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Evaluation of biomarkers of exposure and potential harm in smokers, former smokers and never-smokers

Frazer J. Lowe[1,*], Evan O. Gregg[2] and Michael McEwan[1]

[1] Group Research and Development, British American Tobacco, Southampton, UK
[2] Consultant, ENI Limited, Towcester, UK

Abstract

Background: The objective of this study was to obtain baseline data on biomarkers of exposure (BoE) and biomarkers of potential harm (BoPH) in smokers, former smokers and never-smokers.
Methods: This was a cross-sectional study of 80 healthy male and female volunteers over 21 years old, self-selected for smoking status. Subjects were prescreened by medical staff at an independent clinical research unit, within 1 week prior to a single overnight residential visit and sample collection.
Results: All BoE were able to differentiate between the two smoking groups and smokers from all nonsmokers. There was a strong correlation between cigarettes smoked per day and total urinary nicotine equivalents (TNE; $r=0.85$). TNE correlated better with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol levels than cigarettes smoked per day ($r=0.75$ and $r=0.56$, respectively). Of the BoPH included in this study, seven (11-dehydro-thromboxane B2, 2, 3-dinorthromboxane B2, 8-epi prostaglandin $F_{2\alpha}$, 8-hydroxy-2 deoxyguanosine, cis-thymidine glycol, low-density lipoprotein cholesterol and IgG) were significantly different between the group who smoked more cigarettes per day and never-smokers. These differences became more apparent and extended to the group who smoked 10 or less cigarettes per day, when total urinary recovery values were corrected for creatinine clearance.
Conclusions: While BoE clearly differentiate between groups based on self-declared smoking status, most BoPH examined could not do so in a consistent manner. The dynamics of BoPH levels are not well understood. Future studies of BoPH should eliminate potential confounding factors and increase the number of subjects to allow the investigation of genetic polymorphism in metabolic pathways.
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Keywords: biomarkers; cardiovascular risk; evaluation; potential reduced-exposure products; smoking.

*Corresponding author: Frazer J. Lowe, Research and Development Centre, British American Tobacco (Investments) Ltd., Regents Park Road, Millbrook, Southampton, Hampshire, SO15 8TL, UK
Phone: +44-2380-793647, Fax: +44-2380-793076,
E-mail: frazer_lowe@bat.com
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Introduction

Cigarette smoke is a complex mixture of over 4000 different chemicals, split between a gaseous and a particulate phase (1, 2). Interaction between the smoke chemicals adds further complexity of the mixture. The yields of smoke constituents per cigarette are measured by machine under controlled smoking conditions defined by the International Organization for Standardisation (ISO) (3) or the Federal Trade Commission (4). While these methods are useful for measuring the machine-derived yields of cigarette smoke chemicals, they do not, and were not intended to, reflect human smoke yield (5). Individual smoking behaviour, such as puff volume, puff frequency and depth of inhalation, determines the amount of smoke a given individual takes into the lungs (6).

The health risks of smoking are clear and undisputed (7) and the only way to be certain of avoiding these risks is not to smoke. Much research has been focused on lung cancer, diseases of the respiratory tract (e.g., chronic obstructive pulmonary disease) and diseases of the coronary and vascular systems (cardiovascular disease or ''CVD'' in this paper). However, the relationship between specific tobacco smoke chemicals and mechanistic steps in these diseases remains unclear (8). Since the 1950s, when the risks associated with tobacco products use were becoming established, there have been attempts to modify products to lessen their health risks (9). In 2001, the Institute of Medicine (part of the US National Academy of Sciences) reviewed the scientific basis for tobacco harm reduction. They introduced the term 'potential reduced-exposure products' (PREPs) for modified products which gave a substantial reduction in one or more tobacco toxicants and could reasonably be expected to reduce the risk of one or more specific diseases or other adverse health effects. They concluded that PREPs should be developed under conditions of strict regulation as an appropriate approach to tobacco harm reduction (10). The availability of biomarkers as tools should allow manufacturers and public health scientists to evaluate PREPs more effectively.

The limits of smoking machine measurements of smoke chemical yields, discussed above, point to the need for other systems to measure the exposure to, and potential toxicity of, cigarette smoke. As one of a number of approaches to address this topic, studies of biomarkers were recommended by the Institute of Medicine and by the World Health Organization (11). However, the biomarkers that could be used for such a purpose were not identified by either organisation and so it was decided to commence the validation of potential biomarkers for this use (12). Two distinct

categories of biomarkers are being developed and validated: (1) biomarkers of exposure (BoE), which should confirm the absorption of specific smoke chemicals in a quantitative manner, avoiding the problems of smoking behaviour and machine-yield measurement; and (2) biomarkers of potential harm (BoPH), which should assess biological effects on mechanisms that may be related to smoking-associated diseases. BoPH can be molecules derived from an interaction with a toxicant and can yield information on the physiological functioning of the body in relation to a normal or pathological process.

To cover the range of chemicals present in tobacco smoke, and the major tobacco-associated diseases, a suite of biomarkers will be required. For example, exposure to tobacco smoke is typically estimated by the measurement of nicotine and its metabolites in body fluids (13), but they do not reflect the spectrum of the diverse chemicals that are present in smoke. Ideally, BoE should be related to an exposure of specific interest, such as the measurement of urinary 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) as a biomarker of exposure to 4-(methylnitrosamino)-1-(3-pyridyl)-1 butanone (NNK) (14).

