

## RESEARCH ARTICLE

**Study of cardiovascular disease biomarkers among tobacco consumers, part 1: biomarkers of exposure**Leanne R. Campbell<sup>1</sup>, Buddy G. Brown<sup>1\*</sup>, Bobbette A. Jones<sup>1</sup>, Kristin M. Marano<sup>2</sup>, and Michael F. Borgerding<sup>1</sup><sup>1</sup>R.J. Reynolds Tobacco Company, Winston-Salem, NC, USA and <sup>2</sup>RAI Services Company, Winston-Salem, NC, USA**Abstract**

A study was conducted to evaluate biomarkers of biological effect and physiological assessments related to cardiovascular disease (CVD) among adult male cigarette smokers (SMK), moist snuff consumers (MSC) and non-consumers of tobacco (NTC). Additionally, biomarkers of tobacco and tobacco smoke exposure (BoE) were measured in spot urines and are reported here. Except for the BoE to nicotine and NNK, BoE were generally greater in SMK compared with MSC, and BoE were generally not different in comparisons of MSC and NTC. Results demonstrated that MSC had lower systemic exposures to many harmful and potentially harmful constituents than SMK, which is consistent with epidemiological data that indicate a differential in CVD risk between these groups.

**Keywords**

Cigarettes, clinical study, CVD, harmful and potentially harmful constituents, moist snuff, spot urines

**History**

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**Background**

Cigarette smoking is a leading cause of preventable deaths in USA and significantly increases the risk of developing lung cancer, heart disease, chronic bronchitis, emphysema and other serious diseases and adverse health conditions. No tobacco product has been shown to be safe and without risks, and quitting cigarette smoking significantly reduces the risk for serious diseases. Notably, the health risks associated with cigarettes are significantly greater than those associated with the use of smoke-free tobacco and nicotine products (Nutt et al., 2014; Zeller et al., 2009).

Adult cardiovascular disease (CVD) includes diseases of the heart and/or vascular system (i.e. blood vessels), including hypertension, atherosclerosis and stroke [World Health Organization (WHO), 2013]. According to a 2008 report from the Centers for Disease Control and Prevention, during the period between 2000 and 2004, cigarette smoking was associated with ~128 000 deaths per year from all CVDs, and ~80 000 deaths from coronary heart disease (CHD) alone in the USA (Adhikari et al., 2008). In addition, in USA, the annual mortality from CHD was the third leading cause of smoking attributable death, following lung cancer and chronic obstructive pulmonary disease (COPD) (Adhikari et al., 2008).

Data suggest significant increases in risk for CHD and stroke among cigarette smokers compared to never smokers [US Department of Health and Human Services (USDHHS), 2004]. For example, in the American Cancer Society's Cancer Prevention Study-II (CPS-II), the CHD and stroke mortality hazard ratios among male cigarette smokers were 1.9 (95% CI: 1.8–2.1) and 1.7 (95% CI: 1.5–2.0), respectively (Thun et al., 2000). Despite the relatively high number of smoking-associated CHD deaths, it is notable that, among cigarette smokers, the relative risks for CVD are lower compared to those associated with lung cancer or COPD (Thun et al., 2000). This is likely due to the complexity of CVD as a chronic progressive disease associated with several risk factors including cigarette smoking, lack of physical activity, poor diet, diabetes, obesity, hypertension and dyslipidemia (Gillespie et al., 2013).

Cigarette smoking likely contributes to the progression of cardiovascular events through increased inflammation, increased platelet activation (i.e. thrombosis), endothelial dysfunction and reduced oxygen supply (Benowitz, 2003; Messner & Bernhard, 2014). Notably, adverse effects of these determinants of cardiovascular events have not been observed in consumers of smokeless tobacco (ST). Based on a study of cardiovascular biomarkers, ST use did not affect inflammation (including C-reactive protein levels), endothelial function, platelet activation, oxidative stress, leukocyte counts, fibrinogen or lipid profiles in a manner similar to cigarette smoking (USDHHS, 2010). That is, these biomarkers, and biomarkers related to these events, were not different in comparisons of ST consumers with individuals who had never used tobacco (USDHHS, 2010).

