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RESEARCH ARTICLE

Increases in tobacco exposure biomarkers measured in non-smokers exposed to sidestream cigarette smoke under controlled conditions

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Abstract

National surveys of the exposure of non-smokers to secondhand smoke based on serum cotinine analyses have consistently identified certain groups within the population including children, males and non-Hispanic Blacks as having relatively greater exposure. Although these differences in mean serum cotinine concentrations probably represent differences in exposure of individuals in their daily lives, it is also possible that metabolic or other differences in response might influence the results. To better define the nature of those findings, we have examined the response of 40 non-smokers including both men and women and African-Americans and whites to sidestream (SS) cigarette smoke generated by a smoking machine under controlled conditions. In this study, participants were exposed to aged, diluted SS smoke (ADSS) generated in an environmental chamber with a mean air nicotine concentration of 140 μg m^{-3} and 8.6 ppm CO for 4 h. Salivary cotinine was measured every 30 min, and serum cotinine samples were taken prior to, and 2 h after exposure. Urinary nicotine metabolites and NNAL, a tobacco-specific nitrosamine, and 4-aminobiphenyl (4-AB) haemoglobin adducts were also measured prior to and 2 h following the exposure. Under these uniform, controlled conditions, we found a similar response to ADSS smoke exposure among all the participants. In all cases a significant increase in biomarker concentration was noted following exposure, and the short-term increases in salivary cotinine concentration were quite similar at approximately 12 pg ml^{-1} min^{-1} among the groups. In this small study, no significant differences by gender or race were seen in the mean increases observed in cotinine, NNAL or 4-AB adducts following 4 h of exposure. Thus, our results are most consistent with a relatively uniform response in tobacco biomarker concentrations following short-term exposure to ADSS tobacco smoke, and suggest that biomarker measurements are capable of effectively indicating increases in exposure among groups of non-smokers.

Keywords: *Secondhand smoke; SHS; ETS; nicotine; cotinine; exposure chamber; 4-aminobiphenyl; adducts; NNAL; cigarette smoking; passive smoking*

Introduction

The exposure of non-smokers to secondhand smoke (SHS), also sometimes referred to as environmental tobacco smoke or 'ETS', remains a significant public health concern (DHHS 2006). SHS is composed primarily of a mixture of sidestream (SS) smoke and exhaled mainstream (MS) smoke and is a complex and protean mix of more than 4000 chemicals including many toxicants and carcinogens. SS smoke is the predominant component of fresh SHS and primarily includes emissions from the smoldering cigarette tip between puffs. Mainly because of lower tip combustion temperatures between puffs, this smoke may actually generate higher levels of several toxic compounds than does MS smoke. The exposure of non-smokers to SHS has been declining within the United States over the past decade (Pirkle et al. 2006), especially within communities that have adopted

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more stringent public smoking policies (Pickett et al. 2006). Nevertheless, exposure to SHS continues to be of concern for the population, particularly black people, children and pregnant women. National surveys have consistently found widespread exposure to SHS among non-smokers as determined by the measurement of cotinine, the primary proximate metabolite of nicotine, in people, and have identified certain groups including black people, males and children as being at greater risk based on their increased cotinine levels (DHHS 2006, Pirkle et al 1996, Pirkle et al 2006). However, it remains unclear whether or not these differences specifically reflect only higher exposures, or may in some cases involve metabolic or other differences.

For example, it is well established that black smokers have higher serum cotinine concentrations per cigarette smoked than do white smokers (Wagenknecht et al. 1990, Carballo et al. 1998), and recent studies indicate that blacks may metabolize nicotine differently, contributing to this difference. Specifically, black smokers were found to have lower total and non-renal clearances of cotinine, a slower fractional conversion of nicotine to cotinine, and a longer cotinine half-life, compared with white smokers (Perez-Stable et al. 1998, Benowitz et al. 1999). The extent to which such racial differences might influence the relative concentrations of cotinine observed among non-smokers exposed to SHS is not clear. Furthermore, among smokers, black and white people also differ in their cigarette preferences with black smokers preferring menthol cigarettes to a much greater extent than do white smokers (Gardiner 2004). Mentholated cigarettes have been reported to inhibit nicotine metabolism in smokers (Benowitz et al. 2004), and SHS derived predominately from mentholated cigarettes might conceivably also contribute to a difference in response among non-smokers. Metabolic differences might also influence the cotinine concentration differences observed between male and female non-smokers, although in NHANES (National Health and Nutrition Examination Survey) such differences have narrowed in recent years. In general, these uncertainties concerning potential group differences in response to SHS exposures complicate the interpretation of cotinine concentration differences observed in national surveys.

Biomarker studies of the exposure of non-smokers to SHS have typically relied on the measurement of cotinine, which is currently regarded as the most sensitive and specific marker available for this purpose. Less information is available on responses of certain carcinogen markers in tobacco smoke such as aromatic amines and the tobacco-specific nitrosamines. Two well-established carcinogen markers known to be present in non-smokers exposed to SHS are 4-aminobiphenyl (4-AB), which may be measured as a haemoglobin adduct in humans (Bryant et al. 1987), and the tobacco-specific nitrosamine 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanol (NNAL) which can be measured in urine (Hecht et al. 1993). Both 4-AB and the 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), precursor of NNAL in tobacco smoke, are known to be enriched in SS smoke, and both 4-AB and NNAL tend to be elevated in non-smokers exposed to SHS (Bryant et al. 1987, Hammond et al. 1993, Hecht 2004).

To understand more clearly the biomarker response of non-smokers to SHS among individuals, we exposed non-smoker volunteers for a period of 4 h to aged, diluted SS smoke (ADSS) – a surrogate for SHS – under controlled conditions in an environmental chamber. This study had two main objectives. The first objective was to assess the overall rate and extent of salivary cotinine response when non-smokers are exposed to a defined level of ADSS cigarette smoke under controlled conditions. The second objective was to compare the biomarker response in both male and female non-smokers, in both black and white subjects, and within several biological matrices (serum, saliva, urine), using either mentholated or non-mentholated cigarettes as the source of the SS smoke. In this report we describe the responses observed under these conditions for cotinine and other nicotine metabolites in serum, saliva and urine samples, and similar pre- and post-exposure concentration comparisons of 4-AB haemoglobin adducts and urinary total NNAL.

