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Biomarkers of exposure and potential harm in adult smokers of 3–7 mg tar yield (Federal Trade Commission) cigarettes and in adult non-smokers

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Abstract

The paper reports levels of 24-h urine nicotine and five of its major metabolites (expressed as nicotine-equivalents) and blood carboxyhaemoglobin as biomarkers of exposure to particulate- and gas-phase cigarette smoke, respectively, from an exploratory pilot study of adult smokers of 3.0–6.9 mg tar delivery (Federal Trade Commission (FTC) method) cigarettes. On multiple occasions over 6 weeks, blood high-sensitivity C-reactive protein (hs-CRP), fibrinogen, HDL- and LDL-cholesterol, and 24-h urine 8-*epi*-prostaglandin $F_{2\alpha}$ (8-*epi*-$PGF_{2\alpha}$) and 11-dehydro-thromboxane B_2 (11-dehydro-TxB_2) were also evaluated as biomarkers of potential harm. All the biomarkers examined, except for LDL-cholesterol, discriminated with high sensitivity and specificity between adult smokers and non-smokers overall. Except for HDL-cholesterol, all biomarker medians were greater in adult smokers than in non-smokers: urine nicotine-equivalents 64.514 versus <0.034 nmol mg^{-1} creatinine ($p<0.001$), carboxyhaemoglobin 4.0 versus 0.4% saturation ($p<0.001$), hs-CRP 0.27 versus 0.12 mg dl^{-1} ($p=0.05$), fibrinogen 292 versus 248 mg dl^{-1} ($p<0.001$), HDL-cholesterol 46 versus 53 mg dl^{-1} ($p=0.003$), LDL-cholesterol 119 versus 109 mg dl^{-1} ($p=0.18$), urine 8-*epi*-$PGF_{2\alpha}$ 1935 versus 1034 pg mg^{-1} creatinine ($p<0.001$) and urine 11-dehydro-TxB_2 973 versus 710 pg mg^{-1} creatinine ($p<0.001$). All the biomarkers of exposure and most of the biomarkers of potential harm showed no time of sampling (by visit week) effect.

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Introduction

Scientific evidence of the harm to smokers from tobacco use has accumulated over the decades. The medical and scientific consensus is that cigarette smoking causes lung cancer, heart disease, emphysema and other serious diseases in smokers (Stratton et al. 2001). Smokers are far more likely to develop serious diseases, such as lung cancer, than non-smokers (US Department of Health and Human Services 1989, Nelson et al. 1994). However, the relationship between specific smoke constituents and these diseases is poorly understood. Similarly, the actual amount of smoke

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and smoke constituents to which a smoker is exposed is not well characterized (Stratton et al. 2001).

The number of chemical compounds to which a smoker is exposed when smoking a cigarette has been estimated as 4800 (Green & Rodgman 1996). The rate and amount of exposure to these chemicals are complex functions of cigarette composition and design, rate of smoking, burn temperature and many other factors (Baker 1999). The relative delivery of mainstream smoke constituents (e.g. nicotine and tar) of different cigarettes is typically determined in smoke generated by machines operated under defined smoking regimens (e.g. the Federal Trade Commission (FTC) method) (Federal Trade Commission 1967, Pillsbury et al. 1969). Tobacco smoke uptake by smokers is influenced not only by cigarette composition and design, but also by many smoker-dependent parameters, e.g. ventilation hole blocking (Baker & Lewis 1997), puffing patterns and number of cigarettes smoked (Bridges et al. 1990, Hofer et al. 1991). Therefore, it is difficult to establish dose–effect relationships for toxic smoke constituents in humans, as the dose/uptake is difficult to predict.

The purpose of this single-centre, exploratory, pilot study was to evaluate the study design and logistics for a subsequent large, multicentre study of adult cigarette smokers representing all tar and nicotine categories in the US market. To determine the exposure of US adult smokers to cigarette smoke, a direct evaluation of the levels of smoke constituents or their metabolites in appropriate body fluids was used. In this pilot study, biomarkers of exposure and biomarkers of potential harm (Stratton et al. 2001) were evaluated in adult smokers who regularly smoked cigarette brands of 3.0–6.9 mg tar delivery, as measured by the FTC method. Selecting this lower tar delivery category, smoked by less than 11% of US adult smokers (Maxwell & Fenstermacher 1999), permitted evaluation of the adequacy of the limits of quantification of the analytical methods used to assess biomarker concentrations, and explored whether the selected biomarkers could differentiate between adult smokers and non-smokers.

The selection of biomarkers of exposure was based on modified National Research Council guidelines (Benowitz 1999). We selected biomarkers of exposure that are tobacco smoke constituents or metabolites of tobacco smoke constituents, which are representative of the particulate and gas phases of tobacco smoke. In general, these biomarkers are representative of health-relevant tobacco smoke constituents, require only minimally invasive specimen sampling procedures, are measurable by sensitive and reliable analytical methods, and have concentrations reflective of uptake of cigarette smoke constituents.

Carboxyhaemoglobin (COHb), a product of the interaction of haemoglobin and carbon monoxide (CO), was measured as a representative biomarker of exposure to gas-phase cigarette smoke constituents. CO combines reversibly with oxygen-carrying sites on the haemoglobin molecule with an affinity ranging from 210 to 240 times greater than that of oxygen, which results in decreased oxygen-carrying capacity of the blood. CO also alters the dissociation of oxygen from haemoglobin sites, which compromises the delivery of oxygen to the tissues (Varon et al. 1999, World Health Organization 1999). COHb is fully dissociable, and, once exposure has ceased and the CO is eliminated through the lungs, the haemoglobin reverts to oxyhaemoglobin. The elimination of CO is related to minute ventilation, and the half-life of COHb while breathing room air is 4–6 h (Pace et al. 1950).

Urine nicotine-equivalents, the molar sum of urine nicotine, cotinine, *trans*-3′-hydroxycotinine, and their corresponding glucuronides, measured in a 24-h urine

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collection, provide a measure of the amount of nicotine that enters the body daily and was the biomarker of exposure to particulate-phase cigarette smoke constituents in the present study (Boswell et al. 2000). This molar sum accounts for approximately 80–85% of total nicotine uptake in humans (Curvall et al. 1991, Benowitz et al. 1994, Schepers & Demetriou 2002).

