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RESEARCH ARTICLE

Is 24h nicotine equivalents a surrogate for smoke exposure based on its relationship with other biomarkers of exposure?

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Abstract

Nicotine and its 5 major metabolites (Nicotine equivalents, NE) may serve as a surrogate biomarker for smoke exposure.

Objective: To investigate the relationship between nicotine equivalents (NE) and biomarkers of exposure (BOE) to cigarette smoke.

Methods: Data from nine controlled studies in 916 adult smokers were used. BOEs to nicotine, NNK, pyrene, acrolein, benzene, 1,3-butadiene and CO were used.

Results: Among all the factors investigated (NE, cigarette type, age, gender, BMI and study), NE was the most statistically significant factor for all biomarker relationships. Weak to moderate relationships ($0.32 \leq R^2 \leq 0.65$) were found between NE and the BOEs.

Conclusions: Based on the relationships with BOEs, NE may be considered as a surrogate biomarker of total cigarette smoke exposure.

Keywords: *Nicotine Equivalents; biomarkers of exposure to cigarette smoke; surrogate; biomarker relationships*

Introduction

Thousands of chemical constituents have been identified in tobacco smoke (Rodgman et al. 2000). This complex mixture consists of the chemicals distributed in the particulate phase (PP) or gas vapor phase (GVP) of smoke, some of which have been classified as toxicologically relevant (Kensler & Battista 1963, Hecht et al. 1993, Bombick et al. 1997, Chepiga et al. 2000, Rodgman et al. 2000, Smith et al. 2000, 2001). Due to the vast number of chemical constituents in cigarette smoke, it is impossible to examine the human exposure to all these constituents. The measurement of biomarkers of exposure (BOE) in urine or blood in adult cigarette smokers can provide quantitative estimates of the uptake of selective smoke constituents (Institute of Medicine 2001, Hecht 2002, Hatsukami et al. 2006). The relative uptake and levels in the human body of the smoke constituents may vary depending on the chemical characteristics, absorption, metabolism, excretion and other factors (Patterson et al. 2003, Feng et al. 2007a). This poses a challenge when trying to determine overall cigarette smoke exposure. Several reports exist in which exposure to smoke constituents in smokers of specific cigarette types have been measured (Zacny & Stitzer 1988, Roethig et al. 2007, 2009, Scherer et al. 2007, Mendes et al. 2008, 2009, Sarkar et al. 2008). The relationships between cigarette consumption and selected biomarkers of exposure (Joseph et al. 2005) as well as the relationships between machine-derived smoke yields and biomarkers in cigarette smokers (Scherer et al. 2007) has also been examined.

Since the mid-1980s, studies assessing exposure to tobacco smoke and its biological effects have predominantly used nicotine or its metabolites, particularly

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cotinine, measured either in blood, urine or saliva as a marker of exposure and uptake of tobacco smoke. NE (Nicotine Equivalents which consists of nicotine and five of its major metabolites: nicotine-glucuronide, cotinine and its glucuronide, trans-3′-hydroxycotinine and its glucuronide) is often used to estimate nicotine exposure in clinical studies since it reflects at least 80% of the daily nicotine uptake in smokers (St. Charles et al. 2006, Feng et al. 2007b). Urinary nicotine equivalents may be considered as a surrogate biomarker since it is not only a suitable measure of total nicotine uptake but also is specifically obtained from tobacco, and therefore considered a biomarker of cigarette smoke exposure (Scherer 1999, 2005, Roethig et al. 2005, 2007, 2008, Zedler et al. 2006, Feng et al. 2007b, Mendes et al. 2008, Sarkar et al. 2008). However, no systematic assessment of the relationships between specific biomarkers of exposure, particularly the relationships between nicotine equivalents (NE) and other particulate and gas phase biomarkers, has been published.

The primary objective of this analysis was to investigate the relationships between 24h urinary NE and seven other biomarkers. The BOE included 4-methylnitrosamino-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronide metabolites; 1-hydroxypyrene (1-OHP) and its glucuronides and sulfates; 3-hydroxypropylmercapturic acid (3-HPMA); *S*-phenylmercapturic acid (*S*-PMA); monohydroxybutenyl-mercapturic acid (MHBMA); carboxyhemoglobin (COHb) and plasma cotinine. In addition, the relationships among the other BOE were also explored.