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Not surprisingly then, CVD risk estimates associated with ST use are consistently lower than the CVD risk estimates associated with cigarette smoking (Piano et al., 2010). For example, CHD and stroke mortality hazard ratios from the CPS-II study for male ST users compared with male never tobacco users were 1.26 (95% CI: 1.08–1.47) and 1.40 (95% CI: 1.10–1.79), respectively (Henley et al., 2005). Thus, in accordance with a statement from the American Heart Association (Piano et al., 2010), the risk of CVD among ST users may be increased relative to never tobacco use; however, any risk of CVD associated with ST use is less than that associated with cigarette smoking. It is also notable that in an evaluation of cigarette smokers who switched from cigarette smoking to ST use (also the CPS-II cohort), the relative risks of CHD and stroke were not statistically different from quitting tobacco entirely (Henley et al., 2007).

This study was conducted to evaluate biomarkers of biological effect (measured in blood and urine) and physiological assessments related to CVD among exclusive cigarette smokers (SMK), exclusive moist snuff (i.e. a type of ST) consumers (MSC) and non-consumers of tobacco (NTC). Biomarkers of tobacco and tobacco smoke exposure (BoE) were additionally evaluated and are reported here along with a description of the study design and conduct. Comparisons of biological effect biomarkers and physiological assessments are reported elsewhere (Marano et al., 2015; Nordskog et al., 2015).

## Methods

### Study design and participants

An age-stratified, cross-sectional study was conducted between September 2008 and February 2009 at a single clinical research unit in Lincoln, NE. The study was managed by Celerion (formerly MDS Pharma Services) (ClinicalTrials.gov; identifier: NCT01692353). The study was approved by the MDS Pharma Services Institutional Review Board and was conducted in accordance with Good Clinical Practice, the Declaration of Helsinki and applicable sections of the United States Code of Federal Regulations (21 CFR Parts 50, 54 and 56). All participants provided written informed consent at screening and prior to any study procedures being performed. Participants were free to withdraw from the study at any time and were compensated for their time and participation.

Generally healthy male participants, aged 26–49 years (inclusive), were recruited via radio, print ads, Celerion's website and phone calls to Celerion's database of smokers residing in Lincoln and Omaha, NE. The target completion for this study was 60 participants per group (SMK, MSC, NTC), and 15 participants per age stratum (26–31, 32–37, 38–43 and 44–49 years), for 180 total participants. SMK had smoked at least 15 cigarettes per day with mainstream "tar" yields >6.0 mg, as measured by the Cambridge Filter Method [Federal Trade Commission (FTC), 2008], for at least 3 years prior to study screening and had expired carbon monoxide (ECO) levels between 10 and 100 parts per million (ppm). During the 6 months prior to study enrollment, SMK were required to have exclusively smoked cigarettes and not to have used any other form of tobacco or nicotine replacement

therapy (NRT). Additionally, SMK had limited lifetime usage of other types of tobacco (i.e. <10 cigars, <10 pipes and <10 packs/tins of ST, lifetime). MSC reported using at least two cans of moist snuff (any cut, any style) per week for at least 3 years prior to study screening and had ECO levels  $\leq 5$  ppm. During the 6 months prior to study enrollment, MSC were required to use moist snuff exclusively and not to have used any other form of tobacco or NRT. Additionally, MSC had limited lifetime usage of other types of tobacco (i.e. <20 packs of cigarettes, <10 cigars, <10 pipes and <10 packs/tins of any other ST, lifetime). NTC had limited lifetime usage of tobacco (i.e. <20 packs of cigarettes, <20 cans/packs of any ST, <50 cigars and <50 pipes of tobacco, at time of screening), had never used NRT and had ECO levels  $\leq 5$  ppm. All tobacco products used in this study (i.e. study product) were the participants' usual brand (UB) and were supplied by the subject.