Non-invasively acquired biomarkers that reflect biochemical mechanisms of disease and that differ quantitatively between smokers and non-smokers or former smokers include total cholesterol, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol (Muscat et al. 1991, Eliasson et al. 2001), fibrinogen (Meade et al. 1987, Eliasson et al. 2001, Bazzano et al. 2003), and high-sensitivity C-reactive protein (hs-CRP) (Bazzano et al. 2003). These biomarkers of potential harm, measured in this study, have been validated as surrogate markers for cardiovascular disease outcomes (Gordon et al. 1977, Stamler et al. 1986, Chen et al. 1991, Ridker & Haughie 1998, Koenig et al. 1999, Ridker et al. 2001). Urinary 11-dehydro-thromboxane B_2 (11-dehydro-TxB_2) and 8-*epi*-prostaglandin $F_{2\alpha}$ (8-*epi*-PG$F_{2\alpha}$), markers of platelet activation and oxidative stress, respectively (FitzGerald et al. 1983, Morrow et al. 2002), were also measured. These two arachadonic acid metabolites have not yet been validated as predictive of clinical outcomes.

This pilot study also investigated whether selected biomarkers of exposure to cigarette smoke constituents and selected biomarkers of potential harm are adequately sensitive and specific to discriminate between adult cigarette smokers and non-smokers and determined each biomarker's within- and between-subject variability.

Materials and methods

Study design

This was an exploratory pilot study with two parallel groups (adult smokers and adult non-smokers), and two subgroups (males and females) within each group, with five clinic visits over the 6-week study period. During the study, the adult smokers continued to smoke their usual brand of 3.0–6.9 mg tar (FTC) cigarettes in an unrestricted manner. Subjects provided their own cigarettes. The FTC tar rating of each subject's cigarette brand was taken from the most recent Tobacco Institute Testing Laboratory Market Sample #43 Report (2001).

Study subjects

Eligible participants were community-based adult volunteers at least 21 years of age in good general health. No subjects used any tobacco- or nicotine-containing products other than cigarettes for at least the 3 months before entering the study. For this study, smokers were defined as smoking at least one manufactured cigarette daily for at least the past year and smoking exclusively cigarettes in the 3.0–6.9 mg FTC tar delivery category for at least the past 3 months, with at least 90% of daily cigarette consumption of the same brand. Non-smokers were defined as not smoking for at least the past year. Exclusion criteria included clinically significant renal, liver, metabolic, cardiac or pulmonary disease; evidence of hepatitis B or C or HIV infection; or illicit drug use. Women of childbearing potential were excluded if they were pregnant, lactating or intending to get pregnant during the study.

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Subjects were volunteers recruited through local advertising (Madison, WI, USA) and were paid for their participation. All subjects gave written informed consent, and the study was approved by the Covance Clinical Research Unit Institutional Review Board.

Subject enrolment, study conduct and sample collection

A structured telephone interview was used to determine initial eligibility. At a subsequent screening visit, written informed consent to participate was obtained from each subject followed by a medical history including concomitant medications, tobacco and alcohol use history, and measurement of vital signs (oral temperature, respiratory rate, automated seated resting blood pressure, pulse), 12-lead electrocardiogram, lung spirometry (forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV_1)), clinical blood chemistry, complete blood cell count, urinalysis, urine pregnancy test (females of childbearing potential only), urine illicit drug screen, hepatitis B and C blood tests, and HIV blood test. Eligible subjects returned within 4 weeks for an enrolment visit that included a physical examination with height and weight, and repeat of screening blood and urine tests. A trained interviewer administered a questionnaire (repeated at week 6 visit) about demographic characteristics, exposures to agents that might affect measured biomarkers (i.e. occupational, hobbies, household, solvents), exposure to environmental tobacco smoke, home heating/ventilation systems, dietary/lifestyle habits, physical activity, smoking behaviour (e.g. number of cigarettes smoked per day, depth of inhalation), tobacco product usage, and use of non-tobacco nicotine products.

Subjects returned to the research site 1, 2, 3 and 6 weeks after the enrolment visit for venous blood sampling. They were instructed to fast for 6 h before each visit. At each visit, vital signs, urine pregnancy test (females of childbearing potential only), and hepatitis and HIV blood tests were obtained, and each subject was queried about any change in health status, concomitant medications and smoking consumption or exposure to environmental tobacco smoke over the prior 3 days. During the 72 h before each visit, each adult smoker kept a written diary of smoking consumption, and each adult non-smoker kept a written diary of exposure to environmental tobacco smoke. Smoking subjects collected the packs from all cigarettes smoked during this 72-h period. During the 24-h interval before the week 1, 2 and 3 visits, each subject collected all voided urine in a high-density polyethylene (HDPE) screw-capped specimen container (Support Plastics Ltd, Beloeil, Quebec, Canada) stored in a portable cooler with a gel refrigerant pack. The subjects kept the cooler with them during the 24-h interval as they performed their usual daily routine.

Plasma, serum and urine were stored in aliquots at $-20^{\circ}C$ until used for the various analyses. The 24-h urine collections were analysed for nicotine and five of its major metabolites, 11-dehydro-TxB_2, 8-*epi*-$PGF_{2\alpha}$ and creatinine. At each visit, blood was analysed for COHb. At the week 1, 3 and 6 visits, blood was analysed for fibrinogen, hs-CRP, triglycerides, total and HDL-cholesterol, and LDL-cholesterol was calculated.

Laboratory methods

All biomarkers were analysed using validated analytical methods with appropriate quality controls in compliance with US Food and Drug Association (FDA) Guidelines (2001) at certified clinical laboratories. Analytical performance parameters are shown in Tables I and II.

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Table I. Assay analytical performance: biomarkers of exposure.

Biomarker	Assay performance parameter		
	Lower limit of quantification	Linear range	Inter-assay coefficient of variation (%)
Carboxyhaemoglobin (% saturation)	0.3	0.3–64.7	4.7
Nicotine and metabolites ($ng\ ml^{-1}$)‡	1	1–1000	14.5

‡For nicotine, cotinine and *trans*-3′-hydroxycotinine.

Blood specimens. At the research site, immediately after specimen collection, COHb was determined spectrophotometrically with an IL-682 CO-Oximeter (Instrumentation Laboratory; Lexington, MA, USA). High-sensitivity CRP was measured with a Dade Behring Nephelometer Analyzer System™ (Behring Diagnostics; Westwood, MA, USA). Fibrinogen was analysed by monitoring clot formation with photometric detection by the MLA 1600 (Medical Laboratory Automation; Mt Vernon, NY, USA). Urinalysis, complete blood cell count, clinical blood chemistry, triglycerides, and total and HDL-cholesterol were measured at a certified clinical laboratory using standard clinical laboratory procedures for which acceptable proficiency testing data were provided. LDL-cholesterol was calculated using the Friedewald equation as:

$$\text{LDL-C} = (\text{total cholesterol}) - (\text{HDL-C}) - (\text{triglycerides} \times 0.2)$$

and is invalid for triglycerides $>400\ mg\ dl^{-1}$ (Friedewald et al. 1972).

