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RESEARCH ARTICLE

A correlation study applied to biomarkers of internal and effective dose for acrylonitrile and 4-aminobiphenyl in smokers

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Abstract

The urinary metabolites 2-cyanoethylmercapturic acid and 4-aminobiphenyl have been correlated with tobacco smoke exposure. Similarly, 2-cyanoethylvaline and 4-aminobiphenyl haemoglobin adducts have been used as biomarkers of effective dose for the exposure to acrylonitrile and 4-aminobiphenyl, respectively. Each pair of biomarkers is derived from the same parent chemical; however, the correlation between the urinary and the haemoglobin biomarkers has not been investigated. Using clinical study samples, we report a weak correlation between urinary and haemoglobin biomarkers due to different accumulation and elimination rates. Time course analysis showed that a reduction in exposure was paralleled by a delayed reduction in haemoglobin adducts.

Keywords

4-aminobiphenyl, acrylonitrile, biomarkers, haemoglobin adduct

History

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Introduction

The use of combustible tobacco products is a leading cause of preventable diseases such as lung cancer, cardiovascular diseases and chronic obstructive pulmonary disease (COPD). Cigarette smoke is a complex mixture with over 6000 different chemicals identified (Rodgman & Perfetti, 2013), split between gaseous and particulate phases of which 93 substances have been classified as harmful and potentially harmful constituents by the US FDA (Food and Drug Administration, 2012). The Family Smoking Prevention and Tobacco Control Act gave the FDA the statutory oversight of public health policies in the US relating to tobacco products, including marketing authorisation (Food and Drug Administration, 2012). The FDA guidance on general principles for scientific studies on novel tobacco products is articulated around product chemistry, non-clinical studies and studies with adult human subjects.

Leischow et al. (2012) in a guest editorial identified research priorities needed to inform the FDA on tobacco-related policies. The first priority that was identified by the authors was to ''expand characterisation of biomarkers of exposure and health risks'' and apply these biomarkers to product comparative studies. In 2008, Gyorffy et al. (2008) proposed a model to describe the biomarker continuum for genotoxicants which included biomarkers of internal dose (absorbed dose of toxicant measured in biofluids), effective dose (bioactive dose that reacts with macromolecules, proteins/DNA), and early biological effect (observable change in normal biological processes). This causative biomarker progression line from exposure to disease can also be applied to tobacco smoke chemicals and a variety of methods have been validated to measure internal and effective dose of cigarette smoke toxicants. Throughout this article, we have applied the biomarkers categories proposed by Gyorffy et al. (2008) to define internal dose and effective dose.

It was suggested that the assessment of a biomarker of internal dose combined with a corresponding biomarker of effective dose could give a more accurate representation of tobacco toxicant exposure (Hecht et al., 2010). For instance, an observed reduction in NNAL a urinary metabolite of NNK and IARC class 1 carcinogen, could either indicate reduced NNK exposure or could be due to an increased bioactivation of NNK which then accumulates in the form of DNA and protein adducts (Hecht et al., 2010). Therefore, variability of the metabolic activity in different subjects could potentially lead to mis-interpretation of individual exposure data when using internal dose biomarkers only. The concept that the mass balance is conserved for a given dose of toxicant metabolised through multiple pathways can be easily understood. However, this does not imply that in clinical practice there is a dose correlation between these different metabolites. This is due in part to the relative short half-life of biomarkers of internal dose usually measured in hours compared to the residence time in the body of the biomarkers of effective dose usually measured in weeks or months in the case of protein adducts (Carmella et al., 2009; Maclure et al., 1990). The significant differences in body residence time between internal dose and effective dose biomarkers reside in

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the respective elimination kinetics of the free soluble metabolites and the metabolites that have reacted with macromolecules. The persistence of biomarkers of effective dose will be effectively dependent on the residence time of the target macromolecule. For instance, haemoglobin adducts will have a body residence time of approximately 120 days, corresponding to the persistence of red blood cells in humans of 120 days (Berlin et al., 1959). Few correlation studies between biomarkers of internal dose and biomarkers of effective dose have been conducted thus far and correlations appear to be highly dependent on the matrix used. N^7-methyldeoxyguanosine (N^7-MEG), is a major methylated DNA adduct excreted in urine. It can be formed by the α-hydroxylation pathway of NNK, but is not specific to NNK since adduct can also be formed by other methylating agents. A moderate correlation was reported between free NNAL and N^7-MEG in a group of urothelial carcinoma patients split between smokers and non-smokers (Lee et al., 2008). BPDE-haemoglobin adducts but not the albumin adduct were highly correlated with the urinary polyaromatic hydrocarbon 1-hydroxypyrene (Tzekova et al., 2004). Interestingly another study conducted with smokers and non-smokers reported no correlation between the BPDE–haemoglobin adduct and cotinine whilst a moderate correlation was reported between the BPDE–albumin adduct and cotinine (Pastorelli et al., 2000; Wang et al., 2007). However, none of those studies have looked at the correlation between specific pairs of biomarkers of internal and effective dose derived from the same parent compound. In this report, we studied the correlation between the haemoglobin adducts and the urinary biomarkers of acrylonitrile and 4-aminobiphenyl in smokers at multiple time points over a period of 6 months. Acrylonitrile has been classified as a respiratory toxicant and carcinogen in the FDA list on harmful and potentially harmful constituents in tobacco products and tobacco smoke (HPHCs; Food and Drug Administration, 2012) and a class 2B carcinogen by IARC (International Agency for Research on Cancer, 1979, 1987, 1999). Ranges for mainstream smoke yields were reported to amount to 4.4–11.9 and 7.8–39.1 μg/cigarette when machine smoked with ISO and Massachusetts smoking parameters, respectively (International Agency for Research on Cancer, 2004). Once absorbed in the body, acrylonitrile is metabolised through:

(i) Epoxidation to glycidonitrile, which in turn can form DNA and protein/haemoglobin adducts such as 2-cyanoethylvaline (CEVal; Figure 1) which can be released by a modified Edman degradation procedure.
(ii) Reaction with glutathione (GSH) possibly catalysed by GSTs (Figure 1). As final products of the reaction with GSH, a number of mercapturic acids are formed which are excreted into the urine, the most important of which is 2-cyanoethylmercapturic acid (CEMA; Figure 1; Minet et al., 2011).

4-Aminobiphenyl is a cigarette smoke aromatic amine classified as a class 1 IARC carcinogen and also present in the FDA HPHCs list (Food and Drug Administration, 2012; International Agency for Research on Cancer, 2010). 4-Aminobiphenyl mainstream smoke yields ranged from 0.5 to 3.3 ng/cigarette in 48 commercial cigarettes smoked with three different regimes including ISO (Riedel et al., 2006). N-Hydroxylation of the parent amine is a key step in the activation of aromatic amines into carcinogenic species. N-Hydroxy-4-aminobiphenyl can react with a single cysteine residue (93β) of haemoglobin to form a sulphonamide crosslink (Bryant et al., 1987). The adduct can be hydrolysed from haemoglobin in alkaline conditions which releases 4-ABP (Bryant et al., 1987). For convenience, we have labelled this biomarker 4-ABP-Hb throughout this article. The hydroxylamine can also be transported to the bladder in a stable *N*- and *O*-glucuronidated form. In the acidic environment of the bladder, the glucuronide moiety can be hydrolysed leading to the formation of DNA reactive nitrenium ions (Bryant et al., 1987). The glucuronide can also be hydrolysed in urine samples using glucuronidase allowing the measurement of urinary 4-ABP (Grimmer et al., 2000).