### Study conduct

Eligible participants were admitted to the clinic between 12:00 p.m. and 5:00 p.m. on Day 1. After Day 1 check-in, participant eligibility was reconfirmed and all participants completed relevant health-related questionnaires, including the American Thoracic Society ATS-DLD-78, the Smoking Cessation Quality of Life (SCQoL, including SF-36v2<sup>®</sup>), the National Cancer Institute Diet History Questionnaire (DHQ) and a 1-day diary. In addition, SMK completed the Fagerström Test for Nicotine Dependence (FTND) and MSC completed the FTND-ST. Participants were allowed to use their UB tobacco products *ad libitum* after check-in and before they observed a 45-min tobacco abstinence period. This period of abstinence was followed by a "challenge" (i.e. the use of one unit of UB tobacco product), which for SMK was smoking one UB cigarette in the usual manner and for MSC was using a typical portion of UB moist snuff for 30 min. At 15-min post-challenge, blood and urine were collected, and spirometry (SPIRO), ECO and ankle brachial index (ABI) were measured sequentially. At 30-min post-challenge use, flow-mediated dilation (FMD) was measured. For NTC, completion of study questionnaires served as the reference point for collection of the blood and urine, and ECO, ABI and FMD measurements. After challenge and subsequent testing, participants were allowed to use their UB product *ad libitum* until the start of the overnight tobacco abstinence period, which began at ~9.00 p.m. Participants were confined overnight. On Day 2, blood and urine were collected, and SPIRO, ECO, ABI, FMD and carotid intima-media thickness (CIMT) were measured sequentially. Participants were discharged approximately at noon on Day 2. Only Day 1 BoE results are presented here.

### Biomarkers and analytical methods

Blood and spot urine were analyzed for various biomarkers using validated analytical methods with appropriate quality controls. The tobacco and tobacco smoke BoE and corresponding analytical methods are presented in Tables 1 and 2. Urinary biomarker analyses were performed at Analytisch Biologisches Forschungslabor (ABF, Munich, Germany), except for creatinine, which was analyzed at Celerion. The blood biomarkers nicotine, cotinine and

Table 1. Urinary biomarkers of exposure and bioanalytical methods.