Urine specimens. Nicotine, cotinine and *trans*-3′-hydroxycotinine were extracted from a 500-μl aliquot of the 24-h urine specimen using mixed-mode solid-phase extraction (SPE), then measured by high-performance liquid chromatography (HPLC) with tandem mass spectrometric (MS/MS) detection:

- SPE: IST-HCX-3 cartridge (Jones Chromatography, Hengoed, UK) eluted sequentially with 10 mM ammonium acetate, 5% acetic acid, methanol and 5% NH_4OH in methanol.
- LC/MS/MS (Applied Biosystems™ API 3000, Foster City, CA, USA).

Table II. Assay analytical performance: biomarkers of potential harm.

Biomarkers	Assay performance parameter	
	Lower limit of quantification	Inter-assay coefficient of variation (%)
hs C-reactive protein ($mg\ dl^{-1}$)	0.02	4.8
Fibrinogen ($mg\ dl^{-1}$)	100	5.4
HDL-cholesterol ($mg\ dl^{-1}$)	10	4.5
LDL-cholesterol ($mg\ dl^{-1}$)‡	n.a.	n.a.
8-*epi*-Prostaglandin $F_{2\alpha}$ ($pg\ ml^{-1}$)	10	12.8
11-Dehydro-thromboxane B_2 ($pg\ ml^{-1}$)	14	25.2

‡LDL-cholesterol is calculated from the Friedewald equation as: LDL-C = (total cholesterol) − (HDL-C) − (triglycerides × 0.2) and is invalid for triglycerides $>400\ mg\ dl^{-1}$.

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- HPLC: Series 200 LC pump (PerkinElmer, Wellesley, MA, USA), HTC-PAL autosampler and sample cooler (CTC Analytics AG, Zwingen, Switzerland), 7990 column oven (Jones Chromatography, Lakewood, CO, USA), universal switching valve (Anachem, Inc., Allen, TX, USA), Phenomenex 10 cm $\times$2.1 mm 3 μm Phenyl-Hexyl column operated at 50°C and eluted with ammonium acetate/methanol/triethylamine (65:35:0.002 v/v/v with a flow rate of 0.2 ml min^{-1}).
- MS/MS: heated nebulizer interface; positive-ion mass spectrometry with the deuterated aglycones as internal standards and the following transitions monitored: 163.2 $\rightarrow$ 84.0 (nicotine); 177.1 $\rightarrow$ 80.1 (cotinine); 193.2 $\rightarrow$ 80.1 (*trans*-3′-hydroxycotinine); 166.2 $\rightarrow$ 87.0 (nicotine-d_3); 180.1 $\rightarrow$ 80.1 (cotinine-d_3); 196.2 $\rightarrow$ 80.1 (*trans*-3′-hydroxycotinine-d_3).

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Sources of the reference materials were: (±) nicotine-d_3 salicylate salt and (±) cotinine-d_3 (Sigma-Aldrich Corp., St Louis, MO, USA); (±) *trans*-3′-hydroxycotinine methyl-d_3 (Toronto Research Chemicals (TRC), Ontario, Canada); (–) nicotine hydrogen tartrate salt and (–) cotinine (Sigma-Aldrich); (3′*R*,5′*S*) *trans*-3′-hydroxycotinine (TRC).

For all three aglycones, the lower limit of quantification was 1 ng ml^{-1}, and, for three quality control sample levels, inter-assay precision was better than 12%, and inter-assay accuracy was better than 15%. Nicotine-*N*-glucuronide, cotinine-*N*-glucuronide (both obtained from TRC), and *trans*-3′-hydroxycotinine-*O*-glucuronide were determined indirectly in a 500 μl urine aliquot pretreated for 18–22 h at 37°C with β-glucuronidase (Type H-1 from *Helix pomatia*) (Sigma-Aldrich), then analysed in the same manner. The level of glucuronide present was determined by difference between the aglycone levels before (referred to as 'free aglycone') and after (referred to as 'total aglycone') deconjugation with β-glucuronidase (Covance Laboratories, Harrogate, UK).

Nicotine-equivalents were calculated as the molar sum of total nicotine, total cotinine and total *trans*-3′-hydroxycotinine excreted in the 24-h urine. The concentration of each metabolite was first adjusted by the 24-h urine volume to obtain the total amount excreted in 24 h, then divided by the molecular weight of the metabolite to obtain the total amount of each in moles. The sum in moles was then divided by the total amount of urine creatinine (mg/24 h) to give the 24-h excretion of nicotine-equivalents (nmol mg^{-1} creatinine). To determine the nicotine-equivalents per cigarette smoked per day, the 24-h molar sum of nicotine-equivalents (nmol/24 h) was converted to the 24-h mass of nicotine-equivalents (mg/24 h) by multiplying by the molecular weight of nicotine, then dividing by the number of cigarettes smoked that day as determined from the subject smoking diary (mg per cigarette).

11-Dehydro-TxB_2 and 8-*epi*-$PGF_{2\alpha}$ concentrations (pg ml^{-1}) were determined by microplate EIA immunoassays directly, without preliminary concentration (Cayman Chemical Co., Ann Arbor, MI, USA; Covance Laboratories). The total amount excreted over 24 h was calculated and adjusted for the 24-h urine creatinine. Assay performance was demonstrated to be consistent with specifications listed by the kit manufacturer.

Urine creatinine concentration was measured (Covance Clinical Laboratory Services Inc., Indianapolis, IN, USA) using the rate alkaline picrate (Jaffe) method.

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Completeness of 24-h urine collections

Because of the difficulty in clinical practice of obtaining accurate timed urine collections, we assessed compliance of each subject with complete 24-h collection by evaluating creatinine excretion for each 24 h and across all 24-h periods. We noted 24-h urine collections with creatinine levels below the 75th percentile of the lower limit of the gender-specific reference range (i.e. males <750 and females <500 mg creatinine/24 h) (Burtis & Ashwood 1999). We calculated the mean and SD for each of the subject's three 24-h urine collections and noted the collection with the lowest daily creatinine excretion if the coefficient of variation exceeded 50%.

Statistical methods

SAS® version 8.2 (SAS Institute, Inc., Cary, NC, USA) was used to carry out the statistical analysis of the data. At each time point, only data for evaluable subjects were analysed (i.e. those subjects who continued to fulfil study eligibility criteria at that time point and who had reportable results at baseline and at that time point). For data analysis, biomarker measurements below the lower limit of quantification (BLLOQ) (e.g. nicotine and nicotine metabolites in non-smokers) were set to the lower limit of quantification. Due to the asymmetric distribution of most biomarkers, medians (minimums and maximums) were used to present the data. Biomarker levels were compared between the two groups (i.e. adult smokers and non-smokers), and by gender within groups (e.g. male adult smokers and male non-smokers) using the non-parametric Wilcoxon's rank-sum test. As this was not a hypothesis-driven study, p-values are presented as a guide to the significance of the difference between the adult smokers and non-smokers.

Analytical results for urine biomarkers of potential harm were adjusted for daily creatinine excretion, and analytical results for nicotine-equivalents in adult smokers were adjusted for daily cigarette consumption.