For BoPH, many biomarkers covering a range of possible pathways related to the onset of tobacco-associated diseases will be required. Examples of BoPH include markers of DNA turnover or damage [e.g., 8-hydroxy-deoxyguanosine (15)] and cell signalling [e.g., markers of apoptosis, growth factors and integrins (16)] for carcinogenesis. Those reflecting lipid metabolism [low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol (17)], atherosclerotic plaque formation [e.g., upregulation of adhesion molecules (18) and endothelial dysfunction (19)], coagulation [e.g., factor VII (20)] and cardiac damage [e.g., cardiac troponins (21, 22)] may be useful for CVD. Biomarkers reflecting progressive loss of lung function (e.g., FEV_1) or damage [e.g., desmosine (23)] may be relevant to respiratory diseases, such as chronic obstructive pulmonary disease (COPD). Biomarkers that indicate oxidative stress [e.g., 8-epi prostaglandin $F_{2\alpha}$ (24)] may have utility in a number of conditions.

From a review of the scientific literature it is clear that measurement of biomarkers is not a straightforward, routine clinical or outpatient activity. Conflicting results were often identified, possibly based on differences on measurement techniques or different study populations (10, 12). To achieve the goal of harm reduction in smokers, tools must be available for the assessment of conventional cigarettes and PREPs. Before biomarkers can be used as a part of such an approach, their performance in smokers of conventional cigarettes must be characterised and understood. The objective of this study was to measure BoE for specific smoke chemicals and several candidate BoPH across groups of smokers, former smokers and never-smokers. This will provide a solid grounding for future studies of smokers and for PREP evaluation.

Materials and methods

Primary objective

The primary objective of this study was to compare the levels of various biomarkers in blood and urine samples obtained from four groups of healthy subjects:

- Group A (''never-smokers''): subjects who had never smoked.
- Group B (''ex-smokers'') subjects who smoked an average of 10 or more cigarettes per day of a brand with an ISO tar yield of greater than 6 mg, between 1 and 5 years prior to the study visit. The period of time that the subject smoked must have exceeded the period of time since stopping smoking.
- Group C: subjects who regularly smoked 10 or less cigarettes per day of a specified brand with an ISO tar yield of 6 mg or less.
- Group D: subjects who regularly smoked 20 or more cigarettes per day of a specified brand with an ISO tar level of 9 mg or greater.

Overall study design and plan

A cross-sectional study of 80 subjects in four groups of 20 (described above) was conducted. Each subject attended the Clinical Research Unit (CRU) on two occasions; once for a short non-residential screening visit, within 1 week prior to the study commencing and once for a residential visit. During the residential visit, the subjects remained in the CRU from the morning of Day 1 until the morning of Day 2 (a period exceeding 24 h).

The following information and procedures were recorded and performed as part of the screening assessments: medical history, smoking history, demographic data (gender, ethnic origin and age), salivary cotinine and blood carboxyhaemoglobin assessments. Usual smoking routine (Groups C and D) and environmental smoke exposure (Groups A and B) in the period between the initial screen and Day 1 in the CRU were recorded by each subject using a diary card.

This study was conducted in accordance with Good Clinical Practice and the Declaration of Helsinki. The protocol was approved by the Covance Clinical Research Unit (Covance CRU) Independent Ethics Committee and written informed consent was obtained from all subjects prior to the start of the study.

Selection of study population

Subjects were required to meet the following criteria: males and females who were generally healthy (medical history and independent examination by medical staff within the CRU) and aged 21 years or over. Smokers must have smoked their current brand, from a subset of five market-leading brands, for a period exceeding 1 year prior to study commencement. At screening, each subject provided a saliva sample for measurement of cotinine using an Accutest® NicAlert™ test kit (Jant Pharmacal Corp., Encino, CA, USA). This was used to exclude smokers from the self-reported non-smoker groups. Other exclusion criteria were the use of 1) any cyclo-oxygenase inhibitors within the 2 weeks prior to initial screening, and 2) any other prescribed medication, with the exception of oral contraceptives (Table 1).

Study cigarettes

Prior to commencing screening, the CRU purchased a supply of retail batches of cigarettes of the subjects' declared brands. A sufficient number of cigarettes were purchased to ensure that all subjects within each group smoked the same batch for the period between the screening visit and the end of the study (Day 2).

When subjects attended the screening visit, subjects purchased sufficient cigarettes for the duration of the study from the CRU. Subjects were instructed to use their cigarette supplies according to their normal daily smoking routine and not to smoke any cigarettes other than those purchased from the CRU.

Study restrictions and requirements

Subjects from Groups C ($\leq$10 cigarettes per day, ISO yield from pack of $<$6 mg 'tar') and D ($\geq$20 cigarettes per day, ISO yield from pack 10 mg 'tar') were asked to smoke their usual number and brand of cigarettes during the study. Subjects were free to cease smoking at any time during the study, but they would no longer be eligible for inclusion in the study. There were no drop-outs from the study. Subjects from Groups A and B were asked to remain non-smokers and to avoid excessively smoky atmospheres during the study.

Biomarker assessments

Blood carboxyhaemoglobin (COHb) was determined on the sample taken at the initial screening. The following biomarkers were analysed in serum collected during the CRU study: interleukin 6 (IL-6 – ELISA, R&D Systems, Abingdon, UK), C-reactive protein (CRP – ELISA, ICL, Newberg, OR, USA), triglycerides (Tri – enzymatic colourimetric test, Roche, Indianapolis, IN, USA), serum amyloid A (SAA – ELISA, Tridelta Phase™, Maynooth, Ireland) and cardiac troponin I (cTnI – ELISA, Life Diagnostics, Westchester, IL, USA). Immunoglobulins A, G and M (IgA, IgG and IgM) were measured immuno-turbidimetrically (ABX Pentra, Montpellier, France). LDL/HDL-cholesterol was measured using an enzymatic colourimetric test (Hitachi 917, Indianapolis, IN, USA). Plasminogen activation inhibitor type 1 (PAI-1) was analysed in plasma by ELISA (Technoclone, Vienna, Austria) and factor VII (F7) by a modified prothrombin test (HemosIL, Lexington, KY, USA).