We hypothesised that there is a strong positive correlation between urinary 4-ABP and 4-ABP-Hb and CEMA with CEVal since each pair of biomarkers is derived from a common parent chemical. A clinical study was conducted where urine and haemoglobin samples were collected from control smokers, ex-smokers and non-smokers at multiple time points over a period of 6 months. In addition, samples were also collected from a smoker group who switched to a prototype cigarette with reduced levels of acrylonitrile and 4-ABP compared to the control cigarette. The objective of this article is to look at the correlations between urinary CEMA, 4-aminobiphenyl and their haemoglobin adducts CEVal and 4-ABP-Hb in control smokers. We also assessed whether changes in biomarkers of internal dose was paralleled by changes in biomarkers of effective dose over time when smokers used a low 4-ABP, acrylonitrile cigarette prototype or changed the number of cigarettes smoked. A detailed analysis discussing the risk-benefit of the prototype cigarette compared to control cigarettes and including an extended list of biomarkers will be presented in another publication.

Materials and methods

Clinical study design

A detailed study protocol has been described in Shepperd et al. (2013) and the study was registered in the Current Controlled Trials database under the reference ISRCTN81286286. In brief, never-smokers, ex-smokers and current smokers were recruited from the Hamburg area (Germany). Inclusion criteria for the smoking groups were age 23–55 years, current smokers of between 10 and 30 (6–8 mg) ISO tar cigarettes per day (CPD) and smokers for at least 5 years before screening. Criteria for the ex-smoking group were age 28–55 years; not having smoked for at least 5 years but having been a regular smoker of between 10 and 30 CPD for at least 5 years. Inclusion criteria for the never smoking group were age 28–55 years, never having smoked more than 100 cigarettes during his/her lifetime, and none in the previous 5 years. Groups demography is shown in Table 1. Subjects who participated in the study were monitored over a period of 6 months. Control smokers were supplied with a 7 mg ISO tar Lucky Strike Silver (control cigarette) 12 days prior to their first residential clinic evaluation. The daily

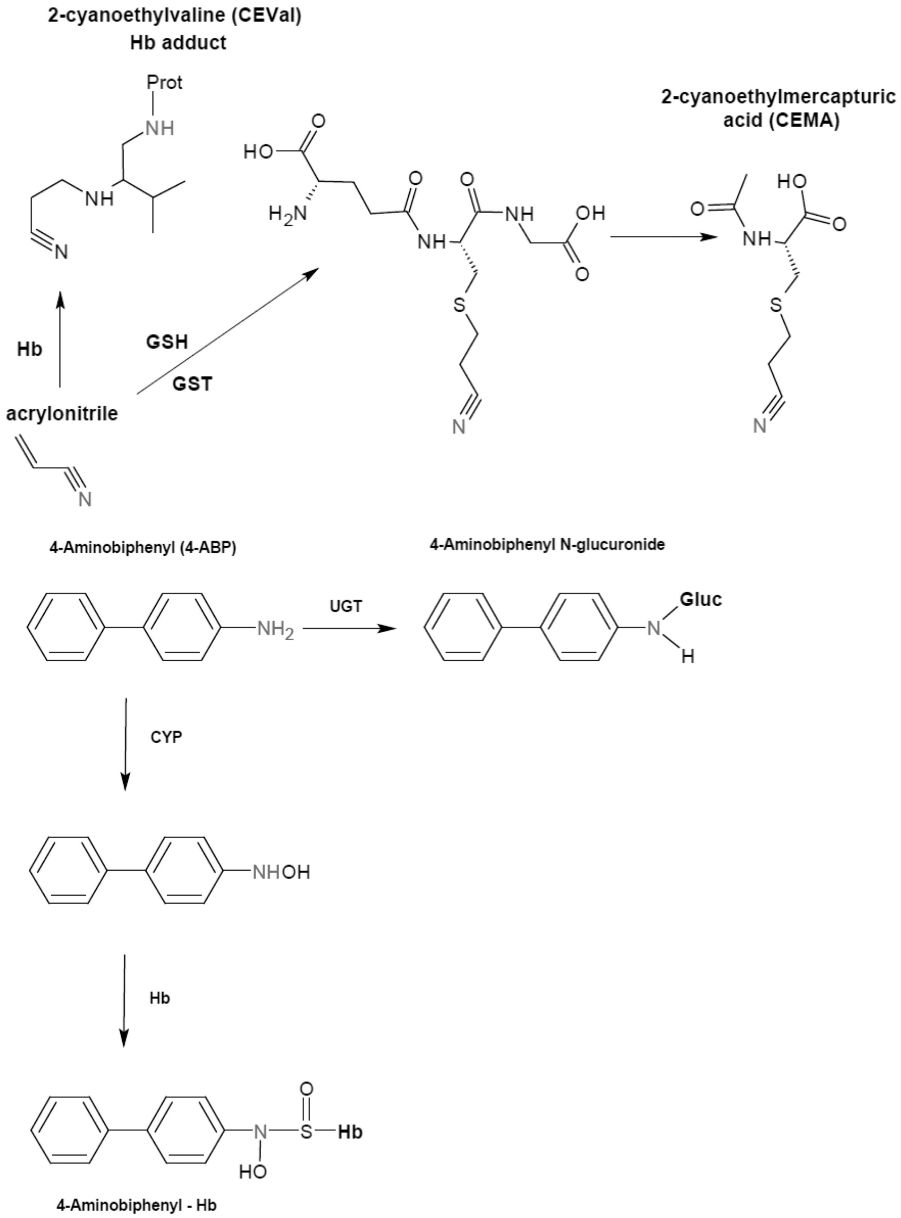

Figure 1. Simplified metabolic pathways leading to the formation of CEMA, CEVal, 4-aminobiphenyl *N*-glucuronide and 4-ABP-Hb.

Table 1. Demography and test product.

	Control smoker group	Prototype cigarette smoker group	Ex-smoker group	Never-smoker group
Enrolled (n)	67 (100%)	76 (100%)	61 (100%)	61 (100%)
Completed (n)	56 (83.6%)	58 (76.3%)	58 (95.1%)	57 (93.4%)
Gender				
Male	33 (49.2%)	36 (47.4%)	31 (50.8%)	32 (52.5%)
Female	34 (50.8%)	40 (52.6%)	30 (49.2%)	29 (47.5%)
Age (± SD) (years)	38.67 (±8.81)	39 (±9.42)	42.48 (±7.73)	41.79 (±7.49)
BMI (± SD) (kg/m^2)	24.57 (±2.73)	24.58 (±3.12)	25.21 (±2.84)	25.02 (±2.69)
CPD average at recruitment (±SD)	19.12 (±5.81)	18.33 (±5.16)	17.13 (±6.24)	NA
Ethnicity				
White	67 (100%)	73 (96.5%)	61 (100%)	59 (96.72%)
Black or African American				1 (1.64%)
Other		3 (3.95%)		1 (1.64%)
Test product				
ISO Tar yield (mg)	7	7		
Acrylonitrile (µg/cig)	5.6	BDL (<0.28)		
4-Aminobiphenyl (ng/cig)	1.4	0.7		