Biomarker <sup>a</sup>	Abbreviation	Chemical Constituent	Method	LLOQ	LOD
Nicotine equivalents	NicEq	Nicotine	NA		
Unconjugated nicotine	NIC-U	Nicotine	LC-MS/MS	10 mg mL <sup>-1</sup>	5 ng mL <sup>-1</sup>
Unconjugated cotinine	COT-U	Nicotine	LC-MS/MS	7.4 ng mL <sup>-1</sup>	2.5 ng mL <sup>-1</sup>
Unconjugated trans-3'-hydroxycotinine	OHCOT-U	Nicotine	LC-MS/MS	12.3 ng mL <sup>-1</sup>	4.1 ng mL <sup>-1</sup>
Nicotine-glucuronide	NIC-G	Nicotine	LC-MS/MS	10 ng mL <sup>-1</sup>	3 ng mL <sup>-1</sup>
Cotinine-glucuronide	COT-G	Nicotine	LC-MS/MS	25 ng mL <sup>-1</sup>	10 ng mL <sup>-1</sup>
Trans-3'-hydroxycotinine-glucuronide	OHCOT-G	Nicotine	LC-MS/MS	21.6 ng mL <sup>-1</sup>	7.2 ng mL <sup>-1</sup>
Cotinine-N-oxide	CNO	Nicotine	LC-MS/MS	10 ng mL <sup>-1</sup>	4 ng mL <sup>-1</sup>
Nicotine-N-oxide	NNO	Nicotine	LC-MS/MS	10 ng mL <sup>-1</sup>	5 ng mL <sup>-1</sup>
Norcotinine	NCOT	Nicotine	LC-MS/MS	10 ng mL <sup>-1</sup>	3 ng mL <sup>-1</sup>
Nornicotine	NNIC	Nicotine	LC-MS/MS	10 ng mL <sup>-1</sup>	4 ng mL <sup>-1</sup>
Total 4-(methylnitrosamino)-1(3-pyridyl)-butanol	NNAL-T	NNK	LC-MS/MS	5 pg mL <sup>-1</sup>	2 pg mL <sup>-1</sup>
Unconjugated 4-(methylnitrosamino)-1(3-pyridyl)-butanol	NNAL-U	NNK	LC-MS/MS	5 pg mL <sup>-1</sup>	2 pg mL <sup>-1</sup>
4-(Methylnitrosamino)-1(3-pyridyl)-butanol glucuronide	NNAL-G <sup>b</sup>	NNK	Calculated	NA	NA
3-Hydroxypropylmercapturic acid	3-HPMA	Acrolein	LC-MS/MS	20 ng mL <sup>-1</sup>	6.2 ng mL <sup>-1</sup>
5-phenylmercapturic acid	5-PMA	Benzene	LC-MS/MS	0.05 ng mL <sup>-1</sup>	0.03 ng mL <sup>-1</sup>
Monohydroxybutenylmercapturic acid	MHBMA	1,3-Butadiene	LC-MS/MS	0.10 ng mL <sup>-1</sup>	0.03 ng mL <sup>-1</sup>
3-Hydroxy-1-methylpropyl-mercapturic acid	HMPMA	Crotonaldehyde	LC-MS/MS	52 ng mL <sup>-1</sup>	17 ng mL <sup>-1</sup>
total 1-hydroxypyrene	1-OHP-T	Pyrene	GC-MS/MS	0.01 ng mL <sup>-1</sup>	0.003 ng mL <sup>-1</sup>
<i>o</i> -Toluidine	<i>o</i> -T	<i>o</i> -Toluidine	GC-MS	2.5 ng L <sup>-1</sup>	0.8 ng L <sup>-1</sup>
2-Aminonaphthalene	2-AN	2-Aminonaphthalene	GC-MS	1.7 ng L <sup>-1</sup>	0.6 ng L <sup>-1</sup>
3-Aminobiphenyl	3-ABP	3-Aminobiphenyl	GC-MS	1.3 ng L <sup>-1</sup>	0.5 ng L <sup>-1</sup>
4-Aminobiphenyl	4-ABP	4-Aminobiphenyl	GC-MS	1.5 ng L <sup>-1</sup>	0.5 ng L <sup>-1</sup>
<i>N</i> -Acetyl- <i>S</i> -(2-carbamoyl)ethyl)cysteine	AAMA	Acrylamide	LC-MS/MS	4.1 ng mL <sup>-1</sup>	1.2 ng mL <sup>-1</sup>
<i>N</i> -acetyl- <i>S</i> -(2-hydroxy-2-carbamoyl)ethyl)cysteine	GAMA	Acrylamide	LC-MS/MS	1.0 ng mL <sup>-1</sup>	0.3 ng mL <sup>-1</sup>
Creatinine	CRE	Creatinine	Picric acid assay	13.1 mg dL <sup>-1</sup>	NA

<sup>a</sup>Analyzed at Analytisch Biologisches Forschungslabor (ABF, Munich, Germany) except for creatinine, which was analyzed at Celerion (formerly MDS Pharma, Lincoln, NE).

<sup>b</sup>Calculated as NNAL-T-NNAL-U

LLOQ, lower limit of quantitation; NA, not applicable; LC-MS/MS, liquid chromatography-tandem mass spectrometry; GC-MS, gas chromatography-mass spectrometry.

Table 2. Blood biomarkers of exposure and analytical methods.