Depending on the biomarker, there were two, three or four sample collections during the 6-week study. To examine whether multiple sample collections or a single sample collection was warranted for a future US study using a large number of subjects, a mixed effects analysis of variance model was used to model the effect over time for each biomarker, comparing a model that included all possible effects (i.e. group, gender, time and all possible interactions) to a model that contained main effects and two-way interactions that were not time-related. Akaike's criterion was used to determine the appropriate covariance structure. Within- and between-subject variability estimates were obtained for adult smokers and non-smokers.

Results

Study subjects

Between May and June 2001, 72 healthy adult smokers of 3.0–6.9 mg FTC tar delivery cigarettes and 68 healthy adult non-smokers were enrolled in the study. All 68 non-smokers and 67 of 72 (93%) adult smokers originally enrolled completed the 6-week study. In the smoking group, 17/67 (25%) were excluded from analysis, mainly due to violation of cigarette smoking eligibility criteria (see above), resulting in 50 evaluable adult smokers (18 males, 32 females). In the non-smokers, 3/68 (4%) were

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excluded from analysis due to violation of non-smoking eligibility criteria (see above), resulting in 65 evaluable non-smokers (30 males, 35 females). Demographic characteristics of the evaluable adult smoking and non-smoking groups were similar at enrolment (Table III), but there were differences between these groups in the frequency of use of certain concomitant medications and supplements (Table IV).

24-h urine collections

Of the 344 24-h urine samples, eight (2%) contained less creatinine than the 75th percentile of the lower limit of the gender-specific reference range. One of the 344 samples (0.3%) had greater than 50% coefficient of variation for serial daily creatinine excretion. We analysed the data with and without these nine samples, and obtained virtually the same results for the biomarkers determined from 24-h urine collections (i.e. nicotine-equivalents, 8-*epi*-$PGF_{2\alpha}$ and 11-dehydro-TxB_2), so all samples are included in the results reported herein.

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Biomarkers of exposure

Comparisons between adult smokers and non-smokers are shown in Table V.

The median COHb level measured in adult smokers overall was tenfold greater than in non-smokers (4.0 versus 0.4% saturation, $p < 0.001$). Male adult smokers had levels ninefold higher than male non-smokers (3.6 versus 0.4% saturation, $p < 0.001$), and female adult smokers' levels were on average 14-fold greater than female non-smokers (4.1 versus 0.3% saturation, $p < 0.001$).

Median nicotine exposure, expressed as nicotine-equivalents, was 64.51 nmol mg^{-1} creatinine in adult smokers overall. By gender, the median level in male adult smokers was 58.04 nmol mg^{-1} creatinine and in female adult smokers 66.34 nmol mg^{-1} creatinine. On a per cigarette basis, adult smokers overall excreted a median of 0.96 mg nicotine-equivalents per cigarette (range 0.03, 3.59) (males 0.89 (range 0.03, 3.59), females 0.96 (0.39, 3.01) mg nicotine-equivalents per cigarette). As can be seen by the ranges of these values, the between-subject variability was large (Table VI).

Of the 105 measurements of urine nicotine and metabolites in female non-smokers (35 evaluable subjects, sampled at weeks 1, 2 and 3), 41% of the total *trans*-3′-hydroxycotinine levels were below the 1 ng ml^{-1} limit of quantification, as were 60% of the total cotinine levels and 87% of the total nicotine levels. For male non-smokers (30 evaluable subjects sampled at weeks 1, 2 and 3), the corresponding percent below the limit of quantification were 19% total *trans*-3′-hydroxycotinine levels, 41% total cotinine levels and 59% total nicotine levels. Coefficients of variability for urine nicotine-equivalents within and between non-smoking subjects are not reported because of the high number of values below the lower limit of quantification. For data analysis purposes, biomarker values below the lower limit of quantification were set to the limit of quantification. Therefore, differences in these very low nicotine-equivalents values, normalized by urine creatinine, were due solely to variations in urine creatinine concentrations.

Means, medians, interquartile ranges and outliers for COHb by smoking status and for urine nicotine-equivalents by smoking status are shown in Figures 1 and 2. Even for the broad dose estimates of zero cigarettes per day, more than 20 cigarettes per day, and less than 20 cigarettes per day, a dose–response was observed for both

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Table III. Baseline characteristics of evaluable subjects.

Characteristic	Overall		Males		Females	
	Adult smokers ($n=50$)	Non-smokers ($n=65$)	Adult smokers ($n=18$)	Non-smokers ($n=30$)	Adult smokers ($n=32$)	Non-smokers ($n=35$)
Age (years):						
Mean (SD)	35.8 (11.1)	36.0 (13.6)	33.1 (10.5)	33.5 (12.4)	37.3 (11.2)	38.1 (14.4)
(minimum, maximum)	(21, 68)	(21, 76)	(22, 57)	(22, 66)	(21, 68)	(21, 76)
Race (n) (%)*						
Caucasian	48 (96.0)	60 (92.3)	18 (100.0)	28 (93.3)	30 (93.8)	32 (91.4)
African-American	2 (4.0)	3 (4.6)		1 (3.3)	2 (6.3)	2 (5.7)
Other[†]	3 (6.0)	2 (3.1)		1 (3.3)	3 (9.4)	1 (2.9)
Weight (lb):						
Mean (SD)	180.2 (46.9)	170 (30.1)	189.5 (46.5)	177.4 (28.9)	174.9 (47.0)	163.6 (29.9)
(minimum, maximum)	(112.2, 307.3)	(117.8, 268.0)	(129.0, 307.3)	(140.0, 268.0)	(112.2, 292.5)	(117.8, 234.0)
Number cigarettes per day (n):						
Mean (SD)	17.6 (8.7)	0	19.6 (9.1)	0	16.4 (8.4)	0
(minimum, maximum)	(3, 40)		(3, 40)		(3, 30)	

*Totals may exceed 100% as some subjects reported more than one race.
[†]'Other' includes Hispanic, Latino, Native American and Asian.

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Table IV. Concomitant medications and supplements consumed by subjects during the study*.

	Males		Females	
Concomitant medication or supplement	Adult smokers ($n=18$)	Non-smokers ($n=30$)	Adult smokers ($n=32$)	Non-smokers ($n=35$)
Aspirin	2 (11%)	5 (17%)	5 (16%)	7 (20%)
Other non-steroidal anti-inflammatory agent	8 (44%)	5 (17%)	12 (38%)	10 (29%)
HMG CoA reductase inhibitor[‡]	1 (6%)	0	0	0
Hormonal contraceptive	n.a.	n.a.	8 (25%)	11 (31%)
Hormone replacement therapy	n.a.	n.a.	1 (3%)	1 (3%)
Vitamins	11 (61%)	10 (33%)	11 (34%)	13 (37%)
Systemic antibiotic	2 (11%)	1 (6%)	6 (19%)	0

*n (%) using at least once during the study period.
‡'Statin'.
n.a., Not applicable.

biomarkers. Not unexpectedly, the ranges of biomarker values are much wider for the smokers than for the non-smokers.

