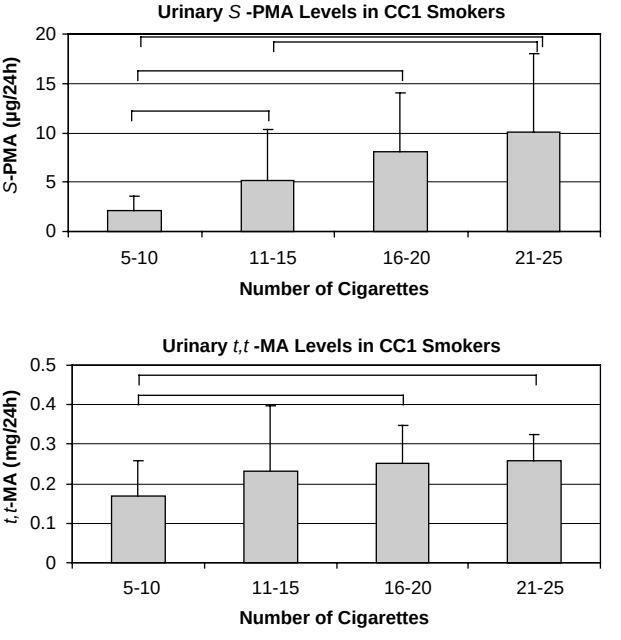

Figure 1. Daily urinary levels of *S*-PMA and *t*,*t*-MA in CC1 smokers categorized by the daily number of cigarettes. Statistically significant differences ($p < 0.05$) between the categories are indicated by the brackets.

smoking CC1 or CC2 cigarettes was a significant source of these two biomarkers, but smoking EHCSS1 or EHCSS2 was not.

At day 8, there were still measurable levels of *S*-PMA and *t*,*t*-MA in the no smoking group, albeit slightly lower than reported levels in non-smokers (Scherer et al. 2001). Both *S*-PMA and *t*,*t*-MA have relatively short elimination half-lives: 12.8 h (Qu et al. 2000, 2003) or 9.1 h (Boogaard & Van Sittert 1996) for *S*-PMA and 13.7 h (Qu et al. 2000, 2003) or 5.0 h (Boogaard & Van Sittert 1996) for *t*,*t*-MA. It can be assumed that the *S*-PMA and *t*,*t*-MA levels in the no smoking group at day 8 were not due to carryover from baseline, but primarily due to other sources of exposure, such as benzene in indoor air for *S*-PMA or both benzene in indoor air and sorbic acid in food for *t*,*t*-MA. It should be noted here that the average percent change of *S*-PMA levels ($90.1 \pm 16.0\%$) in the no smoking group was much greater than the average percent change of *t*,*t*-MA levels ($47.7 \pm 35.6\%$), indicating that *t*,*t*-MA had much higher background levels than *S*-PMA. As shown in Figure 1, *S*-PMA could distinguish the exposure levels much better between the CC1 smokers (at baseline) categorized by the number of cigarettes than *t*,*t*-MA. The statistical comparisons presented in Figure 1 were made using a two-tailed Student's *t*-test.

The variability for *S*-PMA excretion levels was higher than that for *t*,*t*-MA. The inter-individual differences of five groups at baseline ranged by approximately 27–170-fold for *S*-PMA and 3–11-fold for *t*,*t*-MA. As a result, the mean baseline levels of *S*-PMA were more variable than the mean baseline levels of *t*,*t*-MA. The multiple regression analysis showed that the daily number of cigarettes alone and both the daily number of cigarettes and gender accounted for 27 and 35% of the variations in *S*-PMA and *t*,*t*-MA excretion levels, respectively (Table VII). It was reported that in occupationally exposed workers, female subjects excreted significantly higher amount