Urine collections and processing for the analysis of biomarkers

The 24-h collection period commenced after the first morning void on Day 1 and included the first morning void on Day 2. The 24-h urine samples were analysed for the following parameters: nicotine and nicotine glucuronide (total nicotine); cotinine and cotinine glucuronide (total cotinine), hydroxycotinine and hydroxycotinine glucuronide (total hydroxycotinine), NNAL and NNAL-glucuronide (NNAL), 8-epi-prostaglandin $F_{2\alpha}$ (8epiPGF); 8-hydroxy-2'deoxyguanosine (8-OHdG), 11-dehydro-thromboxane B_2 (dTx); 2,3 dinor-thromboxane B_2 (Tx-M), hydroxyproline (OH-P), cis-thymidine glycol (cTG) and creatinine.

NNAL and its metabolite glucuronide were analysed using a solid phase extraction and liquid chromatography with tandem mass spectrometry (LC/MS/MS) method based upon methods by Xia et al. (25) and Pan et al. (26). Urinary nicotine and its metabolites were analysed using a solid phase extraction and liquid chromatography with tandem mass spectrometry as described by St. Charles et al. (27), 8-OHdG and cTG were also analysed using LC/MS/MS. Thromboxane metabolites and 8-epiPGF were analysed using commercially available immunoassay kits (Cayman Chemicals, Ann Arbor, MI, USA). Hydroxyproline was analysed using a commercially available high performance liquid chromatography kit (Bio-Rad, Munich, Germany).

Presentation and statistical analysis

Biomarker results are presented as group median values, with maxima and minima. All biomarker assays were performed in triplicate and a mean of each triplicate was reported. If the coefficient of variation for triplicates was $>$50%, the data were rejected and not included in subsequent statistical analyses. For all urinary biomarkers, the total excretion over 24 h and the creatinine concentration adjusted values are reported.

Analysis of variance on log transformed data was used to establish levels of significance. Where subject biomarker levels were lower than the lower limit of quantification (LLOQ) for the assay, a value of LLOQ/2 was used to establish the group means and in subsequent statistical analysis. In practice, this happened for the biomarkers CRP, SAA and cTnI and because it occurred for a large proportion of these biomarker results, the groups were excluded from statistical analyses, as noted in the appropriate tables. Three values

Table 1 Subject demographics.

	Study group			
	(A) Never-smokers, n=20	(B) Ex-smokers, n=20	(C) $\leq$10 cigarettes per day, n=20	(D) $\geq$ 20 cigarettes per day, n=20
Age				
Mean	30	37	33	41
SD	9.4	13	12.7	9
Median	25	34	28	43
Min	21	22	21	24
Max	45	68	67	66
Gender				
Male, n (%)	8 (40)	12 (60)	10 (50)	10 (50)
Female, n (%)	12 (60)	8 (40)	10 (50)	10 (50)
Ethnicity				
Caucasian, n (%)	18 (90)	20 (100)	19 (95)	20 (100)
Other, n (%)	2 (10)	0 (0)	1 (5)	0 (0)

from the IL-6 data were below the LLOQ (2 from Group C and 1 from Group A).

Results

Biomarkers of exposure

The results of the BoE for the subject groups A–D are shown in Table 2. Urinary biomarkers are expressed as total amounts collected in 24 h and blood COHb as a percent of total haemoglobin.

All BoE were significantly different in smokers compared to never- and former smokers. Group C subjects were also significantly different from Group D subjects ($p < 0.001$ for all BoE), although there was a high degree of variability in BoE levels across these groups (see Figures 1 and 2).

The number of cigarettes smoked per day (as reported by smoking diary cards) had a strong rela-

Table 2 Biomarkers of exposure.