An overall test of time effect between a full and a reduced model containing smoking status, gender and smoking status by gender interaction was performed using a likelihood ratio test. Despite the high within-subject variability, none of the biomarkers of exposure showed a significant time of sampling (by visit week) effect (data not shown).

Biomarkers of potential harm

Comparisons between adult smokers and non-smokers are shown in Table V. Adult smokers, overall and by gender, had higher median levels of hs-CRP, LDL-cholesterol, fibrinogen, urine 8-*epi*-$PGF_{2\alpha}$ and urine 11-dehydro-TxB_2 than non-smokers, and lower levels of HDL-cholesterol.

Median hs-CRP values in females were about twice those in males. Variability within and between subjects was very high for hs-CRP levels, particularly in non-smokers (Table VI). In ten subjects, one of the three measured values was unexpectedly substantially elevated; in seven of these subjects, this was likely due to a mild illness noted by the Investigator at the time of sampling. Strongly elevated hs-CRP values were consistently found in three female adult smokers, two female non-smokers and one male adult smoker. Like hs-CRP, median levels of the other inflammatory biomarker, fibrinogen, were slightly higher in females than in males. However, fibrinogen had far less within- and between-subject variability than did hs-CRP.

The difference in median HDL-cholesterol levels between adult smokers and non-smokers at weeks 1, 3 and 6 was significant in females but not in males. There was no significant difference in median LDL-cholesterol between adult smokers and non-smokers overall, nor by gender subgroups.

For 8-*epi*-$PGF_{2\alpha}$ and 11-dehydro-TxB_2 results, there was moderate within-subject variability in adult smokers and non-smokers that was partly due to inter-assay

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Table V. Biomarkers of exposure and potential harm in adult smokers and non-smokers*.

Biomarker	Body fluid	Overall: Adult smokers ($n=50$)	Overall: Non-smokers ($n=65$)	Overall: p[‡]	Males: Adult smokers ($n=18$)	Males: Non-smokers ($n=30$)	Males: p[‡]	Female: Adult smokers ($n=18$)	Female: Non-smokers ($n=35$)	Female: p[‡]
Biomarkers of exposure:										
COHb (% sat.)	whole blood	4.0 (0.3, 12.2)	0.4 (0.3, 1.3)	< 0.001	3.6 (0.3, 12.2)	0.4 (0.3, 1.3)	< 0.001	4.1 (0.6, 11.0)	0.3 (0.3, 1.1)	< 0.001
NE (nmol mg^{-1} creatinine)	urine	64.514 (0.561,182.343)	< 0.034[†] (0.011,1.222)	< 0.001	58.041 (0.561,150.824)	< 0.034[†] (0.011,1.222)	< 0.001	66.336 (5.950, 182.343)	< 0.039[†] (0.017,0.236)	< 0.001
NE (mg per cigarette)	urine	0.96 (0.03, 3.59)	n.a.	n.a.	0.89 (0.03, 3.59)	n.a.	n.a.	0.96 (0.39, 3.01)	n.a.	n.a.
Biomarkers of potential harm:										
hs-CRP (mg dl^{-1})	serum	0.27 (0.020, 2.300)	0.12 (0.024, 2.267)	0.05	0.11 (0.02, 1.79)	0.07 (0.02, 1.09)	0.25	0.40 (0.02, 2.30)	0.18 (0.03, 2.27)	0.19
Fibrinogen (mg dl^{-1})	plasma	292 (184, 535)	248 (162, 399)	<0.001	268 (184, 358)	226 (165, 296)	0.01	297 (209, 535)	278 (162, 399)	0.04
HDL-C (mg dl^{-1})	serum	46 (26, 81)	53 (29, 85)	0.003	43 (31, 63)	45 (29, 76)	0.42	50 (26, 81)	57 (43, 85)	0.004
LDL-C (mg dl^{-1})	serum	119[§] (70, 219)	109[§] (52, 192)	0.18	122[§] (70, 167)	106[§] (52, 192)	0.15	115[§] (75, 219)	114[§] (67, 179)	0.61
8-*epi*-PGF $F_{2\alpha}$ (pg mg^{-1} creatinine)	urine	1935 (687, 4608)	1034 (579, 2174)	< 0.001	1634 (687, 4608)	991 (579, 2174)	<0.001	2057 (905, 3903)	1110 (701, 1759)	< 0.001
11-DHTx B_2 (pg mg^{-1} creatinine)	Urine	973 (410, 2154)	710 (179, 1261)	< 0.001	838 (429, 2154)	610 (179, 1230)	0.010	1052 (410, 2058)	799 (335, 1261)	< 0.001

*Values are medians (minimum, maximum) and calculated as the average across all collection time points within each subject, then averaged across all subjects within the group.

[†]Many non-smokers' values for nicotine and its five metabolites were below the lower limit of quantification (BLLOQ) (see the Discussion for details). For data analysis purposes, biomarker values below the lower limit of quantification were set to the limit of quantification. Differences in non-smokers' very low nicotine-equivalents values, normalized for urine creatinine, were due solely to variations in urine creatinine excretion.

[‡]p-value from Wilcoxon's rank-sum test.

[§]LDL-cholesterol could not be accurately calculated for two smokers and two non-smokers due to elevated triglycerides (> 400 mg dl^{-1}). COHb, carboxyhaemoglobin; NE, nicotine equivalents; hs-CRP, hs-C-reactive protein; HDL-C, HDL-cholesterol; 8-*epi*-PGF, 8-*epi*-prostaglandin F_{2x}; 11-DHTx B_2, 11-dehydrothromboxane B_2.

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Table VI. Biomarker variability within and between subjects.

Biomarker	Subject variability (%coefficient of variation)			
	Adult smokers		Non-smokers	
	Within	Between	Within	Between
Biomarkers of exposure:				
Carboxyhaemoglobin	64.5	69.3	30.9	68.6
Nicotine equivalents	61.8	64.5	‡	‡
Biomarkers of potential harm:				
hs C-reactive protein	105.8	129.3	114.7	210.6
Fibrinogen	18.0	28.3	15.7	26.2
HDL-cholesterol	23.2	25.2	20.8	22.6
LDL-cholesterol	21.6	25.0	24.4	28.3
8-*epi*-Prostaglandin $F_{2\alpha}$	35.8	47.3	26.0	33.2
11-Dehydro-thromboxane B_2	38.2	48.9	29.6	37.8

‡Because most levels in non-smokers were BLLOQ, differences in these very low urine nicotine-equivalents values, normalized for urine creatinine, were due solely to variations in urine creatinine excretion.