of both *S*-PMA and *t*,*t*-MA than male subjects (Melikian et al. 2002). However, in the present study, we found that *S*-PMA excretion levels were not associated with gender, but *t*,*t*-MA excretion levels were associated with gender, with higher levels in male subjects than in female subjects. As mentioned above, the 1-OHP excretion levels (another biomarker that could be influenced by diet) in males were higher than in females. This could not be explained by the possible gender difference in smoking behaviour or metabolic rate because both *S*-PMA and *t*,*t*-MA would have probably shown gender difference if these were the main reasons. Therefore, it can be assumed that the gender differences in the 1-OHP and *t*,*t*-MA excretion levels might be related to the differences in dietary intake of pyrene or sorbic acid, although the effects of metabolic rate and puffing behaviour cannot be totally ruled out. The fact that only 27% of the variability for *S*-PMA and 35% of the variability for *t*,*t*-MA could be explained by either the number of cigarettes or both the number of cigarettes and gender suggest there are other strong influencing factors which might include smoking behaviour, other sources of benzene exposure, genetic polymorphisms (Rossi et al. 1999, Scherer et al. 2001, Verdina et al. 2001, Wan et al. 2002), or the speed of biotransformation and elimination (Dor et al. 1999). However, the extent to which these factors influence the excretion levels of the two biomarkers is unknown from either this study or from the literature. The inter-individual differences of five groups at day 8 were much tighter for *S*-PMA as compared with those at baseline, ranging between 17- and 25-fold, but were wider for *t*,*t*-MA, ranging between 5.3- and 17-fold.

A moderate but significant correlation ($r = 0.37$, $p < 0.0001$) between *S*-PMA and *t*,*t*-MA was observed. This is in contrast to the much higher correlation coefficients (0.79 and 0.83) between these two biomarkers in occupationally exposed workers reported in the literature (Boogaard & Van Sittert 1996, Melikian et al. 2002, Qu et al. 2003). A possible explanation is the exposure to benzene during smoking is lower than that in occupational settings and therefore the diet may have a more profound influence on the *t*,*t*-MA excretion levels. Both *S*-PMA and *t*,*t*-MA correlated with other urinary biomarkers except 8-OHdG and thioethers with higher correlation coefficients for *S*-PMA (Table VIII).

It is concluded that both *S*-PMA and *t*,*t*-MA are useful biomarkers for cigarette smoking-related exposure. However, *S*-PMA is less confounded and has lower background levels and therefore is a more sensitive biomarker than *t*,*t*-MA.

3-MeAd and 3-EtAd as biomarkers

In this study, the mean baseline excretion levels in five study groups for both 3-MeAd (range 7.14–19.68 μg/24 h) and 3-EtAd (range 124.64–210.53 ng/24 h) were highly variable. At day 8 after switching from the CC1 cigarette, the 3-MeAd excretion levels decreased significantly in the CC2, EHCSS1, EHCSS2, and no smoking groups relative to their respective baselines, whereas no significant change was found in the CC1 group. When compared with the CC1 group, the no smoking group was the only one that showed a significantly lower level ($p < 0.0001$). The no smoking group also had a significantly lower level ($p < 0.0001$) of 3-MeAd than the CC2 group at day 8. The 3-MeAd excretion levels in CC1 and CC2 smokers were comparable with the data for smokers reported by Kopplin et al. (1995), but those in the no smoking group was lower than in non-smokers ('passive smokers') reported by the same authors. For

3-EtAd, the results are difficult to interpret because, first, the levels in the CC1 and no smoking groups did not show any significant change relative to their respective baselines; second, there was no detectable difference between the CC1 and no smoking groups either at baseline or at day 8; third, there was no within subject-correlation between baseline and day 8 for the CC1 group; and forth, the levels in the CC2, EHCSS1 and EHCSS2 groups decreased significantly relative to their respective baseline levels. Compared with the 3-EtAd excretion levels reported by Kopplin et al., the observed levels in this study for the CC1 and CC2 groups appeared higher than their results in smokers and the level in the no smoking group in this study was much higher than the non-smoker ('passive smokers') in Kopplin et al. It is unknown why the 3-EtAd excretion level in the no smoking group at day 8 was so high. There should not be any carryover effect (from baseline to day 8) for either of these two biomarkers. Prevost et al. (1993) reported rapid eliminations of deuterium labelled 3-MeAd and 3-EtAd in human following ingestion: within 24 h, $>90\%$ of 3-MeAd was excreted and 67–74% of 3-EtAd was excreted. In the two reported controlled studies (Kopplin et al. 1995, Prevost & Shuker 1996), immediate rising and falling of excretion levels of these alkyladenines in smokers on alternating smoking and no smoking days were observed, which is consistent with the normally rapid repair mechanisms following DNA alkylation. The high 3-EtAd excretion level in the no smoking group at day 8 could not be explained by dietary confounding either because the menus at baseline and at day 8 were identical. Besides, 3-EtAd was reported to be less confounded by diet than 3-MeAd.