Variable	Study group	n	Mean	SD	Minimum	Median	Maximum	p
Nicotine, mg/24 h	(A) Never-smoker	20	0.00	0.00	0.00	0.00	0.00	
	(B) Ex-smoker	20	0.00	0.00	0.00	0.00	0.00	NS
	(C) ≤10 cigs per day	20	1.11	0.98	0.00	0.80	3.90	<0.001
	(D) ≥20 cigs per day	20	3.74	1.62	1.20	3.60	7.60	<0.001
Nicotine, ng/mg creatinine	(A) Never-smoker	20	0.00	0.00	0.00	0.00	0.00	
	(B) Ex-smoker	20	0.00	0.00	0.00	0.00	0.00	NS
	(C) ≤10 cigs per day	20	778	645	12	644	2560	<0.001
	(D) ≥20 cigs per day	20	2834	1320	723	3064	5715	<0.001
Cotinine, mg/24 h	(A) Never-smoker	20	0.01	0.03	0.00	0.00	0.10	
	(B) Ex-smoker	20	0.01	0.02	0.00	0.00	0.10	NS
	(C) ≤10 cigs per day	20	3.36	2.25	0.10	3.00	7.30	<0.001
	(D) ≥20 cigs per day	20	8.42	2.87	3.20	7.75	14.00	<0.001
Cotinine, ng/mg creatinine	(A) Never-smoker	20	7.14	21.98	0.00	0.00	71.50	
	(B) Ex-smoker	20	2.21	9.87	0.00	0.00	44.14	NS
	(C) ≤10 cigs per day	20	2508	1751	155	2097	6667	<0.001
	(D) ≥20 cigs per day	20	6287	2509	2840	5718	12553	<0.001
OH cotinine, mg/24 h	(A) Never-smoker	20	0.03	0.06	0.00	0.00	0.20	
	(B) Ex-smoker	20	0.03	0.07	0.00	0.00	0.30	NS
	(C) ≤10 cigs per day	20	6.18	4.31	0.70	4.70	15.70	<0.001
	(D) ≥20 cigs per day	20	15.91	6.27	3.30	16.35	29.40	<0.001
3OH cotinine, ng/mg creatinine	(A) Never-smoker	20	18.08	41.10	0.00	0.00	142.68	
	(B) Ex-smoker	20	18.28	39.18	0.00	0.00	132.41	NS
	(C) ≤10 cigs per day	20	4572	3141	560	4424	13433	<0.001
	(D) ≥20 cigs per day	20	11796	5096	4117	10898	20422	<0.001
Total nicotine metabolites, mg/24 h	(A) Never-smoker	20	0.05	0.08	0.00	0.00	0.20	
	(B) Ex-smoker	20	0.05	0.11	0.00	0.00	0.40	NS
	(C) ≤10 cigs per day	20	9.42	5.98	0.70	8.35	22.10	<0.001
	(D) ≥20 cigs per day	20	24.91	7.25	8.00	26.10	36.00	<0.001
Total nicotine metabolites, ng/mg creatinine	(A) Never-smoker	20	36.97	54.20	0.00	0.00	143.00	
	(B) Ex-smoker	20	28.09	60.20	0.00	0.00	176.60	NS
	(C) ≤10 cigs per day	20	6947	4336	723	6555	19275	<0.001
	(D) ≥20 cigs per day	20	18577	6539	7514	16944	32681	<0.001
NNAL, ng/24 h	(A) Never-smoker	20	8.23	6.52	1.91	5.80	24.63	
	(B) Ex-smoker	20	10.69	10.01	2.29	6.32	37.70	NS
	(C) ≤10 cigs per day	20	199.50	153.90	16.00	174.90	569.60	<0.001
	(D) ≥20 cigs per day	20	362.10	150.60	118.80	366.30	596.20	<0.001
NNAL, ng/mg creatinine	(A) Never-smoker	20	0.01	0.01	0.00	0.00	0.02	
	(B) Ex-smoker	20	0.01	0.01	0.00	0.01	0.03	NS
	(C) ≤10 cigs per day	20	0.14	0.09	0.01	0.14	0.30	<0.001
	(D) ≥20 cigs per day	20	0.26	0.10	0.10	0.26	0.45	<0.001
COHb, %	(A) Never-smoker	20	0.35	0.38	0.00	0.30	1.30	
	(B) Ex-smoker	20	0.31	0.22	0.00	0.30	0.80	NS
	(C) ≤10 cigs per day	20	2.02	1.24	0.40	1.50	4.10	<0.001
	(D) ≥20 cigs per day	20	5.69	1.82	2.80	6.15	9.40	<0.001

NS, not significant. All p-values are relative to never-smokers.

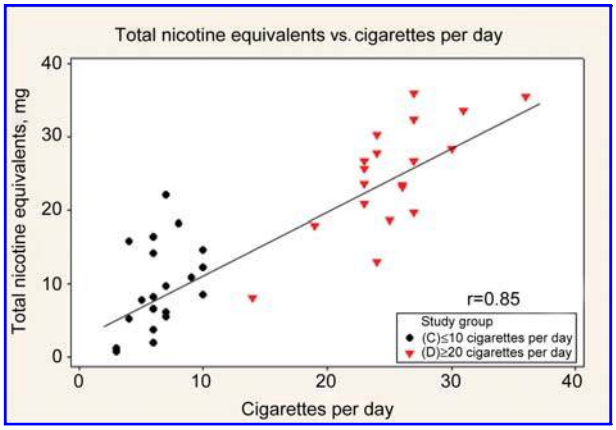

Figure 1 Relationship between 24-h total nicotine equivalents and number of cigarettes smoked per day.

tionship with total nicotine equivalents (nicotine and all measured metabolites added together, taking into account relative molecular mass). Some Group C (≤10 cigarettes per day) smokers had higher levels of urinary nicotine equivalents than some of the group D (≥20 cigarettes per day) smokers, despite smoking fewer cigarettes and the difference in tar yields between the Group C and Group D cigarette brands.

The overlap in total nicotine equivalents between the smoker groups could be explained by smoking behaviour differences from subject to subject. Variables, such as puff frequency and puff duration, can influence how much smoke is taken into the body and absorbed. The number of cigarettes smoked per day does not reflect the way each cigarette was smoked. Therefore, as nicotine is absorbed very efficiently by the body, smoking behavioural differences are more accurately accounted for by using total urinary nicotine equivalents for a more accurate assessment of acute cigarette smoke exposure (27).

A comparison of NNAL and COHb with total nicotine equivalents excretion, as well as cigarettes per day is illustrated in Figure 2.