Materials and Methods

Study Design

The 916 self-affirmed adult smokers (491 males and 425 females) included in this analysis were from nine clinical studies, conducted between 2002 to 2007. These studies utilized similar study designs, which were randomized, controlled, forced switching smoking studies. Adult males and females, 21 years or older, in generally good health, smoking 10 - 30 cigarettes per day were recruited from the general population in Lincoln, Nebraska and Phoenix, Arizona through Institutional Review Board (IRB) approved local newspaper advertisements. In all the nine studies, volunteers were screened according to the same inclusion and exclusion criteria. Interested adult smokers gave written informed consent before enrolling in the studies and were paid for their participation. Subjects were free to stop smoking and continue or discontinue the studies at any time for any reason. During the entire study period, subjects smoked in controlled clinical settings. At Baseline, 225 subjects smoked Marlboro full flavor cigarettes (CC15) in one study, 459 subjects smoked Marlboro Lights cigarettes (CC11) in five studies, and 232 subjects smoked Marlboro Ultra Lights cigarettes (CC6) in three studies. During the post-baseline phases in each individual study, the subjects were switched to different groups that either smoked test cigarettes, continued smoking their own cigarettes or stopped smoking. Additional details about seven of these studies (Table 1) have been published previously

Table 1. Demographic characteristics of the adult smokers from the nine studies.

Variable	Study 1 (N = 98)	Study 2 (N = 50)	Study 3 (N = 84)	Study 4 (N = 110)	Study 5 (N = 100)	Study 6 (N = 120)	Study 7 (N = 100)	Study 8 (N = 29)	Study 9 (N = 225)
Product Used at Baseline	CC6	CC6	CC6	CC11	CC11	CC11	CC11	CC11	CC15
Gender									
Male	50	24	27	55	50	57	50	15	163
Female	48	26	57	55	50	63	50	14	62
Race									
African American	1	0	1	3	1	0	0	0	5
White	95	45	81	99	97	113	97	29	200
Hispanic	0	1	1	2	1	3	1	0	17
Others*	2	4	1	6	1	4	2	0	3
Age (Years)									
Mean ± SD	34.2 ± 11.1	34.5 ± 11.1	34.8 ± 10.2	31.4 ± 10.4	33.5 ± 11.1	32.1 ± 10.3	33.8 ± 10.2	32.5 ± 9.9	35.0 ± 10.5
(min, max)	(21, 63)	(21, 60)	(21, 65)	(21, 58)	(21, 63)	(21, 60)	(21, 59)	(21, 59)	(21, 65)
BMI (kg/m^2)									
Mean ± SD	25.00 ± 3.07	27.56 ± 4.42	27.16 ± 5.33	24.45 ± 3.34	24.59 ± 2.93	24.96 ± 2.91	24.31 ± 3.15	26.94 ± 3.75	26.63 ± 4.42
(min, max)	(19.14, 31.66)	(18.96, 37.23)	(18.02, 40.90)	(17.95, 32.35)	(17.93, 33.22)	(18.26, 32.63)	(17.37, 31.12)	(20.47, 39.47)	(19.31,40.17)

* Included American Indian, Asian, European / Middle Eastern and mixed.

References: Study 1 & 7, Sarkar et al. 2008; Study 3, Frost-Pineda et al. 2008a; Study 4, Roethig et al. 2005; Study 5, Roethig et al. 2007; Study 6, Frost-Pineda et al. 2008b; Study 9, Mendes et al. 2008.

(Roethig et al. 2005 (Study 4), 2007 (Study 5), Frost-Pineda et al. 2008a (Study 3), 2008b (Study 6), Mendes et al. 2008 (Study 9), Sarkar et al. 2008 (Study 1 & 7)). Study 2 utilized a randomized single blind, controlled, forced-switching, 2-way crossover design. Fifty self-affirmed adult male and female smokers of CC6 cigarettes were enrolled. Subjects smoked CC6 cigarettes on the Baseline day (Day -1) then switched to test cigarettes from Day 1 through Day 8. In Study 8, twenty-nine self-affirmed CC11 adult male and female smokers were enrolled. Subjects smoked CC11 cigarettes on the Baseline days (Day -2 and -1, mean of Day -2 and -1 biomarker values for each subject was considered as the Baseline) then switched to a test cigarette from Day 1 through Day 8. All studies were approved by the MDS Pharma Services Institutional Review Board (IRB) and conducted at MDS Pharma Services (Lincoln, Nebraska) in accordance with Good Clinical Practice and the Declaration of Helsinki (International Conference on Harmonization, 1996; World Medical Association, 2000).

Study Products

The three cigarette products used at the Baseline of the nine studies were analyzed for tar, nicotine, and CO yield in mainstream smoke using the standard Cambridge Filter Test method smoking machine protocol (previously known as FTC method, Pillsbury et al. 1969). The smoke machine yields for the CC15 cigarettes were 15 mg tar, 1.1 mg nicotine, and 13 mg CO per cigarette; for the CC11 cigarettes were 11 mg tar, 0.8 mg nicotine, and 11 mg CO per cigarette; and for the CC6 cigarettes were 6 mg tar, 0.6 mg nicotine, and 7 mg CO per cigarette.

Biomarkers of Exposure

Although multiple measurements were taken over the duration of the studies, only Baseline data of eight biomarkers of exposure of either GVP or PP, were used in this analysis. Twenty-four hour urine samples were collected for the determination of urinary biomarker levels. Blood samples were also collected at 19:00 for determination of COHb and plasma cotinine levels.