The inter-individual variations for 3-MeAd excretion levels were higher than those for 3-EtAd. For 3-MeAd, the differences in excretion levels of five study groups ranged between 4- and 11-fold at baseline and between 7- and 249-fold at day 8. For 3-EtAd, the differences ranged between 3- and 5-fold at baseline and between 2- and 36-fold at day 8. Only 12 and 15% of the variations in 3-MeAd and 3-EtAd could be explained by either the number of cigarettes alone (for 3-MeAd) or by both the number of cigarettes and gender (for 3-EtAd), indicating the presence of other strong influencing confounding factors that may include smoking behaviour, diet, the rate of DNA repair and the elimination of the alkylated DNA bases.

Significant correlations between 3-EtAd excretion levels and the daily number of cigarettes or urinary cotinine in two subjects over a 15-day period ($r=0.86$, $p<0.01$ for the daily number of cigarettes and $r=0.60$, $p<0.01$ for urinary cotinine) are reported (Prevost & Shuker 1996). In the present study, 3-EtAd showed weak but significant correlations with the daily number of cigarettes ($r=0.28$, $n=107$, $p<0.0031$) in all CC1 smokers at baseline. At day 8, no significant correlation was observed in all four smoking groups, probably due to smaller sample sizes. Interestingly, as shown in Table VII, 3-EtAd had no correlation with 3-MeAd, but showed significant correlations with all other urinary biomarkers. On the other hand, 3-MeAd showed significant correlations with the daily number of cigarettes not only in all CC1 smokers at baseline but also in three out of the four smoking groups including the CC1, CC2, and EHCSS2 groups at day 8, which all had significantly higher excretion levels than no smoking group. 3-MeAd also showed significant correlations with nicotine equivalents, 1-OHP, *S*-PMA and *t*,*t*-MA, all of which are related to smoking as described above. From the multiple regression analysis on data collected at baseline, the daily number of cigarettes was confirmed to be a positive predictor for both 3-MeAd and 3-EtAd. Gender was also associated with 3-EtAd excretion levels.

These results suggested that the 3-EtAd excretion levels should be associated with cigarette smoking which contradicts the high excretion levels in the no smoking group at day 8.

In summary, 3-MeAd as a biomarker of exposure to methylating agents is capable of differentiating between the two conventional cigarettes smokers and those who stopped smoking, but not capable of differentiating between conventional cigarette smokers and the two EHCSS smokers. It might be only useful in studies where the diet is strictly controlled. Because it is not clear which specific smoke constituent causes the formation of 3-MeAd, it might be used as an index marker of DNA methylation occurring *in vivo* as proposed by other authors (Shuker & Farmer 1992). For 3-EtAd, it is difficult to reach any conclusion because of the contradictory data observed in this study. Further study may be needed to elucidate the utility of this biomarker.