Methods

Subject recruitment and selection criteria

The exposure chamber aspects of this study were conducted in the laboratories of Battelle Memorial Institute in Columbus, OH, USA. Subjects were recruited largely from local colleges and universities by placing advertisements in local and university newspapers, and by providing short informational presentations to groups of prospective volunteers. Participation was limited to healthy non-smokers aged 18 to 55 years, and enrolment was directed towards a target population of 40 participants that was approximately balanced both by race between blacks and whites and by gender. Racial classification was based on self-identification during the interview. To help allay concerns about the exposure of non-smokers to SS tobacco smoke, we limited recruitment to subjects who were routinely exposed to SHS in their daily lives by residing or working with others who smoked in their presence. Self-reports of daily exposure to SHS from all sources (home, work and other) were obtained from 26 of the participants in this study, and averaged approximately 4.8 h. However, we requested that all subjects avoid such incidental SHS exposure to the extent possible for at least 3 days prior to their participation in this study. Subjects with known

illnesses such as haemophilia, respiratory illnesses including asthma, or who were or could be pregnant were excluded from participation, as were those who used prescription medications, tobacco in any form, or any other source of nicotine such as nicotine gum or transdermal patches.

All screened respondents who were found to meet eligibility requirements attended information sessions in which the study and protocol were described in detail, questions were addressed, and those who chose to participate signed consent forms and chose a date for the exposure assessment. Each subject was scheduled and evaluated individually, and sessions were conducted over a period of 6 months from March to August 2003. This study was reviewed and approved by Institutional Review Boards at the Centers for Disease Control and Prevention and at Battelle Memorial Institute (Columbus, OH). All subjects received monetary compensation for their time and inconvenience.

Environmental chamber and SS smoke generation

Exposure assessments were conducted on subjects individually in a room-sized (17.3 m^3) environmental chamber constructed of aluminium and lined on the ceiling and along one side with Teflon® film, to reduce the effects of surface adsorption. The chamber was equipped with six fans for uniform mixing, and temperature, humidity and airflow into the chamber were controlled and continuously recorded. Slightly negative pressure relative to room air was maintained to minimize external contamination. SS smoke entered the chamber via a wall port, and additional ports allowed sampling of the air exchange rate (using sulfur hexafluoride as a tracer gas), nicotine concentration and continuously acquired carbon monoxide levels. A table, chair and laptop with DVD player were provided for the subjects use, and they were all seated at the same position within the chamber. To help assure that the participant remained awake and alert during exposure, DVD movies were provided. Water was also freely available during the period of exposure. After each day's evaluation, the chamber was vented, thoroughly cleaned to avoid build-up of contaminants, and then flushed with fresh air for several hours before reuse.

A 30-position CSM 2072i (Baumgartner-Jaeger; CH Technologies, Westwood, NJ, USA) automated smoking machine was used to generate the cigarette smoke. This device provided for automatic loading and ignition of the cigarettes, automated ejection of the butts, and was capable of separating MS and SS smoke. The MS smoke was vented to an exhaust hood while the SS smoke was introduced into the chamber using a dual bypass arrangement to provide for an initial rapid increase in the smoke level, while maintaining a constant concentration (based on continuous CO measurements) during the remainder of the exposure period. The smoking machine was also carefully cleaned following each day's use. A diagram of the chamber, smoking machine and associated equipment used in this work is given in Figure 1.

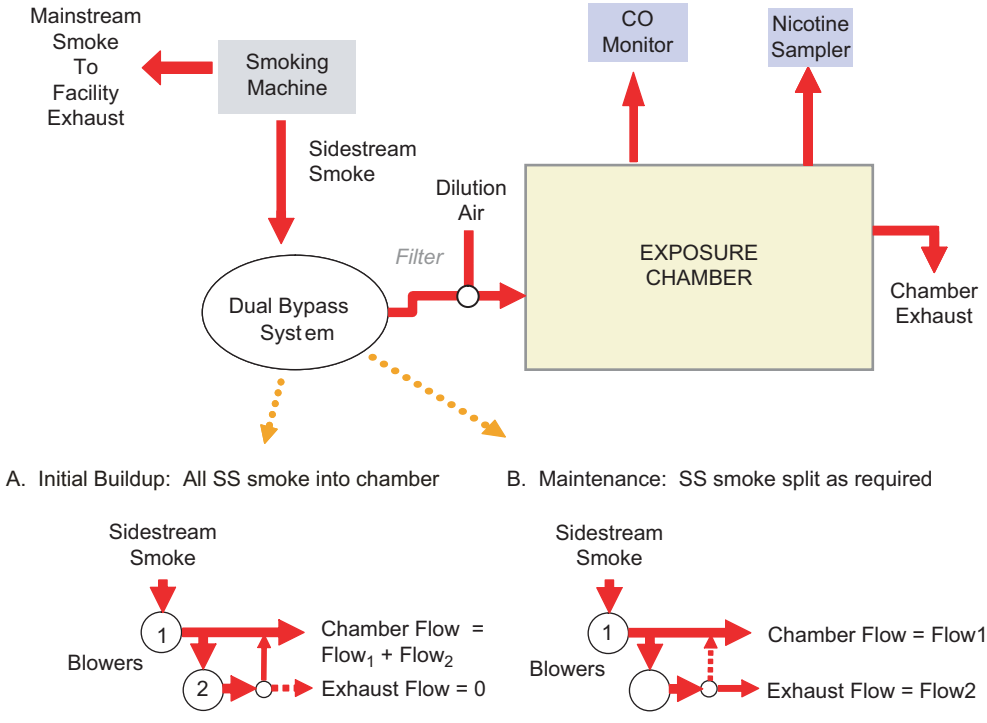

Figure 1. Diagram of the exposure chamber and associated equipment. SS, sidestream smoke.