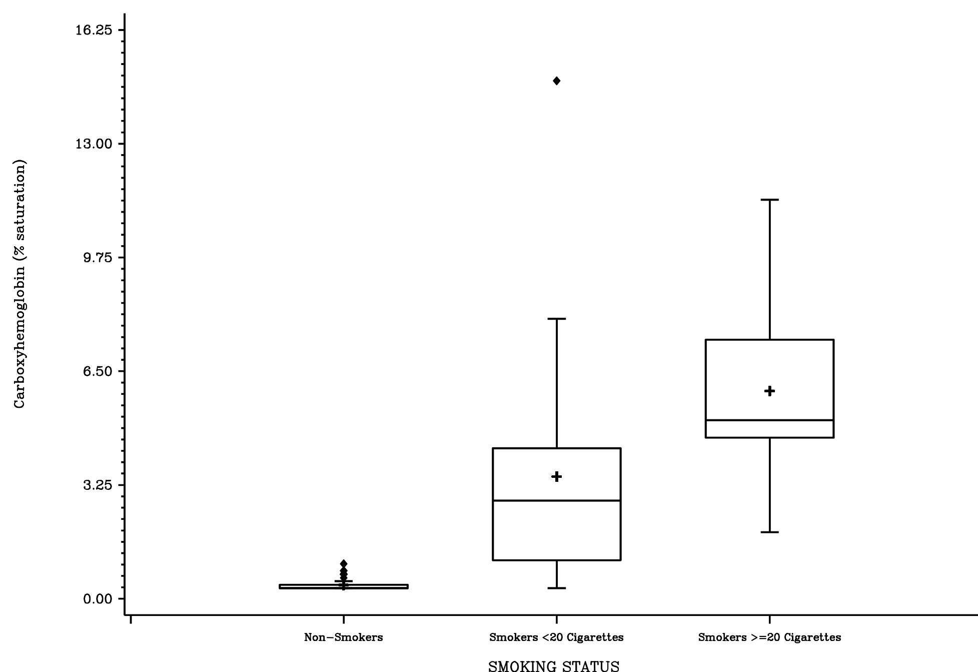

Figure 1. Box-plot of carboxyhaemoglobin (% saturation) by smoking status. Plus signs are means; middle bars are medians; the lower and upper borders of the box are the 25th and 75th percentiles, respectively; the lower and upper whiskers of the box are the minimum and maximum values, respectively, within 1.5 times the interquartile range of (75% value − 25% value); outlying points below the lower whiskers are values less than the 25% value − 1.5 times the interquartile range; outlying points above the upper whiskers are values greater than 75% value + 1.5 times the interquartile range.

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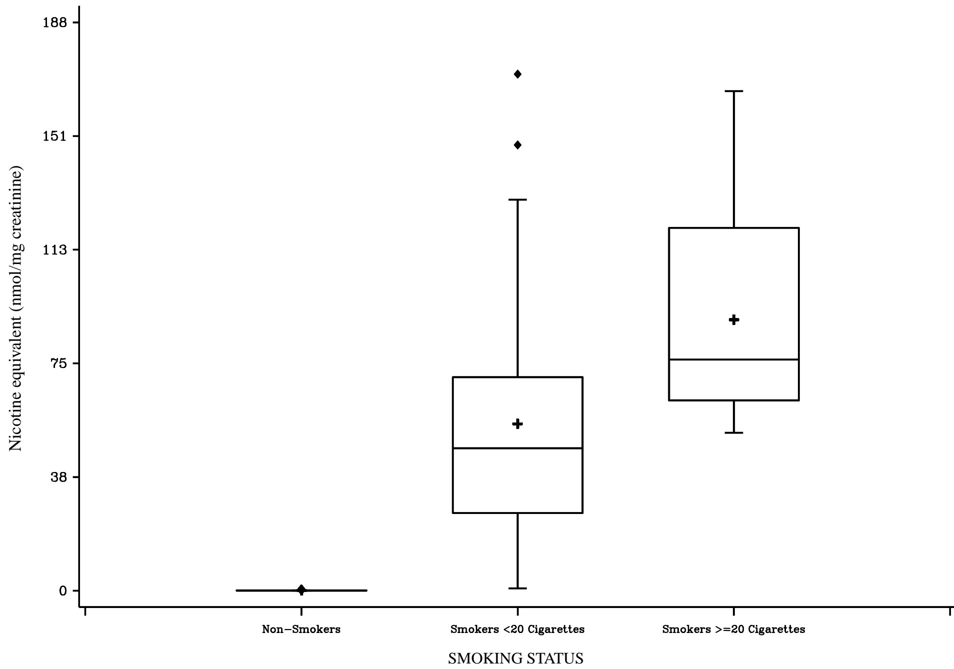

Figure 2. Box-plot of 24-h urine nicotine expressed as nicotine-equivalents (molar sum of nicotine and five metabolites) adjusted for creatinine excretion (nmol mg^{-1} creatinine) by smoking status. The legend is as described for Figure 1.

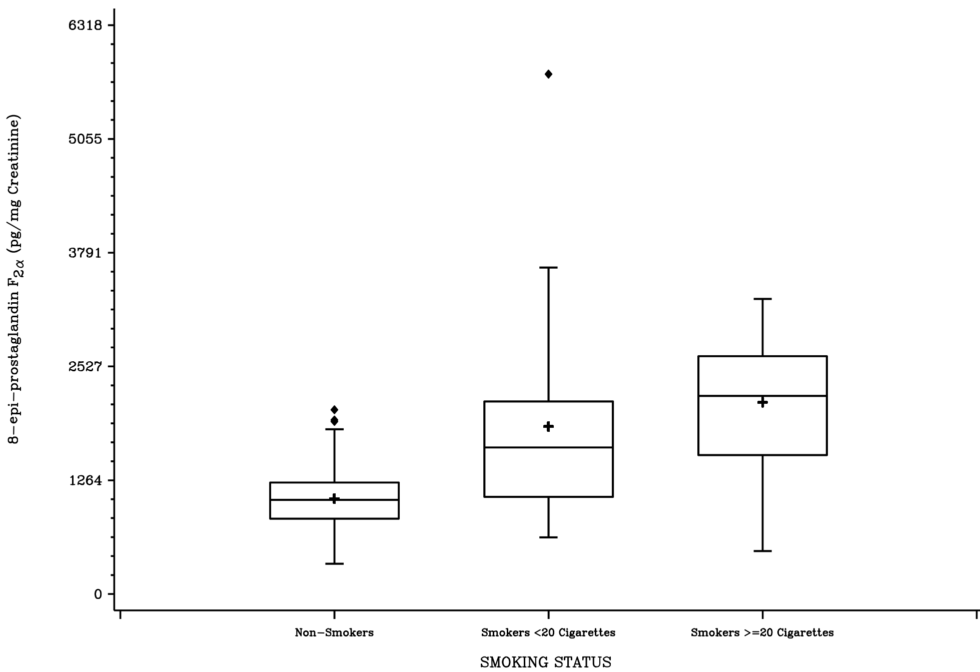

Figure 3. Box-plot of 24-h urine 8-*epi*-$PGF_{2\alpha}$ levels adjusted for creatinine excretion (pg mg^{-1} creatinine) by smoking status. The legend is as described for Figure 1.