From Figure 2, considerable inter-subject variability was again observed for each of these BoE when compared to cigarettes smoked per day. The levels of urinary NNAL were more closely linked to total nicotine equivalents (r=0.75) than to the number of cigarettes smoked per day (r=0.56). There was a linear relationship between these two biomarkers. COHb levels were strongly linked to urinary nicotine metabolites (r=0.77) and to cigarettes per day (COHb vs. cigarettes per day, r=0.75), despite chemical differences between these smoke constituents.

One possible source of variation with the NNAL data was the cigarette brands' nitrosamine yield. However an analysis of covariance of NNAL, total nicotine equivalents, cigarette brand (factor) and cigarettes per day (co-variate) revealed that the cigarette brand had a marginal influence on the correlation between NNAL and total nicotine equivalents (data not shown).

These data suggest that some of the variability in NNAL compared to the number of cigarettes smoked

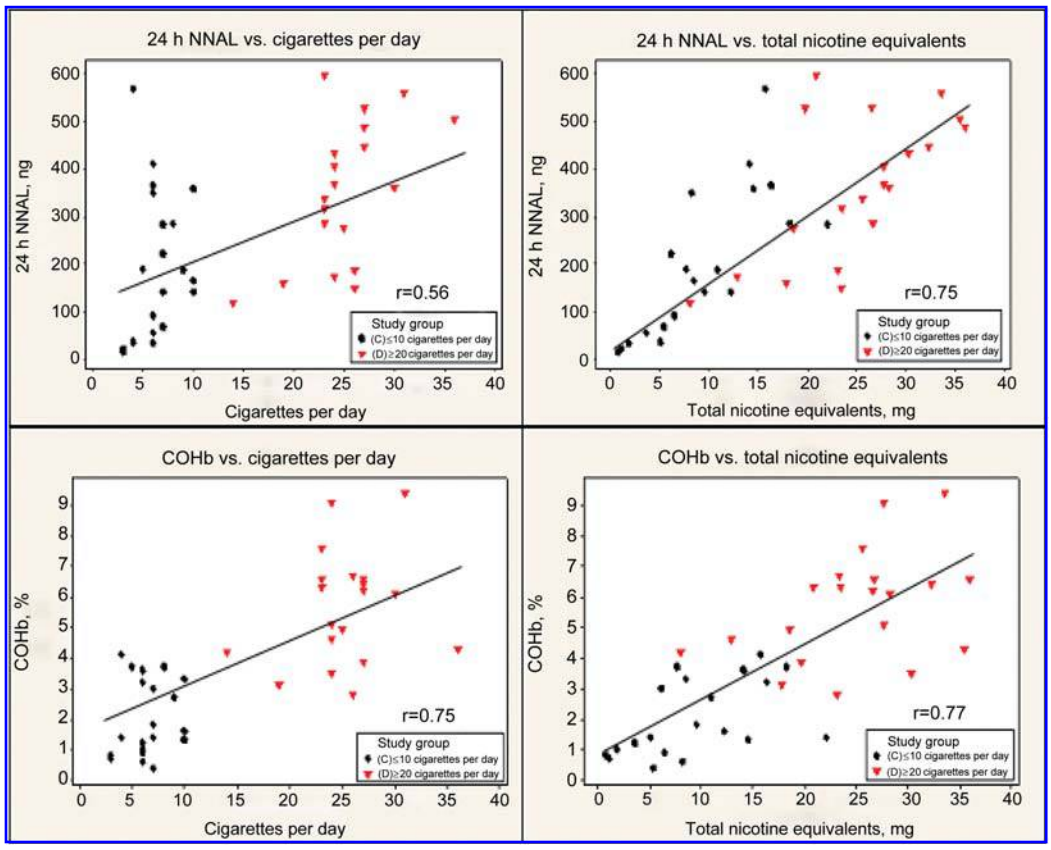

Figure 2 Relationship between NNAL and COHb and total nicotine equivalents in 24-h urine samples of smoking subjects.

Table 3 Biomarkers of potential harm achieving statistical significance relative to never-smokers.