Biomarker <sup>a</sup>	Abbreviation	Chemical constituent	Matrix	Method	LLOQ	LOD
Nicotine	NIC-U	Nicotine	Serum	LC-MS/MS	2.00 ng mL <sup>-1</sup>	NA
Cotinine	COT-U	Nicotine	Serum	LC-MS/MS	20.00 ng mL <sup>-1</sup>	NA
Carboxyhemoglobin	%COHb	Carbon monoxide	Whole blood	Spectrophotometric	0.1 mg dL <sup>-1</sup>	NA

<sup>a</sup>Analyzed at Celerion (formerly MDS Pharma, Lincoln, NE).

LLOQ, lower limit of quantitation; NA, not applicable; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

carboxyhemoglobin were determined at Celerion. For nicotine plus nine metabolites, quantification was performed by modification of the “direct” method (Meger et al., 2002). That is, each nicotine metabolite was converted to an equivalent mass of nicotine using a metabolite-specific molar ratio. Subsequently, converted masses of each metabolite were summed to total nicotine equivalents (NicEq). The analysis for 4-(methylnitrosamino)-1(3-pyridyl)-butanol (NNAL), the urinary biomarker for the tobacco-specific nitrosamine 4-(methylnitrosamino)-1(3-pyridyl)-1-butanone (NNK), was performed by the “indirect” method (Byrd & Ogden, 2003). All urine biomarkers were normalized to creatinine to reduce variability resulting from spot urine collection using the conventional creatinine-ratio-normalization technique (i.e. mass per milliliter converted to mass per milligram of creatinine) (Heavner et al., 2006). ECO levels (ppm) were

measured using the Micro IV Smokerlyzer<sup>®</sup> Breath Carbon Monoxide Monitor (Bedfont Scientific Ltd, Haddonfield, NJ).

### Statistical methods

An analysis of variance (ANOVA) model using least squares (LS) means was used to compare urine and blood biomarkers among SMK, MSC and NTC. Age stratum was included in the model as a classification variable, because age is a presumed confounder of tobacco exposure and CVD outcomes. Tobacco group, age stratum, and the interaction between group and age were fixed effects in the model, and the SAS<sup>®</sup> PROC MIXED procedure was used to perform the analyses. If the interaction between group and age was not statistically significant, the main effect was used to interpret the group results. If the interaction was significant, the simple

Table 3. Demographic summary statistics, SMK, MSC and NTC.

<i>n</i>	SMK 60	MSC 48	NTC 60
Race			
African-American	2	1	6
Caucasian	57	47	45
Hispanic	0	0	5
Other <sup>a</sup>	1	0	4
Age			
Mean (SD)	37.2 (6.9)	36.5 (6.3)	36.9 (7.0)
Min, max	26, 48	26, 49	26, 48
Weight (lbs)			
Mean (SD)	197.0 (35.9)	214.1 (37.5)	194.8 (28.8)
Min, max	124.0, 300.4	149.0, 333.6	138.6, 284.6
Height (inch)			
Mean (SD)	70.6 ( )	70.7 (2.2)	70.3 (2.3)
Min, max	65.5, 77.0	64.5, 75.0	65.8, 75.0
BMI (kg/m <sup>2</sup> )			
Mean (SD)	27.8 (4.9)	30.1 (5.0)	27.8 (4.5)
Min, max	19.1, 39.3	21.5, 49.3	19.9, 46.3
COPD status ( <i>n</i> )			
FEV1/FVC <70	8	1	4
FEV1/FVC ≥70	52	47	56

<sup>a</sup>American Indian/Alaskan Native, Asian/Pacific Islander and European/Middle Eastern. Min, minimum; max, maximum; FEV1/FVC, the forced expiratory volume in 1 s/the forced vital capacity; SD, standard deviation.