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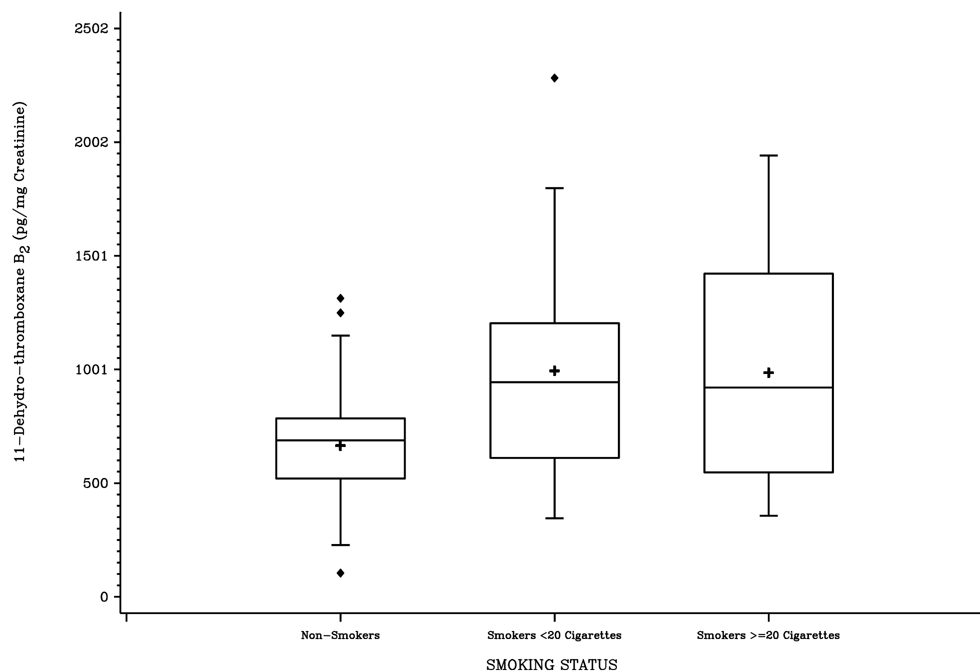

Figure 4. Box-plot of 24-h urine 11-dehydro-TxB_2 levels adjusted for creatinine excretion (pg mg − 1 creatinine) by smoking status. The legend is as described for Figure 1.

precision variability (Tables II and IV and Figures 3 and 4). These were the only biomarkers in the study found to have a significant time of sampling (by visit week) effect ($p = 0.03$ for both 8-*epi*-$PGF_{2\alpha}$ and 11-dehydro-TxB_2; data not shown) by an overall test of time effect in a model containing smoking status, gender and smoking status by gender interaction using a likelihood ratio test.

Discussion

The data presented here are measures of biomarkers of exposure and biomarkers of potential harm in a community-based cohort of adult smokers of 3.0–6.9 mg FTC tar delivery cigarettes and non-smokers in the Madison, WI, USA, metropolitan area over 6 weeks of routine lifestyle (diet, physical activity, leisure and non-leisure activities).

The adult smokers in the present study smoked an average of 16 (females) and 20 (males) cigarettes per day. These values are comparable with the 15 (females) and 17 (males) cigarettes per day reported in a recent survey of adult health behaviours (Schoenborn et al. 2004).

The results of this pilot study are consistent with previous studies of carbon monoxide exposure in adult smokers. The Second National Health and Nutrition Examination Survey (NHANES II) 1976–80 population study of COHb levels in the USA found in cigarette smokers a mean (± SD) of 4.55 (± 2.35)% saturation. In the NHANES II population, 95.8% of non-smokers had levels below 2% saturation, with a mean (± SD) of 0.9 (± 0.96)% saturation (Klesges et al. 1992). The non-smokers in the present study had lower mean COHb levels than in the NHANES II study, possibly because we corroborated self-reported non-smoking status with urine

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cotinine levels. Madison is an urban community with a population of about 400 000, and 94% of the subjects were Caucasian. NHANES II relied on self-report of non-smoking status and found discrepant non-smoker COHb levels greater than 2% saturation more likely in self-reported ex-smokers, males, non-Whites and residents of urban areas with populations of 1 million or greater. As reported previously for this pilot study, the COHb level in blood correlated well with the measurement of carbon monoxide in exhalate (Spearman's $r=0.97$) (Kinser et al. 2002). Importantly for the logistics of tobacco smoke exposure population studies, this strong correlation supports the use of blood COHb measurements instead of the logistically more difficult (i.e. smoking time-dependent) measurement of CO in exhalate (Benowitz 1983).

Consistent with previous reports of nicotine absorption biomarkers (Byrd et al. 1995, 1998, Ueda et al. 2002), a wide variability was found in the concentration of urine nicotine-equivalents per cigarette in this cohort of adult smokers of 3.0–6.9 mg FTC tar delivery cigarettes. The average measured urine nicotine and metabolite 'yield' in the present study was 1.01 mg nicotine-equivalents per cigarette (median of 0.96 mg nicotine-equivalents per cigarette), which is two to three times greater than the FTC method-determined nicotine delivery of the cigarette brands smoked (mean of 0.3–0.6 mg per cigarette, FTC). In addition, the reported nicotine-equivalents include nicotine and five of its major metabolites, which approximate 80 to 85% of total nicotine uptake (Curvall et al. 1991, Benowitz et al. 1994, Schepers & Demetriou 2002). These exploratory results from a limited sample of adult smokers (i.e. 50 adult smokers of 3.0–6.9 mg FTC tar delivery cigarettes) will be expanded in our large, multicentre study currently underway. Byrd et al. (1998) measured nicotine, these five metabolites, plus two additional minor oxide metabolites of nicotine and cotinine, approximating 90–95% of total nicotine uptake, and found similarly elevated ratios of measured versus FTC method-determined nicotine deliveries of 4.8 (for 2 mg tar delivery (FTC) cigarettes) and 1.4 (for 2.1–6 mg tar delivery (FTC) cigarettes). The ratios for higher FTC tar delivery cigarettes in the study by Byrd et al. were closer to unity, with a ratio of 0.9 determined for 6.1 to 1.9 mg tar delivery (FTC) cigarettes and 0.8 for 12 mg or higher tar delivery (FTC) cigarettes. Interestingly, there were no differences between measured nicotine absorption per cigarette among the three lowest FTC nicotine delivery categories in that study (Byrd et al. 1998), and those authors hypothesized that possibly individual smoker-determined parameters (i.e. inhalation and puffing behaviours) may exert a greater influence on the amount of nicotine absorbed during smoking of lower FTC delivery cigarettes than cigarette design parameters.