SS smoke was generated by burning cigarettes of the selected type at a rate as required to maintain the desired exposure level target of approximately 8 ppm CO. This generally required smoking three cigarettes simultaneously during the initial 5–6 min, and then one or occasionally two cigarettes at a time thereafter. The smoking protocol used a target puff volume of 35 ml, a puff duration of 2 s and a puff interval of 60 s. Zero air was used to flush SS smoke from the closed cabinet of the smoking machine and transport it via negative pressure into the chamber through the dual bypass system. All cigarettes were conditioned at 22 ± 1°C and 60 ± 2% relative humidity for 8 days prior to use. We selected two brands of cigarettes for use in this study, Marlboro King Filters, the market leading non-menthol brand, and Newport 100 Menthol Filter, the leading menthol brand. All cigarettes were purchased locally during 2003 in Columbus, OH.

Protocol

Subjects completed an interview form on the morning of their scheduled visit. Baseline samples were then obtained from each participant immediately before entering the chamber, and additional saliva samples were self-collected every 30 min during the exposure interval of 4 h. Investigators outside of the chamber monitored the process continually and reminded participants of each 30-min saliva collection.

Following exposure, each participant changed clothes (to avoid exposure to out-gassing contaminants) and waited in a separate area for 2 h, at which point the post-exposure samples were collected. In all cases, the post-exposure samples were collected 2 h after the completion of the 4-h exposure period. Chamber CO levels were continuously monitored by using a Trace Level Gas Filter Correlation CO Analyzer, (Model 48C, Thermal Environmental Instruments, Waltham, MA, USA), which sampled the ambient CO level once every 2 s throughout the exposure period. Using the dual bypass system, the concentration of CO in the chamber was initially increased rapidly to ca. 8 ppm CO over a period of about 6 min, and then adjusted to hold that target concentration for the remainder of the study. Figure 2 shows a typical response observed over the initial period for one participant, with the total exposure period for that individual indicated in the inset.

A single time-integrated air sample was collected from the chamber for nicotine measurement over the entire 4-h period using a sampling pump to draw chamber air through an XAD-4 sorbent tube at a rate of 1 l min^{-1}. Following collection, each sorbent tube was capped and stored in a freezer for up to 8 weeks until analysis. Previous work established that samples remain stable under these conditions. The samples were analyzed by extraction of the tubes with 0.01% triethylamine

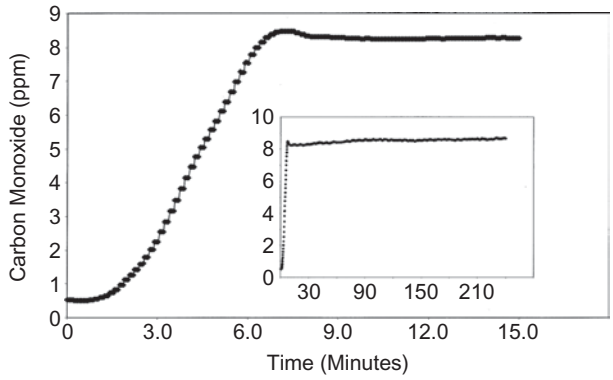

Figure 2. Representative chamber air CO levels measured over time for one subject. The inset shows the entire 4-h exposure period.

in ethyl acetate followed by nicotine analysis by gas chromatography/mass spectrometry using nicotine-D_3 as an internal standard. Samples were separated on a DB-5MS column and analyzed using EI ionization and selected-ion monitoring. The results were calculated as total integrated nicotine concentrations in μg m^{-3}. Chamber temperature and relative humidity were also continuously recorded for each subject and averaged 24.6 ± 0.9°C and 52.3 ± 6.5%, respectively.

The subjects were provided with a set of 12 prelabelled Salivettes at the end of day 1. On the second day following exposure, each participant self-collected one saliva sample each hour from 09.00 to 20.00 h for a total of 12 hourly samples. Each volunteer was asked to avoid further exposure to SHS during the second day, and to record in a diary the time and duration of any exposures that did occur. Valid sample sets were returned by 36 of the participants. After measuring salivary cotinine in each sample, the data were further analyzed by setting each 09.00 concentration to 100% and calculating the percentage remaining for each person at each subsequent time point. The log of these values was regressed on time (hours) to estimate the half-life.

Biomarker measurements

Saliva samples were collected as indicated using Salivettes (Sarstedt, Newton, NC, USA) with the cotton swab inserts. Samples were collected by gently chewing on the cotton swab for about 2 min, with the swab then returned to its container which was subsequently frozen without further treatment until analysis. Blood samples for serum analyses were collected prior to and 2-h post-exposure by venipuncture and collection in 13-ml SST Vacutainer tubes. After clotting, the samples were centrifuged and the serum recovered, transferred to labelled cryovials and frozen for subsequent analysis. Non-coagulated whole blood samples were collected in the same manner for haemoglobin adduct analyses as described below. Urine samples were also collected

pre- and 2-h post-exposure for these assays. The polypropylene bottles used were labelled and frozen directly for later analysis. Periodically, batches of frozen samples were shipped to Atlanta over dry ice where they were thawed and analyzed.

Serum and saliva cotinine were measured by high-performance liquid chromatography atmospheric-pressure chemical ionization tandem mass spectrometry (LC APCI MS/MS) using methods that have previously been described in detail (Bernert et al. 1997, Bernert et al. 2000). Briefly, a 0.5-ml aliquot of the serum or saliva sample was equilibrated with a cotinine-D_3 internal standard for 20 min, extracted with methylene chloride, and further processed as previously described. All samples were analyzed on a Sciex API 4000 tandem mass spectrometer, with the heated nebulizer installed, by measuring selected quantitation and confirmation transition ions in the multiple reaction monitoring (MRM) mode in comparison to a standard curve. The limit of detection (LOD) was 15 pg ml^{-1}. For each subject, one time point was selected at random for duplicate saliva collections. These duplicate salivary cotinine measurements demonstrated close agreement, with a comparison of the duplicate samples yielding $r^2 = 0.9984$.

Nicotine, cotinine, hydroxycotinine and their glucuronides in urine samples were also measured by an LC API MS/MS method as previously described (Bernert et al. 2005a). Briefly, measurements were made by extracting the urine samples following a prior hydrolysis with β-glucuronidase, *Helix pomatia*, type H-1, conducting initial clean-up on ChemElute columns, and analyzing by using gradient elution on a Waters Xterra MS column followed by electrospray ionization and MRM analysis on a Sciex API 4000 tandem mass spectrometer.