consumption was recorded in an electronic diary during ambulatory periods and during residential visits to the clinic. Samples (blood, 24 h urine) were collected at day 13, 14, 46, 77, 109, 182 and 183 in residential clinic visits. A group of smokers initially smoking the 7 mg ISO tar control cigarette was switched at the end of day 14 to a prototype product with a low acrylonitrile and 4-aminobiphenyl yield. On days 1, 31, 62, 95, 124, 144 and 165 smokers received adequate supplies of study cigarettes, calculated from their typical daily consumption, to last until the next ambulatory day plus two packs. The number of cigarettes supplied was based on the self-reported average daily consumption (electronic diary), initially at screening and then based on each previous ambulatory collection period and was caped to a maximum of 60 CPD. If daily consumption increased during the study (including between ambulatory visits), the supply was reviewed with the individual and adjusted accordingly. Non-compliance to study product was rare based on electronic diary records, however this assumes honest feedback from the participants. The increase in cigarette consumption during the course of the study was reported to the independent data safety monitoring board and ethics committee. It was recommended that the study continued, that subjects be asked to complete a questionnaire to ascertain the reasons for the consumption increases, and that consumption should be monitored for 8 weeks following study completion. In addition, the amount and intensity of smoking cessation advice was increased. Cigarette consumption returned to pre-study levels after the study ended. Samples from ex-smokers and non-smokers were collected at day 2, 3, 79, 80, 163 and 164 in residential clinic visits for baseline assessment. Different days were used for the control smoker and switcher groups and the non-smoker, ex-smoker groups for practical reasons including clinic capacity and sample collection.

A full list of the biomarkers measured in this study has been published (Shepperd et al., 2013). For the purpose of this article, we have focused on urinary and haemoglobin biomarkers derived from 4-aminobiphenyl and acrylonitrile. It should be noted however, that the prototype cigarette showed reduced smoke chemistry for a series of smoke toxicants other than acrylonitrile and 4-ABP for which data on biomarkers of internal dose were also collected. A comprehensive data analysis paper including these other biomarkers of internal dose measured in the prototype cigarette smokers is currently under review. This study was conducted in compliance with the ethical principles of the Declaration of Helsinki, Good Clinical Practice and German Law, including informed consent and was approved by the Ethics Committee of Ärztekammer Hamburg, Germany, 29 November 2011 ref: PV3824.

Analytical methods

CEVal Hb adducts

CEVal Hb adducts were determined according to a published method (Van Sittert, 1996), with modifications. In brief, globin from washed and lysed erythrocytes of 5 ml EDTA blood was precipitated, washed and dried. One hundred milligrams of the dried protein were spiked with the internal standard (IS, which is a globin adducted with D_3-CEVal) subjected to a modified Edman degradation procedure using pentafluorophenyl isothiocyanate (PFPITC). After extracting the mixture twice, evaporation and reconstitution in 50 µl acetonitrile, 1.5 µl is injected into a GC-EI-MS/MS system (Trace-GC/TSQ, Thermo-Fisher, Dreieich, Germany). Retention time for the CEVal (analyte) and D_3-CEVal (IS) derivatives was 8.05 min. Mass transitions applied for the analyte were *m/z* 335 → 282 (quantifier; Q1 ion at 335 is obtained due to fragmentation in the source) and 377 → 282 (qualifier) and that for the IS was *m/z* 380 → 338. The mass transitions of the IS which correspond to those of the analyte were found to be interfered by the matrix and could, therefore, not be used for quantification. Both intra-day and inter-day precision of the method were better than 15%. Since no defined and certified reference material is available, the accuracy of the method was determined by spiking non-smoker and smoker globin pools with three levels of *N*-cyanoethyl-valine–leucine–anilide. Accuracy ranged from 93% to 113%. Limit of detection (LOD) and limit of quantification (LOQ) amounted to 0.7 and 2.0 pmol/g. Calibrations were linear in the range of 2.0–500 pmol/g.

4-ABP-Hb adducts

4-ABP-Hb adducts were determined according to a published method (Lewalter & Gries, 2000), with major modifications. In brief, 200 mg dried haemoglobin, derived from washed and lysed erythrocytes of 5 ml EDTA blood were spiked with the IS (4-ABP-D_9) and hydrolysed under alkaline conditions. After solid phase extraction (SPE) of the hydrolyzate on C_{18}-cartridges, the extract was derivatised with hexafluorobutyric acid anhydride (HFBA). The reactions mixture was evaporated in the presence of toluene, re-dissolved in 50 µl *n*-hexane and injected (2 µl) into a GC-MS system (Agilent Technology, Waldbronn, Germany). Retention times for the derivatives of the analyte and IS were 13.14 and 13.10 min, respectively. Mass spectrometric detection was performed after negative chemical ionisation (NCI) applying selective ion monitoring (SIM) mode using the mass traces *m/z* 345 and 354 for the derivatives of the analyte and the IS, respectively. LOD and LOQ levels were 1.7 and 5.0 pg/g Hb, respectively. The modified method was validated according to the FDA guideline (Food and Drug Administration, 2001). Intra- and inter-day precisions are about 15% at low levels and about 5% or better at medium and high levels. Since no defined and certified reference materials are available, the accuracy of the method was determined by spiking non-smokers and smokers haemoglobin pools with three levels of free 4-ABP. The accuracy was found to be in the range of 102–112%. The calibration range amounted to 5–250 pg/g Hb and was linear.

CEMA in urine

Cyanoethyl mercapturic acid (CEMA) reference material was obtained from Synthèse AptoChem, Inc., Montreal, Canada. Stable label internal standard was also obtained from Synthèse AptoChem, Inc., Montreal, Canada. Aliquots of 0.1 mL of urine sample were supplemented with 0.025 mL of working internal standard solution [$^{15}N^{13}C_3$] CEMA.

The samples were pH adjusted with a 1% formic acid solution prior to loading on a mixed-mode solid phase extraction plate. Following aqueous and polar organic wash steps the analytes were eluted with a solution of acetonitrile and ammonium acetate buffer. The extracts were evaporated under an inert gas and reconstituted in an aqueous solution. Sample analysis was performed in a 96-well plate format. Chromatographic separation was achieved with an ACQUITY UPLC™ HSS C_{18}, 150 × 2.1 mm, 1.8 µm analytical column from Waters Corp. A gradient separation was employed utilising mobile phases A: 99:1 v/v water:formic acid and mobile phase B: 50:49.5:0.5 v/v acetonitrile:water:formic acid. The injection is loaded onto the analytical column with 90% mobile phase A followed by a shallow gradient over 3 min with a 1 min wash at 10% mobile phase A. The column was maintained at a temperature of 50 °C. Negative ions (CEMA: 214.9 → 162.0; [$^{15}N^{13}C_3$] CEMA: 219.0 → 166.2) were monitored in multiple reaction monitoring mode on an AB Sciex API 4000TM triple quadrupole mass spectrometer using an electrospray source. LOQ was 0.275 ng/ml with a linear range of 0.275 to 200 ng/ml and an accuracy of 95.6–100.6%.

4-ABP in urine

Urinary 4-ABP was determined by GC-MS-NCI as described earlier (Riedel et al., 2006). Intra-day precision of the method was <3%, inter-day precision <5%. The accuracy was found to be in the range of 95–102%. LOD and LOQ were 0.3 and 1.0 ng/l, respectively. The linear calibration range amounted to 1.0–100 ng/l.

Total nicotine equivalents in urine

Total nicotine equivalents (TNEQ) was measured by LC-MS/MS following the method described by Xu et al. (2004). Measured urinary concentrations for nicotine, cotinine, trans-3*S*′-hydroxycotinine and their glucuronide were multiplied by their respective daily urine volumes and converted based on molecular weights to yield recovery results in nicotine equivalents. The sum of the nicotine equivalents for nicotine, cotinine and trans-3*S*′-hydroxycotinine were calculated for each subject per 24 h urine to give total daily urinary nicotine equivalents in milligrams per 24 h (St Charles et al., 2006).