These biomarkers were measured using validated methods as reported previously (Roethig et al. 2005, 2007, Feng et al. 2006, Zedler et al. 2006, Frost-Pineda et al. 2008a,b, Mendes et al. 2008, Sarkar et al. 2008). The analysis of the samples for the biomarkers was conducted by the same analytical methods and the same laboratories. The validation criteria used for bioanalytical methods ensured that there were no systematic deviations in the analytical measurement.

Nicotine equivalents (NE), a PP biomarker, was calculated as the molar sum of nicotine and its 5 major metabolites in urine, nicotine-*N*-glucuronide, cotinine, cotinine-*N*-glucuronide, *trans*-3′-hydroxycotinine, and *trans*-3′-hydroxycotinine-*O*-glucuronide, is a biomarker of nicotine exposure (Roethig et al. 2005, Zedler et al. 2006, Feng et al. 2007b). **Plasma cotinine** is generally accepted as a PP biomarker and is also considered a surrogate representative of nicotine exposure (Benowitz & Jacob 2001). **Total NNAL (**4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol) a PP biomarker frequently used to estimate exposure to NNK, a tobacco-specific nitrosamine (Hecht 2002), was calculated as the molar sum of free (NNAL) and its conjugated metabolites in urine. **Total 1-hydroxypyrene (1-OHP)**, a PP biomarker, often used as a a surrogate for polyaromatic hydrocarbon (PAH) exposure in urine (Scherer et al. 2000, Hecht 2002, Feng et al. 2006) was obtained by measuring free and conjugated metabolites of pyrene. **Carboxyhemoglobin (COHb)**, a GVP biomarker (Benowitz et al. 1986, Roethig et al. 2005, 2007) was used to estimate carbon monoxide exposure; ***S*-phenylmercapturic acid (*S*-PMA),** a GVP biomarker, a metabolite of benzene, widely used in environmental, occupational, and smoking-related exposure studies (Qu et al. 2000, Hecht 2002, Feng et al. 2006) estimated benzene exposure; **3-Hydroxypropylmercapturic acid (3-HPMA),** a GVP biomarker for smoking-related acrolein exposure (Mascher et al. 2001, Roethig et al. 2007) and **Monohydroxybutenyl mercapturic acid (MHBMA),** a GVP biomarker of exposure to 1,3-butadiene (Sarkar et al. 2008) were measured in these studies (MHBMA was not measured for CC15 smokers).

Statistical Analysis Methods

SAS® version 8.2 (SAS Institute, Inc., Cary, North Carolina, USA) was used to perform the statistical analysis. Descriptive statistics were calculated for the demographic data by study. Even though all subjects in different studies were recruited and screened according to the same inclusion and exclusion criteria, study effect was tested by linear mixed model analysis (SAS® PROC MIXED). If study effect was not statistically significant, the data were pooled by cigarette type. For each cigarette type, Pearson correlation analysis (SAS® PROC CORR) was used to examine the strength of the 28 relationships among the eight biomarkers. Local regression analysis (Loess, SAS® PROC LOESS) was performed to generate scatter plots of NE vs. seven BOE for each cigarette type and helped to select the suitable polynomial terms of NE. The Loess procedure fits simple regression lines to segments of the data and connects the local fits into a smooth curve. In the linear mixed model analysis, each of the seven other BOE was treated in turn as the dependent variable. The independent variables included study (STUDY), interaction of study and NE (STUDY × NE) as random factors, cigarette type (CIGTYPE, CC6 = 1, CC11 = 2, CC15 = 3), interaction of cigarette type and NE

(CIGTYPE × NE) as fixed factors, NE and its quadratic (NE^2) and cubic (NE^3) polynomial terms as covariates based on the findings in the local regression analysis. The final relationship models between NE and the other BOE were developed by multiple regression analysis (SAS® PROC REG). In addition to the retained model terms from linear mixed model analysis, age (AGE), gender (GENDER, Female = 0, Male = 1), Body mass index (BMI) were also included as model terms in the regression. The backward elimination method was used to remove non-significant factors (as defined by $p \geq 0.10$ for interaction terms, and $p \geq 0.05$ for main effects) in both of the linear mixed model and regression analysis. If an interaction term was significant and included in the final model, both factors of the interaction term were also included in the final model. If the higher order terms of the biomarker variable were included, the lower order terms were also included. One of the subjects who smoked CC15 had an NE level of 60.3 mg/24h which was much higher than the NE levels of all other subjects (≤ 43.6 mg/24h). Analyses were conducted by including and excluding this subject in order to determine the impact of this observation.

Based on the relatively large sample size, the Central Limit Theorem was applied and no data transformation was performed. For all statistical analyses, the Type I error rate was controlled at the 0.05 level for two-sided test. The relationship between biomarkers was considered weak if $R^2 \leq 0.33$, moderate if $0.33 < R^2 \leq 0.66$, and strong if $R^2 > 0.66$.