High-sensitivity CRP and fibrinogen values in adult smokers were, as previously reported (Tracy et al. 1997, Yarnell et al. 2000), significantly higher than in non-smokers. Both are acute-phase plasma proteins produced by the liver in response to stimulation by inflammatory cytokines, primarily interleukin 6 (Pepys & Baltz 1983). Consistent with the inflammatory hypothesis of atherogenesis, elevated hs-CRP and fibrinogen are among the most predictive of the evolving atherosclerosis risk factors and independently identify apparently healthy individuals who are at higher risk for vascular events (Ridker & Haughie 1998, Koenig et al. 1999). High-sensitivity assays for CRP allow detection of CRP levels within the normal range for healthy individuals without clinically significant inflammation (i.e. below 1.0 mg dl^{-1}) (Wilkins et al. 1998). Of all the measured biomarkers of potential harm, hs-CRP had the greatest

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within- and between-subject variability, reflecting its short plasma half-life of 19 h (Vigushin et al. 1991) and sensitivity to even mild levels of inflammation (e.g. sporadic mild acute illness). Of note, four of the six subjects with consistently very high values were females (two adult smokers, two non-smokers) taking oestrogen-containing oral contraceptives. Oestrogen has been associated with significantly elevated hs-CRP levels (Cushman et al. 1999a,b). The difference in oestrogen levels may explain the significant gender difference in hs-CRP levels, with the median in females being twice that of males, in both adult smokers and non-smokers. Fibrinogen, an acute-phase reactant with a plasma half-life of 4–5 days (Gill 1995), had much less within- and between-subject variability than hs-CRP.

Significantly higher levels in smokers than in non-smokers or former smokers have been reported for total cholesterol, triglycerides and LDL-cholesterol (7% higher) and lower levels of HDL-cholesterol (14% lower) (Eliasson et al. 2001). Lipid levels in the present study were consistent with the literature, and HDL-cholesterol was the most discriminating of the traditional lipid parameters between adult smokers and non-smokers overall ($p = 0.003$).

Potentially important pathophysiological mechanisms widely implicated in smoking-related tissue injury include enhanced oxidative stress and platelet activation. Cigarette smoke is a potential source of oxidative stress, with each puff containing 10^{14} free radicals (Baker 1999). Consistent with the literature (Chehne et al. 2001, Cracowski et al. 2002), adult smokers' levels of urine F_2-isoprostanes were almost double those of non-smokers in the present study. These prostaglandin-like eicosanoids are biomarkers of lipid peroxidation due to oxidant stress, which can be the trigger of an inflammatory cascade postulated to lead to atherogenesis (Ross 1999).

The median concentrations of 8-*epi*-$PGF_{2\alpha}$ observed in this study were three- to fourfold higher, even in non-smokers, than those reported in the literature. This may be due to different methodologies used (EIA versus GC/MS). Direct quantitative comparison of results obtained using different methods is not recommended (Proudfoot et al. 1999, Bessard et al. 2001) due to uncertainty about whether the same compounds are being measured. A large number of 8-*epi*-$PGF_{2\alpha}$-like regioisomers can be generated from free radical oxidation of arachadonic acid, and some isomers may cross react with the EIA antibody but may be absent from the GC/MS elution peak for 8-*epi*-$PGF_{2\alpha}$ (Cracowski et al. 2002, Morrow et al. 2002, Pilacik et al. 2002). Measurement of F_2-isoprostane levels, particularly by GC/MS methodology, is the more sensitive and accurate means of assessing *in vivo* oxidant stress-induced lipid peroxidation compared with other markers, such as malondialdehyde, and other methodologies, such as EIA (Morrow et al. 2002).

Median levels of 11-dehydro-TxB_2 were 1.4 times greater in adult smokers than non-smokers. Its parent compound, thromboxane A_2, is a cyclooxygenase-dependent arachadonic acid prostaglandin metabolite released by activated platelets. Thromboxane A_2 is one of the most potent platelet-aggregating and vasoconstricting substances known (FitzGerald et al. 1983).

The adequacy of the individual 24-h urine collections was scrutinized by evaluating the 24-h creatinine excretion before accepting the 24-h urine biomarker results. Daily urine volume may vary markedly, but daily formation and urine excretion of creatinine are relatively constant and determined mainly by an individual's muscle mass and renal function. A healthy 70-kg adult male excretes 1000–1850 mg creatinine

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per 24 h, and a similar 60-kg female excretes 660–1200 mg per 24 h, with up to 15–20% between-day variability (Burtis & Ashwood 1999).

There were some limitations in the current study. First, the cohort in this exploratory study was 94% Caucasian; their biomarker results may not apply to non-Caucasian populations. Second, we rejected from analysis the data for about 25% of our adult smoking subjects due to non-adherence with protocol smoking criteria (e.g. smoking cigarettes outside the 3.0–6.9 mg FTC tar category). Byrd et al. (1998) noted similar adherence difficulty in their study of FTC delivery and nicotine absorption in smokers. Third, the levels of some biomarkers of potential harm (i.e. hs-CRP, fibrinogen, 8-*epi*-$PGF_{2\alpha}$, 11-dehydro-TxB_2) may have been affected by concurrent nutritional and vitamin supplements, alcohol, and medications (e.g. aspirin, non-steroidal anti-inflammatory agents, HMG-CoA reductase inhibitors (statins), hormonal contraception and postmenopausal replacement therapy, and systemic antibiotics) that are known to have oxidant stress, antioxidant, thrombotic or antithrombotic properties (Yarnell et al. 2000). In the present study, these were not controlled for nor used in multivariate analysis of biomarker levels.

In summary, this exploratory study confirmed that whole blood COHb and urine nicotine-equivalents discriminate with a high degree of sensitivity and specificity between adult cigarette smokers and non-smokers. Likewise, hs-CRP, fibrinogen, HDL- (but not LDL-) cholesterol, and urine 8-*epi*-$PGF_{2\alpha}$ and 11-dehydro-TxB_2 clearly differentiated adult smokers overall from non-smokers overall. Because none of the biomarkers of exposure and only two of the biomarkers of potential harm (8-*epi*-$PGF_{2\alpha}$ and 11-dehydro-TxB_2) showed a time of sampling (week of visit) effect, a single sampling time point may be considered representative of a subject's biomarker levels in future exposure studies. The measured median nicotine uptake per cigarette (0.96 mg) was higher than that indicated by the FTC method (0.3–0.5 mg). The between-subject variability observed for the biomarkers was a factor in determining appropriate sample size to ensure adequate statistical power of results in the planned large multicentre exposure study.

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