Biomarker	Study group	n	Mean	SD	Minimum	Median	Maximum	Mean ratio	p
dTx, μg/24 h	(A) Never-smoker	20	1.485	0.650	0.890	1.236	3.101		
	(B) Ex-smoker	20	2.111	1.080	0.943	1.923	5.677	1.422	NS
	(C) ≤10 cigsper day	20	2.050	0.813	1.028	1.911	4.110	1.380	NS
	(D) ≥20 cigs per day	20	2.350	1.270	1.077	2.141	6.751	1.582	<0.05
dTx, μg/mg creatinine	(A) Never-smoker	20	1.0504	0.2978	0.536	1.0394	1.7883		
	(B) Ex-smoker	20	1.519	0.746	0.51	1.396	4.025	1.446	0.001
	(C) ≤10 cigsper day	20	1.563	0.651	0.835	1.33	3.09	1.488	0.001
	(D) ≥20 cigs per day	20	1.654	0.644	0.99	1.461	3.872	1.575	0.001
Tx-M, μg/24 h	(A) Never-smoker	18	2.231	1.337	1.187	1.743	6.872		
	(B) Ex-smoker	18	3.364	1.059	1.617	3.065	6.086	1.508	0.001
	(C) ≤10 cigsper day	15	3.098	0.878	1.652	3.051	4.59	1.389	0.001
	(D) ≥20 cigs per day	19	3.487	1.769	1.08	3.318	9.512	1.563	0.001
Tx-M, μg/mg creatinine	(A) Never-smoker	18	1.521	0.437	0.796	1.333	2.735		
	(B) Ex-smoker	18	2.354	0.716	1.328	2.135	3.618	1.548	<0.001
	(C) ≤10 cigsper day	15	2.242	0.524	1.633	2.083	3.32	1.474	<0.001
	(D) ≥20 cigs per day	19	2.44	0.943	1.405	2.104	5.455	1.604	<0.001
8-epiPGF, μg/24 h	(A) Never-smoker	19	0.918	0.638	0.422	0.739	3.315		
	(B) Ex-smoker	19	0.990	0.423	0.415	0.936	2.024	1.079	NS
	(C) ≤10 cigsper day	19	1.346	0.681	0.582	1.241	3.093	1.466	NS
	(D) ≥20 cigs per day	20	1.475	0.724	0.533	1.314	3.354	1.607	<0.01
8-epiPGF, μg/mg creatinine	(A) Never-smoker	19	0.6115	0.2112	0.3929	0.5984	1.3195		
	(B) Ex-smoker	19	0.6959	0.286	0.3702	0.5858	1.4349	1.138	NS
	(C) ≤10 cigsper day	19	0.9617	0.3686	0.5391	0.8505	2.0336	1.573	<0.001
	(D) ≥20 cigs per day	20	1.0354	0.3614	0.5836	0.9183	2.0013	1.693	<0.001
8-OHdG, μg/24 h	(A) Never-smoker	20	3.974	1.85	2.214	3.482	10.616		
	(B) Ex-smoker	20	6.746	2.043	3.758	6.42	11.666	1.698	<0.001
	(C) ≤10 cigsper day	20	5.38	2.835	1.273	4.846	14.207	1.354	NS
	(D) ≥20 cigs per day	20	4.784	1.59	1.817	4.881	7.487	1.204	NS
8-OHdG, ng/mg creatinine	(A) Never-smoker	20	2.81	0.834	1.369	2.52	4.467		
	(B) Ex-smoker	20	4.84	1.382	2.575	4.705	8.009	1.722	<0.001
	(C) ≤10 cigsper day	20	3.796	1.117	1.632	3.699	6.558	1.351	<0.001
	(D) ≥20 cigs per day	20	3.51	1.114	1.406	3.447	5.625	1.249	NS
cTG, μg/24 h	(A) Never-smoker	20	0.808	0.444	0.256	0.670	2.392		
	(B) Ex-smoker	20	1.162	0.519	0.413	1.092	2.178	1.438	<0.05
	(C) ≤10 cigsper day	20	1.240	0.662	0.250	1.139	3.049	1.534	<0.05
	(D) ≥20 cigs per day	20	1.180	0.431	0.383	1.177	2.114	1.460	<0.05
cTG, ng/mg creatinine	(A) Never-smoker	20	0.5639	0.1962	0.2157	0.5273	0.9741		
	(B) Ex-smoker	20	0.8204	0.3415	0.3684	0.7288	1.5121	1.455	<0.01
	(C) ≤10 cigsper day	20	0.8703	0.279	0.2456	0.881	1.4073	1.543	<0.01
	(D) ≥20 cigs per day	20	0.8595	0.2824	0.3544	0.8031	1.2771	1.524	<0.01
LDL-cholesterol, mmol/L	(A) Never-smoker	20	2.354	0.946	1.030	2.230	4.740		
	(B) Ex-smoker	20	2.694	0.911	1.460	2.465	4.000	1.144	NS
	(C) ≤10 cigsper day	20	2.622	1.060	1.230	2.315	5.500	1.114	NS
	(D) ≥20 cigs per day	20	3.100	1.049	1.840	2.985	6.110	1.317	P=0.01
IgG, g/L	(A) Never-smoker	20	12.304	1.887	8.740	12.295	15.040		
	(B) Ex-smoker	20	11.487	1.792	8.180	11.660	15.230	0.934	NS
	(C) ≤10 cigsper day	20	10.040	1.560	6.680	9.965	13.510	0.816	<0.001
	(D) ≥20 cigs per day	20	10.310	1.872	6.730	10.540	12.780	0.838	<0.001

NS, not significant. All p-values are compared to never-smokers. For biomarkers where n<20, triplicate measurements which fell outside of the assay acceptance range could not be considered reliable and were removed.

per day could be due to individual smoking behaviour. Other factors, such as body mass index, lifestyle choices and metabolic differences, may also influence NNAL and other BoE levels.

Biomarkers of potential harm

Of the 18 candidate BoPH that were studied, seven showed significant differences between never-smok-

ers and smokers of ≥20 cigarettes per day. These are shown in Table 3.

In Table 3, the urinary BoPH are shown as total amounts excreted over 24 h with and without correction for creatinine clearance. When the total values without creatinine clearance were considered, levels of dTx, Tx-M, 8-epi PGF, cTG- and LDL were significantly increased, and IgG was significantly decreased in Group D smokers, compared to never-smokers. Although a formal trend analysis was not performed, inspection of the mean ratios across the groups suggests a trend from never-smokers to ex-smokers, Group C smokers and Group D smokers only for the biomarker 8-epiPGF. Interestingly, 8-OHdG levels were significantly higher in the former smoker group compared to never-smokers, and cTG levels were significantly higher in all groups compared to never-smokers. When the total values adjusted for creatinine clearance were considered, dTx, Tx-M, 8-epiPGF, 8-OHdG and cTG were all significantly different, comparing both smoker groups to never-smokers. A trend in the mean ratios was now apparent for 8-epi PGF and dTx; however, an inverse trend across the groups was suggested for 8-OHdG.

None of the other BoPH showed significant differences between the groups (Table 4). Again, there was considerable inter-subject variability across all the groups and this can be seen by comparing minima, medians and maxima across the groups for each biomarker. Although such variability is not unexpected

Table 4 Biomarkers of potential harm not achieving statistical significance relative to never-smokers.