For determination of haemoglobin adducts of 4-AB, whole blood samples were drawn with 10-ml lavender top (EDTA) Vacutainer tubes immediately prior to and again 2-h post-exposure from each subject. The samples were centrifuged shortly after collection to isolate the red cells. The cells were then washed twice with 5 ml of physiological saline, frozen and shipped to Atlanta for analysis. Samples were subsequently analyzed by a capillary on-column gas chromatography tandem mass spectrometry method as previously described (Bernert et al. 2005b). In these analyses the globin samples were hydrolyzed, a nonadeuterated internal standard was added, and free 4-AB was extracted, derivatized with pentafluoropropionyl anhydride, and analyzed by gas chromatography tandem mass spectrometry using a Thermo-Finnigan TSQ 7000 mass spectrometer.

Total NNAL measurements were made by using the method of Xia et al. (2005). In these analyses, the urine samples (5 ml) were hydrolyzed with ß–glucuronidase and total NNAL was measured. Briefly, the assay involved the addition of a $^{13}C_6$-NNAL internal standard followed by a preliminary separation and sample clean-up using specially developed molecularly imprinted polymer columns, and analysis by high-performance liquid chromatography electrospray-ionization tandem mass spectrometry. The LOD for these analyses using 5-ml sample aliquots was 1.6 pg ml^{-1}. Creatinine in urine was measured by a commercial, automated colorimetric enzymatic method (Roche Creatinine Plus) implemented on a Hitachi 912 analyzer.

Quality assurance and statistical analysis

Biomarker measurements were made in groups of samples containing appropriate blank and known (pooled and/or fortified) quality control materials. All results were from analytical runs determined to be in statistical control by the use of standard quality assurance procedures.

Data were analyzed using SAS (version 9.1) software. All exposure and outcome variables were normalized by taking logs of the original variables. Data for all subjects (Tables 1–5) were analyzed by SAS Proc MEANS; adjusted geometric means (Tables 2–5) by gender, race, and cigarette type were analyzed using SAS Proc GLM. For all pre-exposure variables gender, race and cigarette type were used as covariates. For pre-exposure urine variables, urine creatinine (pre-exposure) was also used as an independent variable. For all post-exposure variables, gender, race and cigarette type were again used as covariates, and air exchange rates as well as air nicotine concentrations were included as additional independent variables. For urinary post-exposure variables, post-exposure urine creatinine was added as an independent variable. For all variables defined as differences between post- and pre-exposure, gender, race and cigarette type were used as covariates, and air exchange rates as well as air nicotine were also included as covariates. For the differences between pre-exposure and post-exposure urine variables, both pre-exposure and post-exposure urine creatinine were also included

Table 1. Salivary cotinine response rate.

Group	*n*	Linear estimate[a] (pg ml^{-1} min^{-1})	r^2	Polynomial estimate[b](pg ml^{-1} min^{-1})	r^2
All subjects	40	11.6	0.9885	11.5	0.9978
Males	19	11.0	0.9919	11.3	0.9938
Females	21	12.2	0.9801	13.2	0.9988
Blacks	18	11.2	0.9934	11.6	0.9964
Whites	22	11.9	0.9787	13.3	0.9948
Menthol	21	12.6	0.9887	12.9	0.9921
Non-menthol	19	10.5	0.9754	11.8	0.9961

[a]Assume linear response. Estimated from slope of simple regression curve of salivary cotinine concentration vs time. [b]Estimated from a second-order polynomial curve fit. Rate = calculated concentration (at t = 240 min) divided by 240.

Table 2. Salivary cotinine geometric means. None of the comparisons of increases between groups by gender, race or cigarette type was significant.

Group	*n*	Pre-exposure	Post-exposure[a]	Difference[a]
All subjects	38	0.262 (0.180–0.382)	3.604 (3.268–3.976)	3.062 (2.727–3.440)
Males	18	0.284 (0.163–0.492)	3.677 (3.190–4.238)	3.052 (2.574–3.618)
Females	20	0.234 (0.138–0.395)	3.548 (3.104–4.055)	3.099 (2.640–3.636)
Blacks	18	0.236 (0.134–0.415)	3.851 (3.336–4.445)	3.408 (2.870–4.047)
Whites	20	0.280 (0.168–0.467)	3.387 (2.957–3.880)	2.775 (2.358–3.265)
Menthol	19	0.341 (0.202–0.575)	3.770 (3.297–4.312)	3.135 (2.669–3.682)
Non-menthol	18	0.194 (0.112–0.338)	3.460 (2.999–3.999)	3.017 (2.542–3.579)

Data are data are in ng ml^{-1}, geometric mean (95% confidence interval).[a]Post-exposure results and the difference observed have been adjusted for air nicotine concentrations and air exchange rates.

Table 3. Urinary cotinine geometric means. None of the comparisons of increases between groups by gender, race or cigarette type was significant.

Group	*n*	Pre-exposure	Post-exposure[a]	Difference[a]
All subjects	38	4.082 (2.520–6.610)	29.444 (24.268–35.309)	19.547 (15.953–23.949)
Males	18	4.064 (2.030–8.138)	23.102 (18.540–28.788)	16.341 (12.038–22.182)
Females	20	3.805 (1.937–7.473)	35.542 (29.051–43.485)	22.912 (17.387–30.192)
Blacks	18	2.617 (1.283–5.337)	25.125 (20.142–31.340)	20.580 (15.384–27.531)
Whites	20	5.908 (3.059–11.410)	32.682 (26.690–40.018)	18.192 (13.773–24.028)
Menthol	20	5.125 (2.613–10.054)	29.203 (23.817–35.805)	18.042 (13.751–23.671)
Non-menthol	19	3.017 (1.501–6.064)	28.118 (22.744–34.715)	20.751 (15.607–27.590)

Data are in ng ml^{-1}, geometric mean (95% confidence interval). [a]Post-exposure results and the difference observed have been adjusted for air nicotine concentrations and air exchange rates.

as independent variables. For correlations between nicotine metabolite ratios and the increase in serum cotinine, SAS Proc CORR was used. In all cases a value of $p \leq 0.05$ was regarded as statistically significant.