Statistical analysis

Arithmetic means were calculated and differences over time were tested using the General Linear Model. In cases where the regression assumptions (normal distribution and homoscedasticity) were violated, the natural logarithm was used to transform the data. Tukey's post-hoc test was used to account for multiplicity. The significance level was set at 0.05 (5%). Linear regression and LOESS smoother were used to identify the lines that best describe the relationship between pairs of biomarkers. The measure used to quantify the magnitude of correlation was R^2 (*R*-square). A LOESS smoothing curve was applied to the CPD correlation scatter plots using Minitab 16. The statistical analyses were performed in Minitab 16 (Minitab Inc., Ltd., Progress Way, Coventry, UK), JMP Pro 10 (SAS Institute, Cary, NC) and SAS 9.3 (SAS Institute, Cary, NC).

Results

4-Aminobiphenyl and acrylonitrile biomarkers of internal and effective dose in control smokers, ex-smokers and never-smokers

Urinary biomarkers and haemoglobin adducts of acrylonitrile (CEMA) and 4-ABP were quantified in control smokers, ex-smokers and never-smokers for each time point. All four biomarkers were significantly lower in ex-smokers and never-smokers compared to the control smoker group at 95% confidence interval. There was no statistical difference between never-smokers and ex-smokers at the 5% significance level indicating that the urinary and haemoglobin biomarkers phenotype was reversible. Mean values for each biomarker at each time point for non-smokers, ex-smokers and control smokers are given in Table 2. Detailed examination of CEVal in ex-smokers indicated that the higher mean and standard deviation values (Table 2B) were due to two subjects with high levels of this biomarker. This could be due to non-compliance or occupational exposure. ANOVA general linear model analysis showed an increasing trend between day 46 and day 182 for urinary CEMA and 4-ABP biomarkers in control smokers (Table 2C). This increase was matched by an increase in cigarette consumption and TNEQ (total nicotine equivalents) from day 46 onwards (Table 2C). Overall, levels of CEVal and 4-ABP-Hb in control smokers were significantly lower at the start of the study compared to the latest time points at day 109, 182 and 183 (Table 2C) reflecting a more gradual increase of the haemoglobin adducts when cigarette consumption increases (as would be expected).

Correlation of biomarkers within and between matrices

Pairwise correlations between 4-ABP (urine) versus 4-ABP-Hb and CEMA *vs* CEVal were conducted using data from the control smoking group only, since inclusion of the non-smokers and ex-smokers improved the correlation based on baseline levels. Given that the control smokers increased their consumption in the course of the study and considering the different body residence time of urinary and haemoglobin biomarkers, we performed the correlation at what could be a better approximation of ''steady-state'' level of smoking. For this, (i) a pairwise correlation was performed only with the mean of the data collected at time points 13 and 14 (first in clinic study days; Figure 2A and B). The haemoglobin adducts at that time point reflect the smoking behaviour in the month prior to entering the study. It is assumed that smoking behaviour is in steady-state during this time period since there was no difference between the mean CPD at recruitment and the mean CPD at the first time points indicating little change in smoking behaviour (Tables 1 and 2). (ii) Another correlation was also conducted by averaging for each subject the urinary biomarker across time points in an attempt to approximate the steady-state smoking rate over the 6-month period of the study. Those urine averages were then correlated with the mean haemoglobin biomarkers at the last time points (day 182 and 183; Figure 2C and D) since those are more representative of the smoking behaviour from the previous

Table 2. Summary statistics for urinary biomarkers of acrylonitrile (CEMA), 4-aminobiphenyl (4-ABP) and the haemoglobin adducts CEVal and 4-ABP-Hb for each time point (day of visit) in never-smokers (A), ex-smokers (B) and control smokers (C).

Group	Days of visit	Supplied cigarettes	*N*	CPD	TNEQ (mg/24 h)	CEMA* (μg/24 h)	CEVal* (pmol/g Hb)	4-ABP (ng/24 h)	4-ABP-Hb (pg/g Hb)
A. Never-smokers	2	None	61	0	>0.01	1.3 ± 0.9^{a}	0.3 ± 2.1^{a}	2.2 ± 2.6^{a}	13.4 ± 11.4^{a}
	3	None	60	0	>0.01	1.1 ± 0.8^{ab}	0.3 ± 1.8^{a}	1.6 ± 3.0^{a}	11.4 ± 5.7^{a}
	79	None	59	0	>0.01	1.1 ± 0.9^{ab}	0.3 ± 1.7^{a}	2.9 ± 1.8^{a}	9.2 ± 6.3^{a}
	80	None	59	0	>0.01	1.0 ± 0.8^{b}	0.2 ± 1.5^{a}	2.1 ± 1.3^{a}	9.6 ± 7.5^{a}
	163	None	58	0	>0.01	1.3 ± 1.0^{a}	0.3 ± 1.8^{a}	2.5 ± 2.3^{a}	12.8 ± 15.2^{a}
	164	None	57	0	>0.01	1.0 ± 0.9^{b}	0.3 ± 1.7^{a}	2.1 ± 4.2^{a}	12.2 ± 15.0^{a}
B. Ex-smokers	2	None	61	0	>0.01	1.5 ± 1.1^{a}	1.3 ± 5.3^{a}	2.1 ± 1.7^{ab}	12.5 ± 11.1^{a}
	3	None	60	0	>0.01	1.3 ± 1.2^{a}	1.0 ± 3.8^{a}	1.6 ± 1.4^{a}	12.4 ± 11.5^{ab}
	79	None	59	0	>0.01	1.6 ± 2.1^{a}	1.6 ± 5.9^{a}	3.3 ± 1.7^{d}	8.3 ± 5.2^{c}
	80	None	59	0	>0.01	1.3 ± 2.0^{a}	1.3 ± 6.3^{a}	2.9 ± 1.7^{cd}	8.9 ± 5.6^{bc}
	163	None	58	0	>0.01	1.8 ± 3.2^{a}	1.6 ± 8.1^{a}	2.8 ± 2.0^{bcd}	10.7 ± 5.8^{abc}
	164	None	58	0	>0.01	1.5 ± 2.3^{a}	2.5 ± 12.1^{a}	2.4 ± 1.8^{bc}	10.8 ± 5.7^{abc}
C. Control smokers	13	7 mg ISO control	61	19.3 ± 4.9^{a}	15.9 ± 7.4^{ac}	197 ± 101.3^{ab}	96 ± 49.2^{a}	19.5 ± 8.5^{ab}	61.9 ± 23.2^{a}
	14	7 mg ISO control	61	18.4 ± 5.7^{a}	14.6 ± 6.6^{ab}	179 ± 98.1^{a}	102 ± 60.0^{a}	17.1 ± 7.3^{a}	58.8 ± 22.8^{a}
	46	7 mg ISO control	61	23.3 ± 7.6^{b}	18.2 ± 8.1^{d}	225 ± 113.5^{c}	$137 \pm 59.4^{b\ c}$	22.2 ± 10.4^{c}	59.8 ± 18.9^{a}
	77	7 mg ISO control	$58^{(1*)}$	23.0 ± 6.9^{b}	17.6 ± 7.8^{cd}	237 ± 124.4^{c}	133 ± 56.6^{b}	22.3 ± 9.0^{c}	59.2 ± 21.7^{a}
	109	7 mg ISO control	57	22.6 ± 8.1^{b}	17.4 ± 7.7^{cd}	238 ± 114.7^{c}	162 ± 67.3^{d}	22.3 ± 9.5^{c}	71.2 ± 24.9^{b}
	182	7 mg ISO control	56	21.9 ± 7.9^{b}	16.4 ± 8.4^{acd}	227 ± 132.7^{bc}	160 ± 81.6^{d}	20.6 ± 9.5^{bc}	74.6 ± 26.0^{b}
	183	7 mg ISO control	56	22.0 ± 7.9^{b}	13.4 ± 5.7^{b}	189 ± 101.4^{a}	150 ± 68.3^{cd}	17.1 ± 7.6^{a}	72.8 ± 25.5^{b}