Results

Demographic Characteristics

Demographic data by study are summarized in Table 1. The subjects' ages ranged from 21 to 65 years. The mean ages of subjects in the nine studies ranged between 31.4 and 35.0 years. The majority of the subjects were White (≥ 89%), African Americans, Hispanic and Other ethnic groups were less than 11%. The proportions of males and females were about 50 - 50%, except for Study 3 (68% females) and Study 9 (28% females). The mean BMI values in the nine studies were between 24.3 and 27.6 Kg/m^2.

Summary Statistics of Biomarkers

The summary statistics of the eight biomarkers used in the analysis are shown in Table 2 by cigarette type.

Pearson Correlation Analysis

The results of the Pearson correlation analysis by cigarette type are shown in Table 3. All the Pearson correlation coefficients among the eight biomarkers were positive and statistically significant ($p < .0001$). The correlation

Table 2. Summary statistics of the biomarkers of exposure.

Biomarkers	Cigarette Type	N	Mean	STD	Min	P10	Q1	Median	Q3	P90	Max
NE (mg/24h)	CC6	224	15.62	6.619	2.17	7.24	11.16	15.10	19.54	24.13	38.92
	CC11	458	17.47	7.670	3.07	8.37	12.06	16.60	22.08	28.84	43.24
	CC15	216	22.42	8.292	6.31	12.75	16.42	21.69	27.94	32.56	60.30
Total NNAL (ng/24)	CC6	173	496.79	319.408	0.00	141.47	271.76	422.51	718.56	906.47	2133.51
	CC11	442	562.31	340.254	0.00	218.17	324.77	511.15	741.36	1012.39	2245.12
	CC15	218	606.00	287.869	78.92	289.52	417.38	567.13	765.66	1005.79	2349.93
Total 1-OHP (ng/24h)	CC6	223	172.80	98.700	20.90	77.91	112.78	158.64	212.72	256.81	875.54
	CC11	345	186.20	116.948	0.00	94.62	120.22	162.84	225.42	290.57	1387.75
	CC15	216	228.72	118.086	42.48	105.08	142.97	210.53	282.14	393.49	860.61
Plasma cotinine (ng/mL) [a]	CC6	98	234.23	95.190	54.93	120.58	173.26	215.25	284.13	346.30	575.24
	CC11	308	279.13	108.623	47.69	143.66	203.96	269.33	345.56	422.28	686.26
	CC15	225	321.50	115.963	98.14	179.06	237.98	312.64	386.24	468.46	682.71
COHb (% sat) [a]	CC6	148	5.13	2.034	1.00	2.80	3.85	4.95	6.10	8.40	11.10
	CC11	459	5.81	2.092	1.20	3.00	4.40	5.70	7.20	8.60	12.80
	CC15	225	6.91	1.702	2.70	4.70	5.60	6.90	8.10	9.10	11.10
3-HPMA (ug/24h)	CC6	224	1894.72	941.694	325.60	799.04	1294.83	1744.98	2385.78	2994.66	6479.60
	CC11	347	2033.84	834.637	500.28	1062.81	1466.10	1858.20	2538.00	3152.00	4524.97
	CC15	222	2294.97	916.686	488.40	1145.19	1621.58	2233.50	2913.23	3444.00	5731.75
***S*-PMA (ug/24h)**	CC6	224	6.55	3.784	0.59	2.02	3.92	5.84	9.05	11.57	20.20
	CC11	346	6.99	4.404	0.51	1.88	3.77	5.91	10.08	13.26	23.02
	CC15	220	6.94	4.106	0.75	2.01	3.86	6.46	9.58	12.40	21.68
MHBMA (ug/24h)	CC6	147	4.59	3.311	0.00	0.91	2.16	3.77	6.64	8.68	20.92
	CC11	129	5.88	4.416	0.00	1.05	2.70	5.36	7.51	11.95	26.10

[a] Collected at 19:00 daily.

Table 3. Pearson correlation coefficients (r) between biomarkers by cigarette type.

Cigarette Type	Biomarker	Total NNAL	Total 1-OHP	Plasma Cotinine[a]	3-HPMA	*S*-PMA	MHBMA	COHb[a]
CC6	NE	0.67	0.52	0.75	0.84	0.65	0.62	0.76
	Total NNAL		0.44	0.47	0.66	0.46	0.38	0.60
	Total 1-OHP			0.38	0.50	0.35	0.48	0.42
	Plasma Cot. [a]				0.69	0.57	0.44	0.74
	3-HPMA					0.62	0.56	0.61
	S-PMA						0.88	0.50
	MHBMA							0.37
CC11	NE	0.50	0.51	0.67	0.80	0.56	0.47	0.62
	Total NNAL		0.36	0.47	0.62	0.38	0.34	0.34
	Total 1-OHP			0.35	0.48	0.35	0.34	0.23
	Plasma Cot. [a]				0.66	0.50	0.34	0.59
	3-HPMA					0.53	0.45	0.54
	S-PMA						0.83	0.37
	MHBMA							0.42
CC15	NE	0.72	0.54	0.43	0.69	0.61		0.53
	Total NNAL		0.50	0.32	0.55	0.49		0.42
	Total 1-OHP			0.27	0.52	0.45		0.30
	Plasma Cot. [a]				0.34	0.31		0.65
	3-HPMA					0.48		0.49
	S-PMA							0.40

[a] Collected at 19:00 daily.
$p < .0001$ for all the correlation significance testing.

coefficients (r) for the relationship between the biomarkers were relatively higher for the lower machine-measured tar yield cigarettes (i.e. CC6 vs. CC11 and CC15) in some of the biomarkers relationships (15 out of 28).