Results

Forty participants were included in this study divided approximately equally by race and sex, with 18 black subjects (nine female) and 22 white subjects (12 female). The participant's ages ranged from 18 to 54 years, and approximately half were randomly selected for exposure to SS smoke from menthol cigarettes (53% overall). The mean ± SD age of all participants was 26 ± 8.1 years;

Table 4. 4-Aminobiphenyl haemoglobin adduct geometric means. None of the comparisons of increases between groups by gender, race or cigarette type was significant.

Group	*n*	Pre-exposure	Post-exposure[a]	Difference[a]
All subjects	38	29.253 (22.941–37.302)	36.176 (28.640–45.694)	4.663 (2.887–7.532)
Males	18	26.049 (18.109–37.469)	32.681 (22.872–46.697)	3.119 (1.514 –6.423)
Females	20	32.670 (23.105–46.196)	39.364 (28.094–55.153)	5.518 (2.902 –10.490)
Blacks	16	30.609 (20.839–44.959)	37.047 (25.595–53.624)	3.622 (1.592 –8.238)
Whites	22	27.803 (20.036–38.579)	34.724 (24.901–48.421)	4.752 (2.693 –8.383)
Menthol	20	31.716 (22.492–44.723)	41.388 (29.486–58.096)	6.526 (3.512 –12.127)
Non-menthol	18	26.832 (18.599–38.709)	31.082 (21.699–44.522)	2.637 (1.230 –5.651)

Data are in pg g^{-1} of haemoglobin, geometric mean (95% confidence interval).
[a]Post-exposure and difference results have been adjusted for air nicotine concentrations and air exchange rates.

Table 5. Urinary total NNAL geometric means. Differences by gender and race were not significant.

Group	*n*	Pre-exposure	Post-exposure[a]	Difference[a]
All subjects	40	7.422 (5.352–10.292)	20.093 (16.999–23.750)	11.836 (9.362–14.965)
Males	19	6.022 (3.842–9.437)	18.668 (15.286–22.798)	11.351 (8.487–15.182)
Females	21	8.235 (5.441–12.464)	21.464 (17.798–25.885)	12.342 (9.228–16.507)
Blacks	18	6.498 (4.116–10.258)	19.817 (16.162–24.300)	11.366 (8.543–15.123)
Whites	22	7.631 (5.105–11.407)	20.219 (16.847–24.265)	12.325 (9.264–16.398)
Menthol	21	9.726 (6.458–14.647)	19.733 (16.407–23.735)	9.416 (7.106–12.478)
Non-menthol	19	5.098 (3.251–7.995)	20.304 (16.702–24.684)	14.878 (11.227–19.715)[b]

Data are in pg ml^{-1} of haemoglobin, mean (95% confidence interval). [a]All post-exposure and difference results have been adjusted for air nicotine concentrations and air exchange rates. [b]Difference in response by type of cigarette was significant ($p = 0.0337$).

the black female group was slightly older. All subjects had self-reported routine exposure to SHS at home or at work. However, they were asked to avoid all SHS exposure to the extent possible for 3 days prior to their appointment to minimize the initial background exposure level. A total of 49 participants were examined in this study. After baseline serum cotinine concentrations were measured, six subjects were excluded from the study because of high initial concentrations ranging from 6 to 126 ng ml^{-1} (mean 57.3 ng ml^{-1}). We also excluded one participant with a number of unusual results as an outlier, and two additional subjects who had a substantial number of missing values. Among this group of nine excluded subjects, four were female and seven were black.

A summary of the exposure data is given in Table 6. The carbon monoxide levels in Table 6 represent the time-integrated concentrations observed over the 4-h exposure period for each group, and, as expected, were quite uniform. Integrated nicotine exposures and air exchange rates varied somewhat among the groups although the group differences were not significant. All final biomarker concentrations have been statistically adjusted for each participant's measured air nicotine concentration and air exchange rate as described in the Methods section.

Geometric mean serum cotinine concentrations measured immediately prior to exposure, and again 2 h following exposure to SS smoke are given in Table 7. As indicated in the final column of the table, the difference in post-exposure minus pre-exposure concentrations was significantly higher in all subjects ($p < 0.0001$). However, no significant differences were observed between groups (gender, $p = 0.1013$; race, $p = 0.1834$; cigarette type, $p = 0.2216$). The time course for changes in salivary cotinine concentrations during the exposure period is given in Figure 3, which demonstrates a monotonic rise in concentration throughout exposure. A significant mean increase in salivary cotinine was observable at the earliest time point (30 min), and the mean concentrations continued to increase throughout the 4-h exposure period. As indicated in Table 1, the estimated rate of this increase was similar among all groups, averaging approximately 12 pg ml^{-1} min^{-1} under these exposure conditions. The differences in salivary cotinine measured 2-h post-exposure compared with the initial concentrations are summarized in Table 2, and demonstrate results similar to those observed in serum, although the salivary cotinine concentrations were typically somewhat higher in each case than those observed in serum. The ratio of salivary to serum cotinine concentrations (mean ± SE) was 1.338 ± 0.089 in the pre-exposure samples, and 1.215 ± 0.054 2-h post-exposure, $n = 40$ and 38, respectively. As with serum, no significant differences were observed in the salivary cotinine increases between groups following the 4-h exposure period (gender, $p = 0.8986$; race, $p = 0.0990$; cigarette type, $p = 0.7491$).

Urine total cotinine geometric means are presented in Table 3. For these measurements we adjusted each sample for urinary creatinine by including it as a covariate in the regression in addition to air nicotine concentrations and the air exchange rate. As was observed with both serum and salivary cotinine, the differences between pre- and post-exposure adjusted urinary cotinine concentrations were significantly increased overall ($p < 0.0001$), but no significant differences were observed when comparing the responses by group (gender, $p = 0.1364$; race, $p = 0.5653$; cigarette type, $p = 0.4971$). The (post-exposure–pre-exposure) differences in serum cotinine and in urinary cotinine were also similar in each case.