Number of participants (N), Cigarettes per day (CPD) and TNEQ are also shown. Differences between time points within each group were tested using the General Linear Model and are shown using Tukey grouping at 95% confidence. Means that do not share a letter are significantly different. Groupings marked with an * where based on an ANOVA test using the log transformed data because the data was not normally distributed and or was not homoscedastic. The number of participants (*N*) drops over time due to subjects withdrawing from the study. Numbers in brackets $^{(1*)}$ indicate that at this time points in the control smoker group a subject was an extreme outlier and was removed from the dataset (e.g. non-physiological values reported – possible sample contamination or instrument error).

few months due to different kinetics. In both cases, the coefficient of correlation ($_{L}R^2$) was relatively consistent between 20 and 30% and the correlation was best described by a linear regression (Figure 2A–D). It has to be noted that similar $_{L}R^2$ values (20–30%) were obtained if the correlations were conducted considering all the time points rather than averages (not shown). When comparisons were performed between biomarkers within the same matrix, 43% of the variability of 4-ABP-Hb was accounted for by CEVal with a non-linear regression (Figure 2E, $_{LO}R^2$ values), indicating a better correlation between different smoke chemicals within the matrix rather than between metabolites from the same parent chemical in different matrices. A similar observation was made when urinary 4-ABP was correlated with CEMA, showing a linear correlation with an $_{L}R^2$ value close to 70% for the 24 h urine data (Figure 2F, $_{L}R^2$ value). These observations are in total agreement with the expectations.

Acrylonitrile and 4-aminobiphenyl biomarkers kinetics

One important factor to be considered in the correlation of haemoglobin adducts and urinary biomarkers are their extremely different residence times in the body following exposure. This is of particular importance if no steady-state levels for biomarkers are achieved, which is certainly the case in this study since smokers increased their consumption and this was reflected in the biomarkers of internal and effective dose but at different time points (Table 2C). The half-life of CEMA has been estimated to be 8 h in humans (Jakubowski et al., 1987) whilst haemoglobin adducts have a maximum residence time of 120 days corresponding to the 120 days life time of red blood cells in the human body (Maclure et al., 1990). Therefore, haemoglobin adducts reflect the exposure of the last few of months and do not mirror recent changes in exposure, as long as the exposure is not exceptionally high in the last few days. This can be shown in this study where smokers of a conventional 7 mg ISO Tar yield control cigarette (5.6 μg/cig acrylonitrile) were switched after 14 days to a modified 7 mg ISO Tar prototype cigarette with acrylonitrile levels below a limit of detection of 0.28 μg/cig. Both urinary CEMA and the haemoglobin adduct CEVal were monitored at different time points. The CEMA biomarker level post-switch to the prototype cigarette measured at day 46 was significantly lower compared to the mean baseline values at days 13 and 14, whilst an increase in CPD and TNEQ was noted in this group. There was no significant difference between urinary CEMA from day 46 onwards (Table 3). This suggests that CEMA dropped sharply following the switching at day 14 and had reached a steady-state level at or during the period leading to day 46. A different and more gradual pattern was observed for CEVal. Using the same statistical model, we observed a reduction in CEVal but steady-state level is not achieved before day 109 (Table 3). We did not have a cessation arm in our study to fully assess the body residence time of the biomarker. We also collected the data for 4-aminobiphenyl in switchers. Although the smoke chemistry was showing a 50% reduction in 4-aminobiphenyl at the ISO regime with the prototype cigarette, no such reduction was observed in urinary and haemoglobin adducts in smokers who were switched to the prototype cigarette (Table 3) possibly due to the increased cigarette consumption and the difference between machine smoking regime yields and actual dose delivered when smoked by study subjects.