Local Regression Analysis

Differences were observed in the smooth curves between biomarker relationships as well as among the cigarette types (e.g. total NNAL vs. NE in Figure 1). In the Loess scatter plots, quadratic and even cubic polynomial forms of the Loess smooth curves were observed for most of the biomarker relationships. The intersection of the smooth curves indicated that the interaction of the cigarette type and NE had an effect on the biomarker relationships. The plot in Figure 1A show that the outlier of NE = 60.3mg/24h had no significant impact on the slopes of the curves.

Linear Mixed Model Analysis

The linear mixed model analysis results showed that study effect (STUDY) and its interaction with NE (STUDY × NE) were not statistically significant in any of the seven biomarker relationships. These two model terms were not retained in the multiple regression analysis.

Multiple Regression Analysis

The results of the regression analysis are summarized in Table 4. Weak to moderate relationships ($0.32 \le R^2 \le 0.65$) were found between NE and the BOE, total 1-OHP had the lowest R^2 (0.32) and 3-HPMA had the highest R^2 (0.65). Cigarette type was a statistically significant factor for total NNAL, plasma cotinine, COHb, 3-HPMA, and *S*-PMA. The interaction model term, cigarette type and NE, was statistically significant ($p < 0.10$) for total NNAL, COHb and 3-HPMA. The relationships of total 1-OHP and MHBMA with NE were not affected by cigarette type. Age was a significant factor in all models, but not for *S*-PMA and MHBMA. Gender had a significant effect on total 1-OHP and plasma cotinine. BMI was a significant factor in the models for plasma cotinine and COHb. In the relationship between MHBMA and NE, only NE and its higher polynomial terms (NE^2 and NE^3) were statistically significant. Although cigarette type and demographic variables were statistically significant factors in most models, the linear term of NE contributed the most (79 - 95%) based on the percentage of partial R^2 to overall R^2. Additional regression analysis (data not shown) demonstrated that the relationship between *S*-PMA and MHBMA was relatively strong ($R^2 = 0.74$) and weak to moderate relationships were observed among the other BOE. Furthermore, the exclusion of the subject with high NE level from the analysis revealed that the R^2 of the models did not change. The subject was therefore not excluded in the final models presented in Table 4. Based on the models in Table 4, with age, BMI and NE set to the median values by cigarette type and gender groups, median observed values, model derived values and 99% CI of the dependent biomarkers were shown in Table 5.