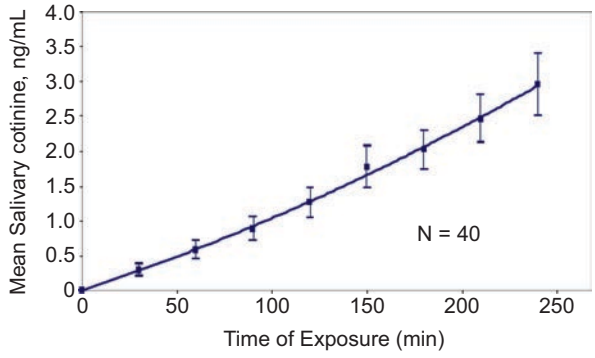

Figure 3. Salivary cotinine time course (all subjects). Vertical bars represent the 95% confidence interval.

Table 6. Summary exposure data.

Group	*n*	Carbon monoxide[a] (ppm•h)	Air nicotine (μg m^{-3})	Air exchange rate (Exchanges h^{-1})
All subjects	40	34.3 (33.9–34.6)	140.2 (127.4–154.3)	0.713 (0.687–0.741)
Males	19	34.2 (33.6–34.8)	133.9 (119.7–149.9)	0.706 (0.667–0.748)
Females	21	34.3 (33.9–34.8)	146.2 (124.8–171.3)	0.720 (0.682–0.760
Blacks	18	34.2 (33.8–34.5)	128.7 (114.3–145.1)	0.704 (0.671–0.738)
Whites	22	34.4 (33.8–35.0)	150.4 (130.0–174.0)	0.721 (0.679–0.766)
Menthol	21	34.8 (34.2–35.3)	137.4 (119.9–157.5)	0.724 (0.680–0.770)
Non-menthol	19	33.7 (33.4–34.1)	143.4 (123.8–166.1)	0.702 (0.671–0.734)

Data are presented as geometric means (95% confidence interval).
[a]Integrated amount (i.e. ppm•h) over 4 h of exposure.

Table 7. Serum cotinine geometric means. None of the comparisons of increases between groups by gender, race or cigarette type was significant.

Group	*n*	Pre-exposure	Post-exposure[a]	Difference[a]
All subjects	40	0.210 (0.141–0.311)	3.068 (2.769–3.400)	2.660 (2.395–2.955)
Males	19	0.224 (0.125–0.401)	2.866 (2.498–3.287)	2.422 (2.113–2.778)
Females	21	0.195 (0.112–0.339)	3.205 (2.815–3.648)	2.843 (2.498–3.236)
Blacks	18	0.217 (0.119–0.394)	2.875 (2.494–3.314)	2.457 (2.132–2.831)
Whites	22	0.201 (0.117–0.345)	3.195 (2.811–3.630)	2.804 (2.468–3.185)
Menthol	21	0.271 (0.156–0.470)	3.306 (2.902–3.766)	2.786 (2.446–3.173)
Non-menthol	19	0.161 (0.090–0.289)	2.780 (2.419–3.190)	2.472 (2.154–2.838)

Data are in ng ml^{-1}, geometric mean (95% confidence interval).
[a]Post-exposure results and the difference observed have been adjusted for air nicotine concentrations and air exchange rates.

Regression of the serum cotinine difference on urine cotinine differences (adjusted for creatinine) yielded an r^2 value of 0.37 ($p = 0.0006$; $n = 37$ pairs). As indicated in Figure 4, similar increases were also noted for hydroxycotinine in each case, whereas the short-term increases in urinary nicotine were higher than either cotinine or hydroxycotinine for each group, and also showed substantial variability. Hydroxycotinine:cotinine ratios were calculated (Dempsey et al. 2004) for all pre-exposure urine samples with a mean 95% confidence interval (CI) of 2.65 (0.53, 6.63), $n = 39$.

When we compared pre-exposure urinary cotinine concentrations with serum cotinine, it made no difference whether or not we regressed either urine cotinine or the cotinine-creatinine ratio (CCR) on serum cotinine, with resulting r^2 values of 0.677 and 0.681, respectively. However, using the CCR rather than the urine cotinine concentrations did make a notable improvement in the regression of the short-term difference in urine cotinine (post-exposure–pre-exposure) on the difference in serum cotinine (urine cotinine, $r^2 = 0.195$; CCR, $r^2 = 0.413$). For the geometric means given in Table 3, the results were adjusted by including urine creatinine in the model rather than by calculating simple ratios, as that is believed to provide a better estimate (Barr et al. 2005).

The half-life for salivary cotinine estimated from the data collected on day 2 (the day after exposure) was 16.7 h ($n = 36$; $r^2 = 0.383$). The half-life estimated separately for black participants was 16.3 h ($n = 16$; $r^2 = 0.3983$), and for whites it was 17.0 h ($n = 20$; $r^2 = 0.3699$). To evaluate the differences by race, we tested the regression lines for blacks and whites for their identity and parallelness (Nester & Wasserman 1974). The two regressions appeared to be indistinguishable ($p = 0.81$) in this evaluation. A larger difference was obtained for comparisons by gender in which the estimated cotinine half-life for the female participants was 14.6 h ($n = 21$; $r^2 = 0.4268$), whereas for men it was 20.4 h ($n = 15$; $r^2 = 0.379$). These regression lines were significantly different ($p = 0.0001$), suggesting that the mean half-life of salivary cotinine for the male participants in this study was longer than that observed for the females.

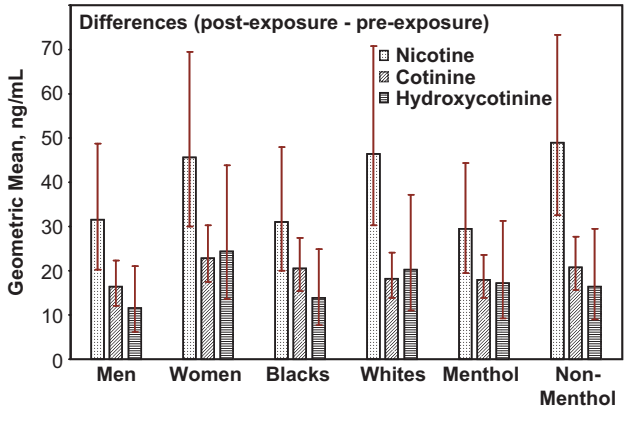

Figure 4. Urinary nicotine metabolite increases following exposure. Vertical bars represent the 95% confidence interval.