8-OHdG as a biomarker

The present study found that the mean 8-OHdG excretion levels in CC1 smokers on both baseline and day 8 ranged from 2.94 ± 1.17 to 3.75 ± 1.10 μg/24 h (equivalent to 10.39 ± 4.13 to 13.25 ± 3.89 nmol/24 h) as compared with 24.9 ± 8.2 nmol/24 h in smokers reported by Pilger et al. (2001). The mean 8-OHdG excretion level in the no smoking group at day 8 was 2.81 ± 0.93 μg/24 h (equivalent to 9.93 ± 3.29 nmol/24 h) as compared with 20.5 ± 7.3 nmol/24 h in non-smokers reported by Pilger et al. There are two possible reasons for the difference between the observed 8-OHdG excretion levels in this study and those reported in the literature: first, different analytical methods were used and it is not known whether the two different methods would produce the similar results from the same samples; second, the study populations were different.

The 8-OHdG excretion levels in the two groups investigated in this study varied by a factor of approximately 3 for the CC1 group and 4 for the no smoking group at baseline, and 8 for the CC1 group and 4 for the no smoking group at day 8. Reportedly, a number of factors including smoking, age, gender, BMI, diet and exposure to ionizing radiation can influence the excretion of 8-OHdG in humans (Loft et al. 1992, 1993). In the present study, the mean 8-OHdG excretion level decreased significantly in the CC1 group from baseline to day 8. However, there was no change in the no smoking group. There was also no significant difference between the CC1 and no smoking groups either at baseline or at day 8. No significant correlation between 8-OHdG excretion levels in CC1 smokers and the daily number of cigarettes was observed. With other urinary biomarkers measured in this study, 8-OHdG significantly correlated with 1-OHP, 3-EtAd and thioethers, but not with nicotine equivalents, *S*-PMA, *t*,*t*-MA and 3-MeAd. Gender was associated with the 8-OHdG excretion with higher mean excretion levels in males than in females, which is consistent with the findings reported by Loft et al. (1992). Gender alone explained 22% of the variability in the 8-OHdG excretion levels. The gender effect on 8-OHdG excretion has been attributed to the fact that men have a higher metabolic rate than women (Loft et al. 1992). However, in contrast to the literature, BMI and age were not associated with the 8-OHdG excretion in the present study.

Overall, from the results observed in this study, 8-OHdG excretion levels do not seem to be associated with exposure due to cigarette smoking and therefore 8-OHdG may not be a useful biomarker for cigarette smoking induced oxidative DNA damage.

Urinary excretion of thioethers as a biomarker

In the present study, average urinary thioethers excretion levels were about 160 μmol/24 h for the subjects smoking CC1 at baseline and day 8, but significantly decreased to 72.48 μmol/24 h in the no smoking group at day 8. Surprisingly, neither the correlation between thioethers excretion levels and the daily number of cigarettes nor the within subject-correlation between the two time points in CC1 group was significant. This is probably because the background excretion levels of thioethers were high due to strong dietary influence and the sample size was not large enough. When compared with other biomarkers measured in this study, thioethers only correlated with 3-EtAd and 8-OHdG, the two biomarkers that, based on this study, have questionable relationships with cigarette smoking-related exposure. The inter-individual differences of the thioethers excretion levels ranged between 4- and 8-fold in CC1 smokers. However, for no smoking at day 8, the difference reached a factor of 16. In the multiple regression analysis, the number of cigarettes was again not a significant determinant for the thioethers excretion levels, even when age, gender, and BMI were included in the analysis. No effects of age, gender, or BMI of the CC1 smoker could be observed. In a study on the influence of diet and other factors on urinary levels of thioethers (Aringer & Lidums 1988), it was also reported that no effects of gender and age of the subjects could be found.

In summary, urinary excretion of thioethers as a biomarker for cigarette smoking-related exposure lacked sufficient sensitivity. A clear dose–response response relationship between the excretion levels and exposure to cigarette smoke could not be established, at least in the CC1 smokers. It might be only useful as an index biomarker in monitoring reduced exposure to electrophiles in the no smoking group if diet is controlled. More specific mercapturic acids from known smoke constituents might be more useful. Certainly, several biomarkers of similar nature would be needed to reflect the exposure to different electrophiles.