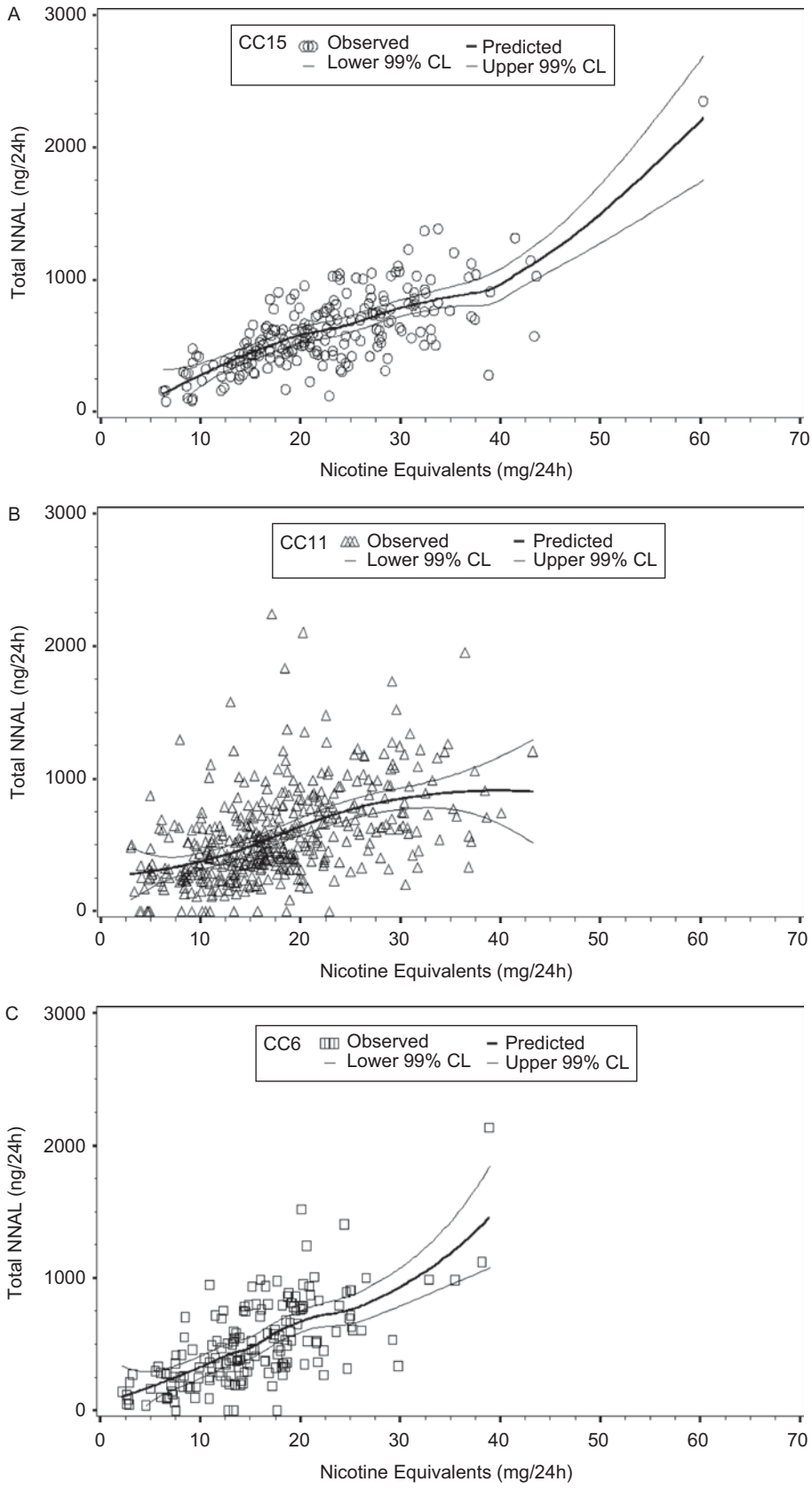

Figure 1. Local Regression plots of total NNAL vs. NE. (A) Cigarette type CC15 with the outlier (NE = 60.3mg/24h), (B) Cigarette type CC11 (C) Cigarette type CC6.

Table 4. Multiple regression analysis result for the relationship between the biomarkers of exposure and NE.

Dependent Variable	Model Term	Estimate	*p*-value	Partial R^2	Overall R^2
Total NNAL	Intercept	-119.35	0.1728		0.359
	NE	48.38	< .0001	0.3399	
	NE^2	-0.86	0.0281	0.0000007	
	NE^3	0.01	0.0092	0.0061	
	CigType	42.02	0.2611	0.0043	
	CigType × NE	-4.02	0.0318	0.0031	
	Age	2.25	0.0098	0.0052	
Total 1-OHP	Intercept	4.78	0.7242		0.319
	NE	7.22	< .0001	0.2972	
	Age	1.15	0.0004	0.0083	
	Gender	27.39	0.0001	0.0130	
Plasma Cotinine [a]	Intercept	175.30	< .0001		0.485
	NE	16.73	< .0001	0.3822	
	NE^2	-0.20	< .0001	0.0321	
	CigType	13.96	0.0071	0.0027	
	Age	0.83	0.0087	0.0018	
	Gender	25.22	0.0003	0.0070	
	BMI	-7.73	< .0001	0.0594	
COHb [a]	Intercept	0.29	0.5903		0.524
	NE	0.38	< .0001	0.4225	
	NE^2	-0.0042	< .0001	0.0561	
	CigType	0.74	0.0005	0.0089	
	CigType × NE	-0.02	0.0357	0.0024	
	Age	0.03	< .0001	0.0245	
	BMI	-0.05	< .0001	0.0093	
3-HPMA	Intercept	-186.37	0.2871		0.652
	NE	95.36	< .0001	0.5998	
	NE^2	1.89	0.0250	0.0228	
	NE^3	-0.04	0.0005	0.0043	
	CigType	130.26	0.0775	0.0082	
	CigType × NE	-12.90	0.0005	0.0048	
	Age	9.28	< .0001	0.0117	
S-PMA	Intercept	1.38	0.0238		0.360
	NE	0.45	< .0001	0.3328	
	NE^2	-0.0028	0.0229	0.0036	
	CigType	-0.90	< .0001	0.0237	
MHBMA	Intercept	3.08	0.0486		0.340
	NE	-0.43	0.1111	0.3001	
	NE^2	0.04	0.0015	0.0087	
	NE^3	-0.00079	0.0004	0.0310	

[a] Collected at 19:00 daily.
Cigtype: Cigarette type, CC6 = 1, CC11 = 2, CC15 = 3;
Gender: female = 0, male = 1.