Biomarker	Study group	n	Mean	SD	Minimum	Median	Maximum	Mean ratio	p
OH-P, mg/24 h	(A) Never-smoker	20	34.310	15.260	14.430	33.350	66.720		
	(B) Ex-smoker	20	31.010	9.340	14.460	30.130	55.360	0.904	NS
	(C) ≤10 cigs per day	20	33.510	12.070	15.920	32.560	69.170	0.977	NS
	(D) ≥20 cigs per day	20	27.560	7.650	13.210	27.570	38.540	0.803	NS
OH-P, μg/mg creatinine	(A) Never-smoker	20	23.45	5.81	15.9	22.52	36.24		
	(B) Ex-smoker	20	22.12	5.69	12.89	21.27	33.48	0.943	NS
	(C) ≤10 cigs per day	20	24.452	3.678	18.33	25.219	31.928	1.043	NS
	(D) ≥20 cigs per day	20	19.867	3.947	14.096	20.321	30.142	0.847	NS
HDL-cholesterol, mmol/L	(A) Never-smoker	20	1.570	0.453	0.850	1.580	2.400		
	(B) Ex-smoker	20	1.468	0.472	0.820	1.330	2.360	0.935	NS
	(C) ≤10 cigs per day	20	1.744	0.443	0.820	1.740	2.640	1.111	NS
	(D) ≥20 cigs per day	20	1.417	0.467	0.670	1.350	2.480	0.903	NS
IgA, g/L	(A) Never-smoker	20	2.177	0.827	1.310	1.880	4.770		
	(B) Ex-smoker	20	2.468	0.895	1.080	2.335	5.080	1.134	NS
	(C) ≤10 cigs per day	20	1.974	0.775	1.120	1.545	3.920	0.907	NS
	(D) ≥20 cigs per day	20	2.309	0.641	1.080	2.405	3.640	1.061	NS
IgM, g/L	(A) Never-smoker	20	1.270	0.606	0.580	1.055	2.420		
	(B) Ex-smoker	20	1.265	0.687	0.410	1.095	3.540	0.996	NS
	(C) ≤10 cigs per day	20	1.182	0.703	0.310	1.060	2.870	0.931	NS
	(D) ≥20 cigs per day	20	1.028	0.453	0.350	0.975	2.010	0.809	NS
Triglycerides, mmol/L	(A) Never-smoker	20	1.086	0.510	0.430	0.965	2.440		
	(B) Ex-smoker	20	1.367	0.789	0.450	1.205	3.500	1.259	NS
	(C) ≤10 cigs per day	20	1.231	0.551	0.480	1.155	2.930	1.134	NS
	(D) ≥20 cigs per day	20	1.425	0.644	0.700	1.220	3.520	1.312	NS
PAI, ng/mL	(A) Never-smoker	20	4.840	1.521	4.500	4.500	11.300		
	(B) Ex-smoker	20	7.630	6.220	4.500	4.500	28.800	1.576	NS
	(C) ≤10 cigs per day	20	5.170	2.172	4.500	4.500	13.300	1.068	NS
	(D) ≥20 cigs per day	20	6.000	4.290	4.500	4.500	22.400	1.240	NS
IL-6, pg/mL	(A) Never-smoker	20	1.208	0.453	0.3	1.165	2.04		
	(B) Ex-smoker	19	1.681	0.931	0.69	1.39	3.8	1.392	NS
	(C) ≤10 cigs per day	20	0.929	0.3197	0.3	0.955	1.43	0.769	NS
	(D) ≥20 cigs per day	19	1.494	0.541	0.65	1.41	2.65	1.237	NS
Factor VII, %	(A) Never-smoker	20	96.040	20.400	56.200	95.100	129.800		
	(B) Ex-smoker	20	105.750	20.810	71.700	110.500	139.400	1.101	NS
	(C) ≤10 cigs per day	20	109.240	17.370	80.500	106.800	139.400	1.137	NS
	(D) ≥20 cigs per day	20	105.700	20.940	66.700	107.450	141.100	1.101	NS

NS, not significant. All p-values are compared to never-smokers. Note: data for C-reactive protein (n=33 out of 80), serum amyloid A (n=40 out of 80) and cardiac troponin I (n=0 out of 80) were incomplete due to levels falling below the lower limit of quantification for their respective assays, and are not shown. For other biomarkers where n<20, triplicate measurements which fell outside of the assay acceptance range could not be considered reliable and were removed.

based on the likely differences in smoking behaviour, the design of this study deliberately sought to minimise within group differences and maximise between group differences by recruiting smokers with widely different current smoking routines (i.e., $\leq$10 cigarettes per day with a $<$6 mg ISO 'tar' yield compared to $\geq$20 cigarettes per day with a 10 mg ISO 'tar' yield). However, during the study, some of the smokers who were recruited into the $\geq$20 cigarettes per day group did smoke fewer than this number of cigarettes (see Figures 1 and 2) and this may have contributed to the overlap in biomarker levels across the groups.

Discussion

The primary objective of this study was to establish baseline data on biomarkers in groups that were clearly separated based upon acute smoking exposure in comparison to never-smokers and former smokers.