Conclusion

It can be concluded that under controlled study conditions: (1) 1-OHP, *S*-PMA and *t*,*t*-MA are useful biomarkers that could differentiate exposure between smoking conventional cigarettes and EHCSS cigarettes or between smoking conventional cigarettes and no smoking; between *S*-PMA and *t*,*t*-MA, the former appeared to be more sensitive; (2) 3-MeAd could only differentiate between smoking conventional cigarettes and no smoking; the results for 3-EtAd were inconclusive; (3) 8-OHdG had a questionable association with smoking-related exposure and therefore the utility of this biomarker for smoking-related exposure could not be established; and (4) urinary excretion of thioethers as a biomarker lacked sensitivity to demonstrate a clear dose–response relationship in conventional cigarette smokers, although it could differentiate the excretion levels between those subjects who smoked a conventional cigarette and those who stopped smoking.

References

Aringer L, Lidums V. 1988. Influence of diet and other factors on urinary levels of thioethers. International Archives of Occupational and Environmental Health 61:123–130.

Benowitz NL. 1983. The use of biologic fluid samples in assessing tobacco smoke consumption. NIDA Research Monograph 48:6–26.

Benowitz NL, Jacob P, III, Fong I, Gupta S. 1994. Nicotine metabolic profile in man: comparison of cigarette smoking and transdermal nicotine. Journal of Pharmacology and Experimental Therapeutics 268:296–303.

Boogaard PJ, Van Sittert NJ. 1996. Suitability of S-phenyl mercapturic acid and trans-trans-muconic acid as biomarkers for exposure to low concentrations of benzene. Environmental Health Perspectives 104(Suppl. 6):1151–1157.

Bos RP, Van Poppel G, Theuws JL, Kok FJ. 1992. Decreased excretion of thioethers in urine of smokers after the use of beta-carotene. International Archives of Occupational and Environmental Health 64:189–193.

Brzeznicki S, Jakubowski M, Czerski B. 1997. Elimination of 1-hydroxypyrene after human volunteer exposure to polycyclic aromatic hydrocarbons. International Archives of Occupational and Environmental Health 70:257–260.

Buckley TJ, Lioy PJ. 1992. An examination of the time course from human dietary exposure to polycyclic aromatic hydrocarbons to urinary elimination of 1-hydroxypyrene. British Journal of Industrial Medicine 49:113–124.

Byrd GD, Davis RA, Caldwell WS, Robinson JH, deBethizy JD. 1998. A further study of FTC yield and nicotine absorption in smokers. Psychopharmacology (Berlin) 139:291–299.

Castano-Vinyals G, D'Errico A, Malats N, Kogevinas M. 2004. Biomarkers of exposure to polycyclic aromatic hydrocarbons from environmental air pollution. Occupational and Environmental Medicine 61:e12.

Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA. 1992. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G-T and A-C substitutions. Journal of Biological Chemistry 267:166–172.

Cocco P, Tocco MG, Ibba A, Scano L, Ennas MG, Flore C, Randaccio FS. 2003. trans,trans-Muconic acid excretion in relation to environmental exposure to benzene. International Archives of Occupational and Environmental Health 76:456–460.

Dor F, Dab W, Empereur-Bissonnet P, Zmirou D. 1999. Validity of biomarkers in environmental health studies: the case of PAHs and benzene. Critical Reviews in Toxicology 29:129–168.

Dube MF, Green CR. 1982. Methods of collection of smoke for analytical purposes. Recent Advances in Tobacco Science 8:42–102.

Eissenberg T, Adams C, Riggins EC III, Likness M. 1999. Smokers' sex and the effects of tobacco cigarettes: subject-rated and physiological measures. Nicotine and Tobacco Research 1:317–324.

Hecht SS. 1999. Tobacco smoke carcinogens and lung cancer. Journal of the National Cancer Institute 91:1194–1210.

Hecht SS. 2002. Human urinary carcinogen metabolites: biomarkers for investigating tobacco and cancer. Carcinogenesis 23:907–922.

Hecht SS. 2003. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nature Reviews in Cancer 3:733–744.