Discussion

This integrated analysis on data from 916 adult conventional cigarette smokers demonstrated that based on the relationship between NE and seven other biomarkers of exposure to smoke constituents, NE may be considered a suitable surrogate marker of overall cigarette smoke exposure. Nicotine and its metabolite cotinine have already been suggested as a surrogate marker for the determination of the number of cigarettes rather than self-reported values of number of cigarettes smoked (Whitehead et al. 2009). However, nicotine uptake, as estimated through urinary excretion of nicotine and five of its major metabolites, has not systematically been compared against biomarkers of exposure for different PP and GVP smoke constituents. The data used from nine studies conducted over a period of five years in different smokers could be pooled because the clinical protocols for the study conduct, i.e. the inclusion and exclusion criteria, were identical. Furthermore the linear mixed model result showed that "study" was not a statistically significant factor in any of the biomarker relationships.

Table 5. Quantitative assessment of suitability of NE as surrogate marker: Model derived biomarker values and 99% CI based on the final models in Table 4.

Dependent Variable	Cigarette Type	Gender	Median Age	Median BMI	Median NE	Model Derived Value	99% Prediction Interval	Median Observed Value
Total NNAL	CC6		32		15.10	513.6	(464.4, 562.8)	422.51
	CC11		29		16.60	522.9	(490.3, 555.5)	511.15
	CC15		34		21.69	601.3	(556.2, 646.5)	567.13
Total 1-OHP		Female	33		15.40	153.9	(141.0, 166.9)	147.88
		Male	29		18.94	202.3	(189.7, 214.8)	194.01
Plasma Cotinine [a]	CC6	Female	32	24.93	13.96	218.0	(199.1, 236.9)	207.30
	CC6	Male	31	26.57	17.49	266.7	(245.3, 288.1)	232.98
	CC11	Female	33	24.24	15.56	255.5	(242.0, 269.0)	241.14
	CC11	Male	27	24.65	17.89	296.1	(281.7, 310.4)	309.58
	CC15	Female	35	25.27	18.57	293.2	(274.9, 311.4)	281.05
	CC15	Male	33	26.33	23.28	348.1	(332.8, 363.5)	332.14
COHb [a]	CC6		32	25.51	15.10	5.14	(4.87, 5.41)	4.95
	CC11		29	24.39	16.60	5.82	(5.66, 5.99)	5.70
	CC15		34	26.00	21.69	7.06	(6.81, 7.31)	6.90
3-HPMA	CC6		32		15.10	1787.4	(1694.1,1880.7)	1744.98
	CC11		39		16.60	1847.4	(1779.4,1915.3)	1858.20
	CC15		34		21.69	2254.8	(2162.3,2347.3)	2233.50
***S*-PMA**	CC6				15.10	6.62	(6.11, 7.14)	5.84
	CC11				16.60	6.27	(5.92, 6.62)	5.91
	CC15				21.69	7.11	(6.55, 7.66)	6.46
MHBMA					17.51	5.07	(4.43, 5.71)	4.40

[a] Collected at 19:00 daily.

The relationships between NE and biomarkers representative of non-tobacco specific constituents, e.g. total 1-OHP, MHBMA and S-PMA were of similar magnitude as the relationship between NE and the biomarker of exposure to tobacco-specific constituents (total NNAL). Although acrolein is often cited as a GVP biomarker (Sarkar et al. 2008), a strong relationship was observed between its metabolite, 3-HPMA and NE ($R^2 = 0.65$). The reason for the relatively stronger relationship between 3-HPMA and NE compared to that for the other GVP constituents could be due to similar uptake characteristics. It has been reported that nicotine and low-molecular aldehydes are highly absorbed in the lung (Baker & Dixon 2006, Feng et al. 2007a).

The Pearson correlation coefficients were relatively high ($r = 0.67$ for CC6, $r = 0.50$ for CC11 and $r = 0.72$ for CC15) for the relationship between the tobacco-specific constituents, total NNAL and NE. Even though both these constituents are tobacco-specific, the regression model only explained 36% of the variability. It is possible that differences in metabolic handling (Benowitz et al. 2006, Brown et al. 2007, Gallagher et al. 2007) could be one of the reasons for the remaining variability that was not accounted in this regression model. Perhaps if a measure of the metabolic handling (e.g. genotypic or phenotypic measures) were included as a variable in the model, the remaining variability could be better explained and may improve the predictability of the model. Nevertheless, based on the partial R^2 in this model, NE was still the most important factor and accounted for 95% of the predictability in the model for total NNAL.