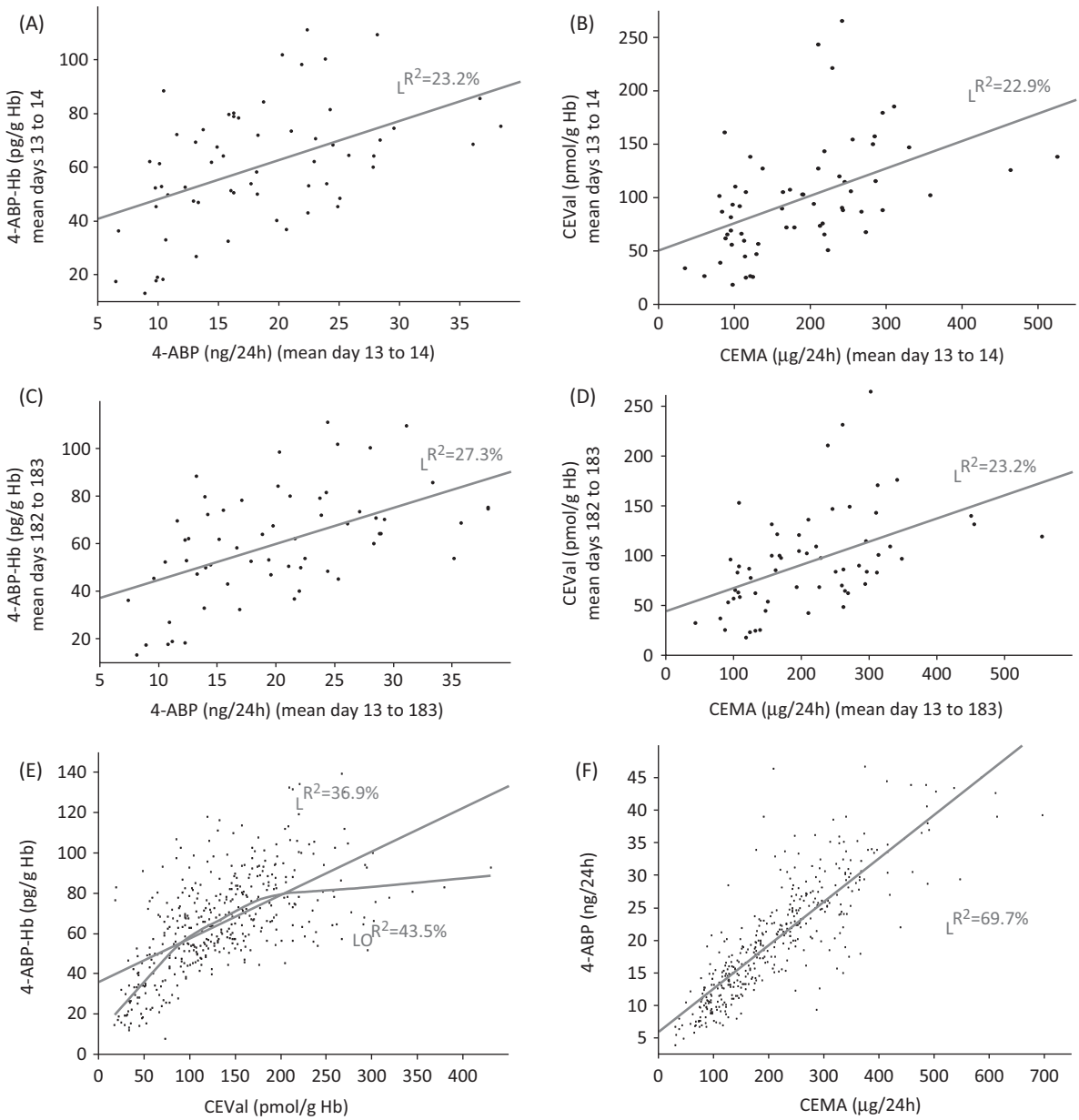

Figure 2. Scatter plots of biomarkers pairs with linear and LOESS regression line in control smokers. (A) Mean 4-ABP-Hb (pg/g Hb) day 13 and 14 versus mean 4-ABP (ng/24 h) day 13 and 14; (B) mean CEVal (pmol/g Hb) day 13 and 14 versus mean CEMA (µg/24 h) day 13 and 14; (C) mean 4-ABP-Hb (pg/g Hb) day 182 and 183 versus mean of all 4-ABP (ng/24 h) time points; (D) mean CEVal (pmol/g Hb) day 13 and 14 versus mean of all CEMA (µg/24 h) time points; (E) 4-ABP (ng/24 h) versus CEMA (µg/24 h), all time points; and (F) 4-ABP-Hb (pg/g Hb) versus CEVal (pmol/g Hb), all time points. Linear regression lines and when appropriate LOESS non-linear smoother are shown of the graphs together with their corresponding $_{L}R^2$ (Correlation coefficient linear regression) and $_{LO}R^2$ (Correlation coefficient LOESS smoother).

Acrylonitrile and 4-ABP biomarkers level as a function of CPD

It has been previously shown that different biomarkers of internal dose and effective dose do not have a linear correlation with CPD and that a biomarker dose plateau is reached in subjects with the highest cigarette consumption (Dallinga et al., 1998; Joseph et al., 2005). Therefore, the correlation between urinary biomarkers and their haemoglobin counterparts can potentially be impacted by different degrees of accumulation when cigarette consumption increases. To test this latter hypothesis, we plotted the relationship between mean adduct levels and mean cigarette consumption per subject and a LOESS regression smoother was applied to the correlation (Figure 3) following the method described by Joseph et al. (2005). For this, data from the control smokers group and the prototype switcher group at days 13 and 14 were used since they both smoked the 7 mg control product during this period which would give a larger dataset to work with. Biomarkers are assumed to be at a relative steady state since no statistical change was observed between mean CPD prior to joining the study and the first clinical time points. As shown in Figure 3(A), a positive correlation between dose and mean CPD is observed but a plateau is reached for smokers with a mean cigarette consumption of 20+CPD for 4-ABP-Hb. A less pronounced trend was observed for 4-ABP and CEMA where the slope of the regression appeared shallower at higher CPD

Table 3. Summary statistics for urinary biomarkers of acrylonitrile (CEMA), 4-aminobiphenyl (4-ABP), and the haemoglobin adducts CEVal and 4-ABP-Hb for each time point (day of visit) in smokers of a 7 mg ISO tar control product switched at the end of day 14 to a low acrylonitrile, 4-ABP cigarette prototype.

Group	Day of visit	Supplied cigarettes	*N*	CPD	TNEQ (mg/24 h)	CEMA* (μg/24 h)	CEVal* (pmol/g Hb)	4-ABP (ng/24 h)	4-ABP-Hb (pg/g Hb)
Prototype cig smokers	13	7 mg ISO control	67	18.5 ± 5.8^{a}	15.2 ± 7.3^{ab}	197 ± 124.0^{a}	114 ± 78.0^{a}	18.3 ± 8.8^{a}	63.8 ± 32.3^{a}
	14	7 mg ISO control	67	19.4 ± 6.6^{a}	13.7 ± 6.8^{a}	174 ± 116.9^{a}	114 ± 72.8^{a}	16.3 ± 7.8^{ab}	63.2 ± 34.9^{ab}
	46	7 mg ISO Prototype	66	23.0 ± 8.9^{b}	19.5 ± 9.3^{d}	73 ± 54.8^{b}	96 ± 62.0^{ab}	15.7 ± 7.4^{bc}	53.3 ± 28.6^{bc}
	77	7 mg ISO Prototype	63	22.0 ± 7.8^{b}	19.3 ± 8.3^{d}	76 ± 68.7^{b}	81 ± 58.2^{bc}	15.4 ± 7.2^{bc}	53.1 ± 31.7^{c}
	109	7 mg ISO Prototype	60	23.6 ± 9.2^{b}	18.1 ± 9.3^{cd}	74 ± 61.0^{bc}	73 ± 55.9^{c}	15.3 ± 7.6^{bc}	61.9 ± 37.2^{ab}
	182	7 mg ISO Prototype	58	22.3 ± 8.1^{b}	17.5 ± 8.1^{cd}	75 ± 66.0^{b}	71 ± 63.6^{c}	13.6 ± 6.8^{cd}	61.8 ± 42.6^{ab}
	183	7 mg ISO Prototype	58	22.3 ± 9.3^{b}	16.6 ± 8.5^{bc}	62 ± 50.4^{c}	69 ± 60.1^{c}	12.4 ± 6.3^{d}	60.9 ± 39.1^{abc}

Number of participants (N), Cigarettes per day (CPD) and TNEQ are also shown. Differences between time points within each group were tested using the General Linear Model and are shown using Tukey grouping at 95% confidence. Means that do not share a letter are significantly different. Groupings marked with an * where based on an ANOVA test using the log transformed data because the data was not normally distributed and or was not homoscedastic. The number of participants (N) drops over time due to subjects withdrawing from the study.