Heinonen T, Kytoniemi V, Sorsa M, Vainio H. 1983. Urinary excretion of thioethers among low-tar and medium-tar cigarette smokers. International Archives of Occupational and Environmental Health 52:11–16.

Henderson PT, Van Doorn R, Leijdekkers CM, Bos RP. 1984. Excretion of thioethers in urine after exposure to electrophilic chemicals. IARC Scientific Publications, 173–187.

Heudorf U, Angerer J. 2001. Urinary monohydroxylated phenanthrenes and hydroxypyrene — the effects of smoking habits and changes induced by smoking on monooxygenase-mediated metabolism. International Archives of Occupational and Environmental Health 74:177–183.

IARC. 2004. IARC monographs on the evaluation of carcinogenic risks to humans, Vol. 83, Tobacco smoke and involuntary smoking. Lyon: IARC Press.

Jongeneelen FJ. 1997. Methods for routine biological monitoring of carcinogenic PAH-mixtures. Science of the Total Environment 199:141–149.

Jongeneelen FJ, Anzion RB, Leijdekkers CM, Bos RP, Henderson PT. 1985. 1-hydroxypyrene in human urine after exposure to coal tar and a coal tar derived product. International Archives of Occupational and Environmental Health 57:47–55.

Jongeneelen FJ, Anzion RB. 1990. 1-Hydroxypyrene. In: Angerer J, editor. Analyses of hazardous substances in biological materials. Weinheim: VCH. p. 151–169.

Jongeneelen FJ, Bos RP. 1990. Excretion of pyrene and hydroxypyrene in urine. Cancer Letters 51:175–179.

Kasai H. 1997. Analysis of a form of oxidative DNA damage, 8-hydroxy-2′-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. Mutation Research 387:147–163.

Kopplin A, Eberle-Adamkiewicz G, Glusenkamp KH, Nehls P, Kirstein U. 1995. Urinary excretion of 3-methyladenine and 3-ethyladenine after controlled exposure to tobacco smoke. Carcinogenesis 16:2637–2641.

Loft S, Fischer-Nielsen A, Jeding IB, Vistisen K, Poulsen HE. 1993. 8-Hydroxydeoxyguanosine as a urinary biomarker of oxidative DNA damage. Journal of Toxicology and Environmental Health 40:391–404.

Loft S, Vistisen K, Ewertz M, Tjonneland A, Overvad K, Poulsen HE. 1992. Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index. Carcinogenesis 13:2241–2247.

Meijer GA, Westerterp KR, Saris WH, ten Hoor F. 1992. Sleeping metabolic rate in relation to body composition and the menstrual cycle. American Journal of Clinical Nutrition 55:637–640.

Melikian AA, Qu Q, Shore R, Li G, Li H, Jin X, Cohen B, Chen L, Li Y, Yin S, Mu R, Zhang X, Wang Y. 2002. Personal exposure to different levels of benzene and its relationships to the urinary metabolites S-phenylmercapturic acid and trans,trans-muconic acid. Journal of Chromatography B: Biomedical Sciences and Applications 778:211–221.

Nerurkar PV, Okinaka L, Aoki C, Seifried A, Lum-Jones A, Wilkens LR, Le Marchand L. 2000. CYP1A1, GSTM1, and GSTP1 genetic polymorphisms and urinary 1-hydroxypyrene excretion in non-occupationally exposed individuals. Cancer Epidemiology, Biomarkers and Prevention 9:1119–1122.

Nia AB, Van Schooten FJ, Schilderman PA, De Kok TM, Haenen GR, Van Herwijnen MH, Van Agen E, Pachen D, Kleinjans JC. 2001. A multi-biomarker approach to study the effects of smoking on oxidative DNA damage and repair and antioxidative defense mechanisms. Carcinogenesis 22:395–401.

Ong CN, Lee BL. 1994. Determination of benzene and its metabolites: Application in biological monitoring of environmental and occupational exposure to benzene. Journal of Chromatography B: Biomedical Sciences and Applications 660:1–22.