The relationship between the BOE provided some mechanistic insights into the metabolic pathways. The relatively strong relationship ($R^2 = 0.74$) between the metabolites of the two gas phase biomarkers, benzene and 1,3-butadiene could be suggestive of the involvement of the similar pathways in the formation of mercapturic acid metabolites. It is well established that the glutathione *S*-transferase (GST) isoenzymes GSTT1 and GSTM1 play a role in the biotransformation of both benzene and 1,3-butadiene (Fustinoni et al. 2002, Dougherty et al. 2008). The strong association between these two metabolites suggests that probably both the mercapturic acid metabolites are being formed by the same enzymes. This is in contrast to the weak relationship between the mercapturic acid metabolites of 1,3-butadiene and acrolein, consistent with the literature reports that different GST enzymes may be involved in these pathways. The formation of 3-HPMA from acrolein is primarily through GST-pi (Van Iersel et al. 1997) compared to the involvement of GSTT1 and GSTM1 in the formation of MHBMA. The weak to moderate relationships among the other BOE remain unclear and may warrant further exploration.

Contrary to the expectation of a strong relationship between the metabolite and parent compound, plasma cotinine showed a moderate relationship with NE ($R^2 = 0.49$). This difference possibly reflects the influence of genetic polymorphisms on cotinine metabolism (Benowitz

& Jacob 2001, Benowitz et al. 2006). Nevertheless these observations are in line with the literature on the relationship between salivary cotinine levels and number of cigarettes, where not only similar R^2 (0.52) was observed but also age was an important variable in the model (Swan et al. 1993). The contribution of age, BMI and gender (for plasma cotinine) to the overall R^2, although statistically significant, was rather small (partial $R^2 < 0.0594$) in our models. It was interesting to note that age appeared to play a statistically significant role in some of the biomarker relationships, perhaps due to age related differences in the metabolic handling of the different smoke constituents.

The results of the local and multiple regression analyses showed that relationship between most of the BOE and NE is not only dependant on linear terms but also higher order terms, i.e. NE^2 and NE^3. In order to assess the appropriateness of NE as a surrogate for overall smoke exposure, a 99% prediction interval was generated for each BOE using the models shown in Table 4. Since the median observed values for most of the BOEs were within the prediction interval (Table 5), NE appears to be an appropriate surrogate for overall smoke exposure.

A limitation of this analysis is that the relationships between NE and other BOE were investigated from studies conducted under controlled clinical settings. However, it is unlikely that the direction of these relationships should change much for observations collected from studies under ambulatory settings, because even if the absolute amounts of smoke exposure was different, the relationships between the biomarkers may still be the same. Nevertheless, the findings from this study were corroborated in a multi-center, cross-sectional study with 3,585 adult smokers where the relationship of NE with other BOE was investigated (Roethig et al. 2009). The authors report similar relationships e.g. the regression analysis showed a strong linear relationship of NE with 3-HPMA ($R^2 = 0.48$) relative to that observed in this study (Table 3, $R^2 = 0.65$). The improvement in the R^2 in our study could possibly be due to the additional terms in the model as well as due to the study being conducted in a controlled clinical setting. This also suggests that NE might be a reasonable surrogate for short-term exposure changes. Due to the relatively short half-life of some of its components (half-life of nicotine and its metabolites ranges from 2-18 hrs, Benowitz & Jacob 2001) changes in exposure due to variations in daily number of cigarettes smoked should be reflected in the NE levels. The suitability of a BOE with a longer half life, e.g. total NNAL has a half-life of ~10-18 days (Goniewicz et al. 2009) should be considered when there is a need for assessing chronic exposure. Another aspect that needs to be considered is the differences between the constituents that might arise due to the differences in the physicochemical properties which might impact the absorption. For example, although nicotine retention has been reported to be >90% when smoke is inhaled into the lung, some degree of retention in the mouth also seems to occur which is highly variable (0-62%), (Baker & Dixon 2006).

NE may be an appropriate surrogate for overall smoke exposure from conventional cigarettes with similar design characteristics. Smoking machine yields of the cigarette must be characterized and the relationship between the different smoke constituents and nicotine must be investigated before applying this concept. In some instances the specific constituents might be altered without impacting the nicotine levels, for example reduction in gas vapor phase constituents with carbon filtered cigarettes (Sarkar et al. 2008). In such cases NE might not be a representative surrogate for total smoke and the specific constituent might need to be investigated in addition to NE.

The three cigarette types examined in this analysis represent different machine measured tar levels among the majority of the cigarettes smoked. Interestingly, for most of the biomarker relationships, the model term cigarette type had a significant effect in the models, however, the relative contribution on the overall model was low (0.5-7%). These observations suggest that in humans, the relationship between nicotine and some of the smoke constituents is not the same across the different cigarette types. These results could have a bearing when comparing constituent levels normalized by nicotine, based on machine measured yields, due to the differences in human exposure across the different cigarette types.

Finally, it can be concluded that based on the relationship demonstrated between NE and the other BOE, NE may be considered as a surrogate biomarker of overall cigarette smoke exposure over a short term period. Here we propose models that could be used to estimate levels of biomarkers of exposure from smoking conventional cigarettes. The robustness of these models will need to be confirmed in future studies.

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Declaration of interest

All authors are or were employees of Philip Morris USA Inc. / Altria Services Inc.

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