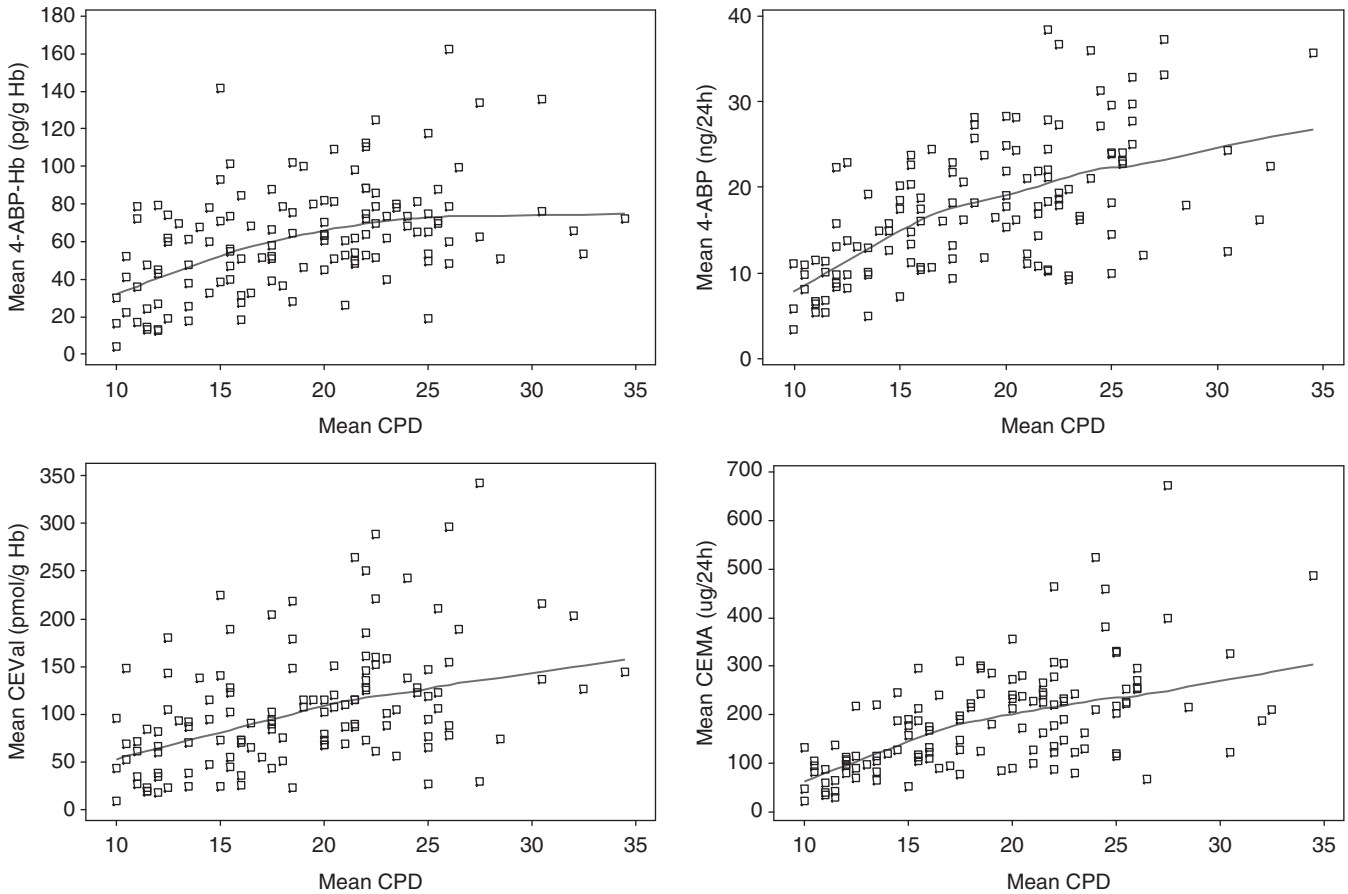

Figure 3. Mean cigarette per day (CPD) per subject in relation to their mean adduct levels and mean urinary biomarker level. Data was taken from the control smoker group and the prototype smoker group prior to switching at day 13 and 14 where both groups used the 7 mg ISO tar control cigarette. (A) 4-ABP-Hb (pg/g Hb), (B) CEVal (pmol/g Hb), (C) 4-ABP urinary biomarker (ng/24 h), (D) CEMA (μg/24 h). Non-linear LOESS regression was applied (Joseph et al., 2005) and regression curve is shown for each graph.

(Figure 3B and C). The relationship between CEVal and CPD appears linear in this study group (Figure 3D).

Discussion

The US EPA has defined a biomarker as ''measurable substances or characteristics that can be used to monitor the presence of a chemical in the body, biological responses or adverse health effects'' (U.S. Environmental Protection Agency, 2014). Due to their mechanistic association with macromolecule damage (in particular DNA), biomarkers of effective dose rank higher in the disease causation scale than biomarkers of internal dose since they reflect the capability of the organism to metabolically activate chemicals into toxic intermediates.

It can be hypothesised that measures of biomarkers of internal dose and effective dose derived from the same parent chemical are not independent from each other and might be correlated. Therefore, we studied the correlation between the biomarker of effective dose CEVal and 4-ABP-Hb and internal dose CEMA and 4-ABP derived from their respective parent compounds, acrylonitrile and 4-aminobiphenyl. The biomarkers were quantified in non-smokers, ex-smokers and control smokers of 7 mg ISO tar yield cigarettes for a period of 6 months at multiple time points. A further group of smokers of the 7 mg ISO tar yield control cigarette was switched after day 14 to a low acrylonitrile, low 4-aminobiphenyl yield cigarette prototype.

As expected, the smokers are clearly discriminated from the never-smokers and the ex-smokers with significantly higher levels of urinary and haemoglobin biomarkers for both acrylonitrile and 4-ABP at all time points (Table 2). The overall mean levels of haemoglobin 4-ABP adducts in smokers (65 pg/g Hb) was consistent with previously reported exposure of 73 pg/g Hb (Seyler et al., 2010) and 43.1 pg/g Hb in a large American cohort of smokers (Roethig et al., 2009). Two other studies have reported mean levels of 4-ABP haemoglobin adducts between 154 and 176 pg/g Hb (Bryant et al., 1987, 1988), however the background level in non-smokers (28–51 pg/g Hb) was 2–5 times higher than in our study (11.4 pg/g Hb) and in the Roethig et al.'s (2009) study (11.4 pg/g Hb). Nevertheless, the adduct ratio between smokers and non-smokers remains consistent across all those studies (3- to 5-fold increase in smokers compared to non-smokers). The mean CEVal value in our study (133 pmol/g Hb) was similar to another study showing mean levels of 112 pmol/g Hb for 264 smokers and 6.5 pmol/g Hb for 100 non-smokers (Scherer et al., 2007) and half of what was reported in an earlier assessment (217 pmol/g Hb) in pregnant smoking mothers (Tavares et al., 1996). However, this study was conducted in a relatively small group of 13 heavy smoker females and the ISO Tar yield of the product was not specified. The mean urinary CEMA in smokers recruited for our study was 212.9 μg/24 h. We previously reported mean values of 139.3 and 214.8 μg/24 h in smokers of 4 and 10 mg ISO Tar yield cigarettes, respectively (Minet et al., 2011). The mean urinary 4-ABP (20.2 ng/24 h) in smokers was also consistent with values reported in a method development paper performed on only 10 smokers (15.3 ng/24 h; Riedel et al., 2006).

An increase in cigarette consumption was noted during the course of our 6-month assessment from an average of 18–19 CPD at screening and at day 13 and 14 then increasing to 22–23 CPD at the subsequent time points (Tables 2C and 3). It is likely that smokers have altered their behaviour in the course of this study. Based on a questionnaire, the participants indicated that the increase was primarily due to the provision of free cigarettes in both the control and switching group, and a perception in the switcher group that the prototype cigarette did not last as long as the control cigarette and that the prototype cigarette might be less harmful. We cannot rule out that some participants were either sharing their cigarettes with others or stockpiling cigarettes during the ambulatory period. However, cigarette butts were collected during this period and generally corroborated consumption levels reported in electronic diaries. In this article, we used the CPD values from the residential clinic visit. Those were more reliable since cigarettes were provided by the clinic staff on request and cigarette butts were also collected. This increased consumption was reflected in an increase in urinary TNEQ (Tables 2C and 3). A similar trend was observed for the acrylonitrile and 4-aminobiphenyl biomarkers in the control smoker group, however the dynamic of the change differed between urine and haemoglobin adducts. The urinary biomarkers response paralleled the increase in cigarette consumption and TNEQ (Table 2) with a maximum increase reached at day 46 and sustained until day 182. The maximum increase in haemoglobin adducts was delayed compared to urinary biomarkers and was reached at day 109 for both acrylonitrile and 4-aminobiphenyl and was maintained thereafter (Table 2).