BoE data showed considerable variability in the smoking groups. Despite this variability, all BoE with the exception of NNAL showed good correlations with daily cigarette consumption. A stronger correlation was observed between total urinary nicotine metabolites and both NNAL and COHb. The use of total urinary nicotine metabolites helps to reduce variability associated with smoking behaviour. Residual variability in the correlation between NNAL and total urinary nicotine metabolites could reflect metabolic differences in the study subjects and other confounding factors, such as diet and lifestyle. As previously mentioned, it is important to recognise the role that differential smoking behaviour could have on smoking status and BoPH levels. ''Pack years'' is traditionally used in smoking studies to evaluate life-time smoke exposure. However, this estimate does not take smoking behavioural parameters, such as inhalation depth, inhalation intensity and puff frequency, into account. Thus, this may lead to a subject's smoking status being misclassified and making BoPH data more variable and more difficult to interpret. An exposure biomarker approach should help to overcome some of the heterogeneity associated with smoking behaviour. It is possible to measure the actual absorption of various cigarette smoke constituents by the body over a number of days prior to BoPH measurement. Such measurements would provide a better insight into an individual's smoking routine and thus help with smoking classification for BoPH studies.

From these BoE data, it is apparent that total urinary nicotine metabolites provide a better indication of acute smoke exposure than cigarettes per day. Total urinary nicotine metabolites should be included in future studies of smoking subjects as an estimate of recent smoke constituent exposure. The reliability of total urinary nicotine metabolites as biomarkers of exposure in smokers has been established over many years. After initial concern that the metabolism of nicotine is highly variable in populations, at least partly due to polymorphism in the major metabolic enzymes, measurement of total nicotine metabolites is now widely accepted as the method of choice (13, 28). However, it is likely that there will be polymorphism in the metabolism of many smoke constituents and this will add to the variability in results obtained. Furthermore, there is a possibility that metabolic polymorphism leading to exposure variation will have an effect on the subsequent levels of BoPH detected. This situation is known for other exposure situations and several groups have developed physiologically based pharmacokinetic (PBPK) models in an attempt to understand the process and minimise variability in results (29, 30). Future studies may be able to apply a PBPK approach to cigarette smoke exposure to reduce variability in BoPH levels.

Previous reviews of the scientific literature had suggested a number of candidate BoPH for which there should be significant differences between smokers and non-smokers (12). These BoPH were examined in this study. Although the BoPH showed considerable inter-subject variability, several were significantly different between smokers and non-smokers, in agreement with previous literature reports (Table 3). For dTx, levels in never-smokers were lower than in smoking subjects (31–33). A similar difference between groups was also observed for Tx-M. These data are in agreement with previous studies in the literature which report significantly higher levels of Tx-M in smokers compared to non-smokers (34–36).

IgG was significantly decreased in smokers, whereas IgA and IgM were not significantly different in any of the study groups (see Table 4). Studies measuring immunoglobulins in smokers and non-smokers confirm decreases in IgG levels in smoking subjects (37–39); however, there are mixed data on IgA and IgM (37, 38).

With respect to levels of 8-epiPGF, both smoker groups were significantly different from never-smokers, when creatinine adjusted values were compared, supporting the findings of previous studies in the literature (31, 40, 41). The levels of 8-epiPGF in former smokers were similar to never-smokers (although not significantly different from either smokers or never-smokers), suggesting that the smoking effect on this biomarker may be reversible with smoking cessation.

cTG levels were significantly different in all study groups compared to never-smokers, suggesting an irreversible categorical difference between ever- and never-smokers, but there is currently little data available on this biomarker in smokers in the literature and further work would be needed to establish this.

Levels of 8-OHdG in Group C smokers and former smokers were significantly higher than never-smokers, when creatinine adjusted values were compared. These data are in agreement with data reported by Loft et al. (15, 42) and Chuang et al. (43).

Although LDL-cholesterol was significantly higher in group D smokers compared to never-smokers, HDL and triglycerides were not significantly different in all study groups (see Table 4). There are reports in the literature that HDL levels are decreased in smokers (31, 44) and that LDL-cholesterol and triglycerides are elevated in smokers compared to non-smokers (45, 46).

Some biomarkers known for their ability to predict future CVD severity, such as CRP (47, 48) and SAA (47), were not increased above the lower limit of quantification for most subjects in this study. The subjects included in this study did not have any clinical symptoms of CVD and thus it is unlikely that they would have elevated levels of these latter stage CVD biomarkers. Furthermore, cTnI is elevated following actual cardiac damage and these subjects would not have been expected to have detectable levels of this biomarker (49).

This study has identified some potentially useful biomarkers for future clinical research, namely 8epiPGF, 11-dehydrothromboxane B_2, 2, 3 dinorthromboxane B_2, IgG and cTG. From these observations, it is apparent that further work will be required before these and other BoPH can be used to predict the risk of disease in smokers or to assess the potential of PREPs for harm reduction. Other studies have shown that large numbers of subjects are often required to detect events that are based on low penetrance or highly polymorphic genes (50, 51) and so future studies should aim to increase the number of subjects involved to increase statistical power to detect small changes. The metabolic pathways for the biomolecules used in this study as biomarkers may also be polymorphic and future studies could also address this point.

More research is needed to understand the dynamics of the biomarker levels, especially in smokers who are actively trying to stop smoking. The properties of an ideal biomarker still remain to be identified. However, it would be desirable to have a suite of biomarkers that include the following properties: 1) specific for a defined process involved with disease initiation or progression; 2) showing clear differences between non-smokers and smokers; 3) showing a graded response with cumulative smoke exposure; and 4) showing reversibility on smoking cessation. The data presented in this manuscript should contribute to this discussion. Therefore, there is a need to discover and develop biomarkers with the desirable properties discussed above.

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