Patskan G, Reininghaus W. 2003. Toxicological evaluation of an electrically heated cigarette. Part 1: Overview of technical concepts and summary of findings. Journal of Applied Toxicology 23:323–328.

Pezzagno G, Maestri L, Fiorentino ML. 1999. Trans,trans-muconic acid, a biological indicator to low levels of environmental benzene: some aspects of its specificity. American Journal of Industrial Medicine 35:511–518.

Phillips DH. 2002. Smoking-related DNA and protein adducts in human tissues. Carcinogenesis 23:1979–2004.

Pilger A, Germadnik D, Riedel K, Meger-Kossien I, Scherer G, Rudiger HW. 2001. Longitudinal study of urinary 8-hydroxy-2′-deoxyguanosine excretion in healthy adults. Free Radical Research 35:273–280.

Prevost V, Shuker DE. 1996. Cigarette smoking and urinary 3-alkyladenine excretion in man. Chemical Research in Toxicology 9:439–444.

Prevost V, Shuker DE, Friesen MD, Eberle G, Rajewsky MF, Bartsch H. 1993. Immunoaffinity purification and gas chromatography-mass spectrometric quantification of 3-alkyladenines in urine: metabolism studies and basal excretion levels in man. Carcinogenesis 14:199–204.

Prieme H, Loft S, Klarlund M, Gronbaek K, Tonnesen P, Poulsen HE. 1998. Effect of smoking cessation on oxidative DNA modification estimated by 8-oxo-7,8-dihydro-2′-deoxyguanosine excretion. Carcinogenesis 19:347–351.

Qu Q, Melikian AA, Li G, Shore R, Chen L, Cohen B, Yin S, Kagan MR, Li H, Meng M, et al. 2000. Validation of biomarkers in humans exposed to benzene: urine metabolites. American Journal of Industrial Medicine 37:522–531.

Qu Q, Shore R, Li G, Jin X, Chen LC, Cohen B, Melikian AA, Eastmond D, Rappaport S, Li H, et al. 2003. Validation and evaluation of biomarkers in workers exposed to benzene in China. Health Effects Institute Research Report, 1–72.

Renner T, Fechner T, Scherer G. 2000. Fast quantification of the urinary marker of oxidative stress 8-hydroxy-2′-deoxyguanosine using solid-phase extraction and high-performance liquid chromatography

with triple-stage quadrupole mass detection. Journal of Chromatography B: Biomedical Sciences and Applications 738:311–317.

Roethig HJ, Kinser RD, Lau RW, Walk RA, Wang N. 2005. Short-term exposure evaluation of adult smokers switching from conventional to first generation electrically heated cigarettes during controlled smoking. Journal of Clinical Pharmacology 45:133–145.

Rossi AM, Guarnieri C, Rovesti S, Gobba F, Ghittori S, Vivoli G, Barale R. 1999. Genetic polymorphisms influence variability in benzene metabolism in humans. Pharmacogenetics 9:445–451.

Ruppert T, Scherer G, Tricker AR, Rauscher D, Adlkofer F. 1995. Determination of urinary trans,trans-muconic acid by gas chromatography-mass spectrometry. Journal of Chromatography B: Biomedical Sciences and Applications 666:71–76.

Scherer G, Doolittle DJ, Ruppert T, Meger-Kossien I, Riedel K, Tricker AR, Adlkofer F. 1996. Urinary mutagenicity and thioethers in nonsmokers: role of environmental tobacco smoke (ETS) and diet. Mutation Research 368:195–204.

Scherer G, Frank S, Riedel K, Meger-Kossien I, Renner T. 2000. Biomonitoring of exposure to polycyclic aromatic hydrocarbons of nonoccupationally exposed persons. Cancer Epidemiology, Biomarkers and Prevention 9:373–380.

Scherer G, Meger M, Kossien I, Pachinger A. 2001. Biological monitoring of the tobacco smoker-related exposure to benzene. Proceedings of the American Association for Cancer Research 42:150.