4-AB haemoglobin adduct results are given in Table 4. As expected, there were significant concentrations of these 4-AB adducts present in the subjects prior to exposure. Although tobacco exposure is a major source of this toxicant in people, there are clearly other sources from the air and diet (Bryant et al. 1987), and significant background concentrations are typically observed. Nevertheless, following the 4-h exposure period, the mean concentrations were clearly increased although there was substantial variability in the response among individuals as indicated by the relatively wide 95% CI. However, for all of the subjects as a group and for each of the subgroups, the increase in haemoglobin adduct concentrations of 4-AB following the 4-h SS smoke exposure compared to pre-exposure levels was statistically significant ($p < 0.0001$), but again no significant differences in the response were noted among the group comparisons (gender, $p = 0.2397$; race, $p = 0.5962$; cigarette type, $p = 0.0757$).

A similar result was seen in urinary NNAL concentrations following exposure (Table 5). Because of the low concentrations involved, only total NNAL concentrations, i.e. those measured following hydrolysis with ß–glucuronidase, were monitored in each case. As with urine cotinine, for these analyses we adjusted for urinary creatinine in addition to air nicotine concentration and air exchange rate differences. For all subjects, the increase in NNAL concentration measured 2-h post-exposure was statistically significant ($p < 0.0001$), although no differences in the response were seen on the basis of either gender ($p = 0.3305$) or race ($p = 0.8877$). For this marker, however, there was a statistically significant difference in the response according to the type of cigarette used, with a larger increase in NNAL observed in the group exposed to smoke generated by the non-menthol cigarette ($p = 0.0337$).

Discussion

In this study we have investigated the response of tobacco associated biomarkers in 40 non-smokers exposed to ADSS smoke under controlled conditions in an environmental chamber. All the subjects in this study demonstrated significant increases in several tobacco exposure biomarkers following exposure to 140 μg m^{-3} of air nicotine and approximately 8.6 ppm CO for 4 h, with consistent responses among the participants.

Several prior studies have examined the response to cigarette smoke generated under controlled conditions in environmental chambers among both healthy and asthmatic adults and children. However, only limited

biomarker response data for these exposures have been available. For example, our mean exposure concentrations of 8.6 ppm CO and 140 μg m^{-3} are quite similar to the 'low level' exposure conditions used by Stankus et al. (1988) in a study of adult asthmatics. However, that study focused on FEV_1 measurements, and no tobacco exposure biomarkers were included. In a more recent study by Willes et al. (1998), 23 non-smoker adults were exposed to SS smoke at 15 ppm CO (air nicotine was not reported) in an environmental chamber for 2 h. The 24-h urinary CCR was reported to increase approximately 10-fold from 11.4 to 123 ng cotinine mg^{-1} creatinine under those conditions, which is similar to the 5–10 fold increases in the (adjusted) spot urine cotinine concentrations that we observed in the present study.

Johnson et al. (1985) examined salivary cotinine responses in ten healthy adult non-smokers who were exposed to a high SS smoke concentration of 25–30 ppm CO and approximately 260 μg m^{-3} air nicotine for 3 h. At that exposure level, the subjects required the use of goggles to avoid excessive eye irritation. The median salivary cotinine concentration measured by RIA in that study increased about 3-fold between 0 and 3 h of exposure, from a rather high initial value of 6 ng ml^{-1}, to approximately 18 ng ml^{-1}at the end of exposure. A further small post-exposure increase was noted with a maximum of about 20 ng ml^{-1} 3-h post-exposure, followed by a gradual decline. The authors observed considerable interindividual variation in this response, which is consistent with our findings.

Jarvis et al (1983) in a non-controlled study of the exposure of seven non-smokers who remained for 2 h in a smoky pub (peak CO 13 ppm) also found significant mean increases in saliva, plasma and urine cotinine among the participants. These findings are in agreement with our results indicating that acute passive exposure to tobacco smoke can be reliably detected and quantified among groups by the measurement of cotinine in serum, saliva and urine.

The salivary cotinine time course in our study indicated that under controlled conditions of exposure, a quantitative mean cotinine response can be measured as a function of exposure time. Furthermore, the general uniformity in this response suggests that such measurements are capable of reliably indicating differences in exposure within the population, although this response is transient as the elimination half-life of cotinine is relatively short at about 16–18 h (DHHS 2006). The latter limitation, however, may be largely minimized in large epidemiological studies in which most participants are presumably at steady-state.

Steady-state concentrations of metabolites like cotinine also may be influenced by differences in elimination rates. We found an apparent difference between the estimated elimination half-life for men and women in this study. The half-life of salivary cotinine in women was significantly shorter, in agreement with a prior report (Benowitz et al. 1999), which would be consistent with a lower serum cotinine concentration for women in surveys such as NHANES, although the observed difference in serum cotinine between men and women in recent surveys has been relatively small (Pirkle et al. 2006).

Conversely, we did not find any significant differences in the estimated cotinine half-life in this study when comparing groups by race. This was surprising and may have reflected the rather small study size. It is possible that a larger study would have detected racial differences in elimination as have been reported previously. One of the reviewers of this paper made the interesting observation that our exclusion of some participants (mainly black) because of higher than expected initial cotinine concentrations may have inadvertently excluded those with slower metabolism and thus biased our results in that regard. Perhaps such an effect contributed to our findings in this case

Salivary cotinine was measured on both the day of exposure and on the following day. The mean salivary cotinine concentration at the end of the exposure period for the half-life evaluation group on day 1 was 3.41 ± 1.62 ng ml^{-1} (mean ± SD, $n = 36$); 24 h later the mean salivary cotinine concentration was 3.10 ± 1.42 ng ml^{-1}, range 1.05–6.75 ng ml^{-1}. In most individuals, the concentration observed 24-h later was nearly the same as or slightly higher than that observed at the end of the exposure period, and in all cases the post-exposure salivary cotinine concentration was much higher than the initial, pre-exposure concentration, consistent with a reliable indication within groups of recent, prior exposures by using cotinine measurements. It should be noted, however, that a quantitative estimate of the prior exposure (ca. 140 μg m^{-3} nicotine for 4 h) on an individual basis would be limited by metabolic differences as indicated in the range of 24-h concentrations observed in this case, from 1.05 to 6.75 ng ml^{-1}.