Our initial hypothesis was that matching pairs of biomarkers of internal dose and effective dose from the same parent chemical should have some degree of correlation. Due to the very large differences in body residence time between the urinary and Hb-related biomarkers and smoking behaviour changes observed in the course of the study, we decided to perform internal dose and effective dose correlations with selected time points that would offer a better approximation of smoking behaviour over time. This included (i) a correlation using the data from the first days of the clinical study in the control smoker group (days 13 and 14) when the subjects had presumably not changed their CPD, potentially representing a steady-state level approximation for the biomarkers (Figure 2A and B); (ii) the last time point for Hb biomarkers correlated with the average urinary biomarkers from all time points for each subject (Figure 2C and D), reflecting exposure over the 6-month duration of the study. Our results show that in both cases the correlation was linear with consistent ${}_{L}R^2$ values comprised between 20 and 30% (Figure 2A–D).

Although pairs of biomarkers of internal and effective dose derived from the same toxicant have not been directly compared in the context of previous tobacco smoke exposure studies, those results are consistent with other correlation studies. For instance, only a moderate linear correlation was found between 4-ABP-Hb and plasma cotinine (ng/ml; ${}_{L}R^2$ 33%; Maclure et al., 1990), which is very similar to the value we obtained when performing a linear correlation between 4-ABP-Hb and 4-ABP (expressed in ng/L; ${}_{L}R^2$ 30%) or 4-ABP-Hb with TNEQ (μg/mL; R^2 24%). A moderate correlation (${}_{L}R^2$ 40%) was reported for acrylonitrile haemoglobin adducts and CPD in the Tavares et al.'s (1996) study. In our study, a similar comparison gave only a 19% correlation, but our smoker group was much larger. HOEtVal, an haemoglobin adduct derived from ethylene oxide which is found in cigarette smoke was also moderately correlated with cotinine with a correlation coefficient of 39% (Bono et al., 2002). In contrast, a good correlation (${}_{L}R^2$ 55%) was observed between acrylamide haemoglobin adduct and TNEQ (Urban et al., 2006). Subjects for this study were selected from a field study and it can be assumed that the smokers are in an approximate steady-state condition with respect to their smoking behaviour.

One likely contributing factor in the quality of the correlation between haemoglobin and urinary biomarkers is that their percentage of the absorbed daily dose differs by multiple orders of magnitude. For instance, considering an average of 70 pg/g Hb of 4-ABP-Hb × 750 g Hb (Total human blood Hb) with an erythrocyte life time of 120 days, we obtain a dose of 4-ABP-Hb of 0.4 ng/day. That is 2% of the amount found in urine in the same period (≈20 ng/24 h). When the same approach is used for acrylonitrile (≈150 pmol CEVal/g Hb) an approximated dose of 0.94 nmol/24 h CEVal is obtained, which is 0.1% of urinary levels of CEMA (≈200 µg/24 h; equivalent to 1 µmol/24 h). Therefore, correlations are likely to be markedly affected if the biomarkers are not measured at steady-state level due to the large difference in dose-range and body residence time. In addition, it needs to be taken into account that levels of the short-term urinary biomarkers are significantly influenced by the actual behaviour on the day of sampling (which might by chance deviate from the usual behaviour), whereas the levels of the long-term Hb adduct biomarkers represent an accumulated dose and is much less sensitive to random behaviour changes.

The question has been raised whether a reduction in internal dose biomarkers accounts for a reduction in exposure (Hecht et al., 2010). Based on our data, a reduction in CEVal (≈2-fold) and CEMA (≈3-fold) was observed when a group of smokers was switched to a cigarette prototype with a reduced acrylonitrile yield despite a significant increase in cigarette consumption and TNEQ (Table 3). Smoke chemistry analysis demonstrated that the test product had levels of acrylonitrile below the limit of detection of 0.28 µg/cigarette, yet the biomarker levels reached at day 183 is still significantly higher than levels observed in non-smokers and ex-smokers. This could indicate an inconsistency between the acrylonitrile cigarette yields when smoked by a machine at an ISO regime compared to actual human smokers or an LOD too high to measure residual acrylonitrile in the new product. We cannot exclude that other smoke volatiles might have been a source of CEMA and CEVal and we have to consider the impact of the increased cigarette consumption as another factor. In this group, the steady-state level for CEVal was reached in the period between days 46 and day 77 whilst it was reached between days 14 and 46 for CEMA (Table 3). The half-life of CEMA has been estimated to be between 7 and 9 h (Jakubowski et al., 1987) and therefore it would be anticipated that a change in exposure dose would be reflected within 24 h in the urine sample. The general assumption for haemoglobin adduct biomarkers is that their body residence time is identical to the life span of red blood cells which is approximately 120 days (Berlin et al., 1959; Maclure et al., 1990). No cessation arm was included in our study, so we cannot confirm the residence time of CEVal, however our results confirmed that there is a delayed dose–response relationship between urinary CEMA and CEVal. Nevertheless, this marked difference in kinetics is likely to have an important impact on the strength of the correlation between urinary biomarkers and haemoglobin biomarkers. Similar dose–response relationship spanning over a period of 6–8 weeks have also been reported for 4-ABP-Hb in groups of quitters (Bryant et al., 1987; Maclure et al., 1990). However, in our study, despite a measurable moderate reduction in 4-ABP based on smoke chemistry, we did not observe a similar reduction in urinary 4-ABP and 4-ABP-Hb possibly due to the increased cigarette consumption.

Finally, the moderate to weak correlation between haemoglobin adducts and urinary biomarkers can potentially be explained in part not only by a variety of other factors including interindividual differences in metabolic activity but also by different accumulation levels observed for the biomarkers based on the number of CPD. The relationship between mean haemoglobin-adduct level and urinary biomarkers at steady-state level of smoking was correlated with the mean CPD for each participant smoking the control cigarette at days 13 and 14 (Figure 3). A plateau effect was observed for 4-ABP-Hb for smokers of an average in the region of 20 or more CPD (Figure 3A). Our result is in agreement with what was previously reported for 4-ABP-Hb by Dallinga in a group of 55 smokers (Dallinga et al., 1998). In the Dallinga et al.'s discussion, the plateau effect was attributed to a different smoking behaviour in the higher cigarette consumption group leading to a stable dose. For instance, heavy smokers might not inhale smoke with the same intensity compared to moderate smokers. Therefore, 4-ABP-Hb would appear to be a less responsive biomarker when the cigarette number increases compared to the other biomarkers and this could have an impact on pairwise correlations. On the other hand, no clear-cut trend towards a plateau was observed for CEVal, CEMA and 4-ABP (Figure 3B–D). Additional data points with smokers of 30 or more CPD would give a more definitive answer regarding the number of CPD where a plateau effect would also be observed for CEMA, CEVal and urinary 4-ABP.

Conclusion

4-ABP, 4-ABP-Hb, CEMA and CEVal levels could be discriminated between smokers and non-smokers. All four biomarker phenotypes were reversible since no statistical differences could be detected between non-smokers and ex-smokers. Pairwise comparisons showed better correlation between biomarkers derived from different parent chemicals but present in the same matrix (e.g. CEVal versus 4-ABP-Hb) as opposed to inter-matrix comparisons between biomarkers derived from the same parent chemical (e.g. CEVal versus CEMA). This is likely to be due to the large differences in residence time of the two types of biomarkers. A marked plateau effect was observed for the 4-ABP haemoglobin adducts biomarker in the heaviest smokers, this was less conclusive for the other biomarkers in this study. We demonstrated with acrylonitrile that, as expected, a reduction in exposure leads to a reduction in both internal dose (urinary CEMA) and effective dose (CEVal Hb adduct), but that the biomarkers dynamic response to the change was slower for the haemoglobin adduct. A similar observation could not be confirmed with 4-ABP.

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Declaration of interest

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