Scherer G, Renner T, Meger M. 1998. Analysis and evaluation of trans,trans-muconic acid as a biomarker for benzene exposure. Journal of Chromatography B: Biomedical Sciences and Applications 717:179–199.

Shuker DE, Farmer PB. 1992. Relevance of urinary DNA adducts as markers of carcinogen exposure. Chemical Research in Toxicology 5:450–460.

Sithisarankul P, Vineis P, Kang D, Rothman N, Caporaso N, Strickland P. 1997. Association of 1-hydroxypyrene-glucuronide in human urine with cigarette smoking and broiled or roasted meat consumption. Biomarkers 2:217–221.

Stabbert R, Voncken P, Rustemeier K, Haussmann HJ, Roemer E, Schaffernicht H, Patskan G. 2003. Toxicological evaluation of an electrically heated cigarette. Part 2: Chemical composition of mainstream smoke. Journal of Applied Toxicology 23:329–339.

Stratton K, Shetty P, Wallace R, Bondurant S, editors. 2001. Clearing the smoke: assessing the science base for tobacco harm reduction. Washington, DC: National Academy Press.

Strickland P, Kang D, Sithisarankul P. 1996. Polycyclic aromatic hydrocarbon metabolites in urine as biomarkers of exposure and effect. Environmental Health Perspectives 104(Suppl. 5):927–932.

Van Doorn R, Bos RP, Leijdekkers CM, Wagenaas-Zegers MA, Theuws JL, Henderson PT. 1979. Thioether concentration and mutagenicity of urine from cigarette smokers. International Archives of Occupational and Environmental Health 43:159–166.

Van Doorn R, Leijdekkers CM, Bos RP, Brouns RM, Henderson PT. 1981. Detection of human exposure to electrophilic compounds by assay of thioether detoxication products in urine. Annals of Occupational Hygiene 24:77–92.

Van Rooij JG, Veeger MM, Bodelier-Bade MM, Scheepers PT, Jongeneelen FJ. 1994. Smoking and dietary intake of polycyclic aromatic hydrocarbons as sources of interindividual variability in the baseline excretion of 1-hydroxypyrene in urine. International Archives of Occupational and Environmental Health 66:55–65.

Van Schooten FJ, Jongeneelen FJ, Hillebrand MJ, Van Leeuwen FE, de Looff AJ, Dijkmans AP, Van Rooij JG, Den Engelse L, Kriek E. 1995. Polycyclic aromatic hydrocarbon-DNA adducts in white blood cell DNA and 1-hydroxypyrene in the urine from aluminum workers: relation with job category and synergistic effect of smoking. Cancer Epidemiology, Biomarkers and Prevention 4:69–77.

Verdina A, Galati R, Falasca G, Ghittori S, Imbriani M, Tomei F, Marcellini L, Zijno A, Vecchio VD. 2001. Metabolic polymorphisms and urinary biomarkers in subjects with low benzene exposure. Journal of Toxicology and Environmental Health A 64:607–618.

Viau C, Diakite A, Ruzgyte A, Tuchweber B, Blais C, Bouchard M, Vyskocil A. 2002. Is 1-hydroxypyrene a reliable bioindicator of measured dietary polycyclic aromatic hydrocarbon under normal conditions? Journal of Chromatography B: Biomedical Sciences and Applications 778:165–177.

Wan J, Shi J, Hui L, Wu D, Jin X, Zhao N, Huang W, Xia Z, Hu G. 2002. Association of genetic polymorphisms in CYP2E1, MPO, NQO1, GSTM1, and GSTT1 genes with benzene poisoning. Environmental Health Perspectives 110:1213–1218.

Weaver VM, Buckley T, Groopman JD. 2000. Lack of specificity of trans,trans-muconic acid as a benzene biomarker after ingestion of sorbic acid-preserved foods. Cancer Epidemiology, Biomarkers and Prevention 9:749–755.

World Health Organization. 2000. Human exposure assessment. Environmental Health Criteria No. 214. Geneva: WHO. p. 230–254.