In addition to the increases in cotinine, we also found significant increases in urinary total NNAL at 2-h post-exposure. Although the concentrations of this analyte were consistently quite low, we were able to measure NNAL above our analytical detection limit in 34 of the 40 pre-exposure urine samples (85%), and all results were well above our limit of detection in the 2-h post-exposure samples. After adjusting for differences in urinary creatinine, the post-exposure NNAL value was increased over the pre-exposure measurement in each of the subjects.

These results clearly indicate that non-smokers even briefly exposed to SS tobacco smoke are also exposed to measurable concentrations of tobacco nitrosamines. NNAL, and NNK from which it is derived, are systemic pulmonary carcinogens that are specific for tobacco exposure (Hecht 2002). This specificity and known

carcinogenicity makes NNAL a particularly important tobacco exposure biomarker to monitor in both smokers and non-smokers. Several reports have established that adult non-smokers with regular exposure to SHS have significantly increased concentrations of NNAL in their urine (Hecht et al. 1993, Anderson et al. 2002, Hecht 2004), and this biomarker has also been confirmed in urine from elementary school-aged children with exposure to SHS (Hecht et al. 2001). In the present chamber study, although the concentrations remained low, adjusted urinary NNAL concentrations were consistently and significantly increased following 4 h of exposure to SS smoke, with no differences in this response noted on the basis of either race or gender.

Although neither race nor gender was influential on the NNAL results, there was a statistically significant difference according to cigarette type. Participants exposed to ADSS smoke from the menthol cigarette had a significantly lower mean increase in NNAL following exposure compared with the group exposed to non-menthol cigarette smoke. It is doubtful that this resulted from inherent differences in the nitrosamine content of the cigarettes because the content of NNK in SS smoke has been reported to be approximately the same for these two brands of cigarettes, with concentrations of 56 ng/cigarette and 63 ng/cigarette for Marlboros and Newports, respectively (Borgerding et al. 2000). The difference in NNAL following exposure resulted from similar post-exposure concentrations in both groups and a significantly lower initial (pre-exposure) concentration in the non-menthol exposure group. Therefore, the extent of increase following 4 h of exposure was greater in the group exposed to SS smoke from non-menthol cigarettes. It is possible that menthol may have inhibited the formation of NNAL from NNK in those exposed to SS smoke from the Newport cigarettes, as menthol has been reported to influence nicotine metabolism in smokers (Benowitz et al. 2004). However, no such effect was noted in the adjusted differences in serum, salivary or urinary cotinine, and the physiological significance, if any, of this difference in NNAL concentrations remains unclear.

Increases were also demonstrated in the concentration of 4-AB adducted to haemoglobin in blood. The difference in concentration in these adducts following exposure was typically low and variable, but the geometric mean differences were consistently increased for each group. Although other exposure sources besides tobacco exist for 4-AB, adducts of this bladder carcinogen are known to be increased in non-smokers exposed to SHS (Bryant et al. 1987), and a recent study has suggested that, at least among women, increased concentrations of 4-AB adducts may help to identify those at increased risk of cancer related to SHS exposure (Airoldi et al. 2005). Our results indicate that significant increases in adducted 4-AB can be measured in non-smokers following short-term, acute exposure to ADSS smoke, and as with most of the other markers examined in this study, no significant differences in response were noted based on race, gender or cigarette type.

We had expected that individual differences among subjects in their formation of cotinine following exposure to ADSS smoke might be associated with metabolic differences in CYP2A6 activity as CYP2A6 is a P_{450} enzyme that is believed to have an important role in the metabolism of nicotine and in the formation of both cotinine and hydroxycotinine (Dempsey et al. 2004, Hukkanen et al. 2005). That expectation appeared to be realized in this study as estimates of CYP2A6 activity based on the ratio of pre-exposure urinary hydroxycotinine to cotinine as proposed by Dempsey et al. (2004) demonstrated a significant correlation with the increase in serum cotinine observed post-exposure, with $r = 0.4822$ ($p = 0.0019$; $n = 39$).

Overall, our study has several limitations. We used ADSS smoke which is not identical to SHS as it omits the admixture of mainstream smoke exhaled by the smoker. Also, our exposure conditions using a (partly) Teflon-lined stainless steel chamber did not mimic the adsorptive and re-emission behaviour of natural environments (Singer et al. 2002), although that should not affect the group comparisons in this study because all of the subjects were exposed under the same conditions. An important limitation was that a total of only 40 participants were included in this study which limited its overall power; because of the complexity and expense of the study, a larger sample size was regarded as infeasible. In addition, the concentration of ADSS used in this study was relatively high, and in general much higher than would normally be encountered today in a natural setting, even in a rather smoky environment. Finally, although the subjects were exposed to a significant level of SS smoke for 4 h, the exposure was on only a single occasion whereas SHS exposure of non-smokers typically is a continuing process leading to the development of steady-state concentrations of biomarkers in people over time. Thus, our results primarily reflect the initial exposure responses of the participants rather than the steady-state conditions normally encountered in population studies. Nevertheless, this approach did allow us to precisely define and determine the environmental conditions and exposure experienced by each person, as well as to control the type (brand) of cigarette smoke to which they were exposed.

Our results indicate that each of the biomarkers evaluated in this study was significantly increased following exposure to SS smoke, and that the responses

were relatively uniform among these non-smokers. In addition, no consistent differences were found when comparing two types of cigarettes (menthol and non-menthol) used to generate SS smoke in this study. Thus, our results are consistent with a similar acute response to SHS exposure among most non-smokers and suggest that differences in mean biomarker concentrations observed in epidemiological studies such as NHANES are probably reflective of true differences in SHS exposure experienced by these population groups.

Acknowledgments

***Declaration of interest*:** The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention. Use of trade names and commercial sources is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services or the Centers for Disease Control and Prevention.

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