(i.e. group-by-age) effects were used to interpret the results. LS means by age stratum are not presented except when necessary to interpret simple effects. Statistical significance was indicated at  $p \leq 0.05$ , and observed  $p$  values were adjusted for multiple comparisons using the Bonferroni method. Biomarker values reported as below the limit of detection (LOD) for individual metabolites were replaced with one-half of the LOD for the statistical analysis.

## Results

### Demographics and product usage

One hundred sixty-eight males were enrolled and completed the study (SMK,  $n = 60$ ; MSC,  $n = 48$ ; NTC,  $n = 60$ ). Demographics and tobacco product usage are summarized in Tables 3–5, respectively. The majority of participants in all three groups were Caucasian, and the mean body mass index (BMI) values within each age stratum and tobacco group were all  $>26$ , suggesting that approximately half of the participants would have been considered overweight (i.e. BMI  $>25$ ). Mean years of tobacco product use within age stratum were generally consistent between SMK and MSC. More broadly, for the SMK group, smoking years ranged from 7 to 33 years, and the MSC group ranged in moist snuff use from 7 to 29 years. FTND scores were generally similar between SMK and MSC within age stratum, ranging between 4.5 and 5.9, overall.

### Adverse events

Seventeen of the 168 subjects reported 25 adverse events (AEs) during the confinement period (for SMK, 5 subjects and 8 AEs; for MSC, 9 subjects and 10 AEs; for NTC, 3 subjects and 7 AEs). None of the AEs were considered serious (i.e. a serious adverse event, SAE) by the Principal Investigator (PI). All AEs were mild (20) or moderate (4) in severity, with the exception

of a single severe case of lightheadedness. The PI considered 5 of the 25 AEs (e.g. pain in extremity, hyperhidrosis) not to be related to study product (i.e. the subject's UB of cigarettes or moist snuff) and the remaining 20 AEs to be possibly related. Headache (11) and lightheadedness (dizziness) (5) were the most commonly reported AEs. Headache was reported in 11 subjects, with eight episodes considered mild in severity. All but one of the episodes of headache was considered possibly related to study product. Dizziness was reported by a total of four subjects, with three episodes considered mild in severity, one considered moderate in severity and one considered severe. The PI initially considered four of the five episodes of lightheadedness possibly related to study product. However, a review of the source data regarding these episodes revealed that three episodes of lightheadedness occurred during or immediately after venipuncture procedures, and one of these subjects was in the NTC group. Therefore, these episodes were deemed related to the study procedure and not study product use. No subjects withdrew from the study due to an AE. All AEs were followed to resolution regardless of whether the subject was still on-study or had completed the study.

### Biomarkers

Concentrations of urinary BoE are presented in Table 6 and blood BoE concentrations are presented in Table 7. Both SMK and MSC had statistically significantly greater serum unconjugated nicotine (NIC-U) levels ( $p < 0.001$ ) and urine NicEq ( $p < 0.0001$ ) than NTC. For nicotine (NicEq), the age main effect and age-by-group interactions were statistically significant. Further analysis indicated that in the 44–49-year age group, NicEq was statistically significantly greater in MSC compared with SMK [LS means  $\pm$  standard error (SE) of  $18.74 \pm 1.86$  for MSC,  $9.53 \pm 1.31$  for SMK and  $0.04 \pm 1.27$  for NTC, units in picogram per milligram CRE]. For serum NIC-U, MSC and SMK were not statistically significantly different ( $p = 0.07$ ). Similarly, a statistically significant age main effect and age-by-group interactions were detected for unconjugated cotinine (COT-U), and further analysis indicated that levels were statistically significantly greater in MSC compared to SMK in the 38–43 years ( $p = 0.0102$ ) and 44–49 years ( $p < 0.0001$ ) age strata.

Concentrations of urinary NNAL in MSC were statistically significantly greater than the levels measured in both SMK and NTC, and were statistically significantly greater in SMK compared with NTC (LS means  $\pm$  SE of  $1594 \pm 85.6$  for MSC,  $656 \pm 72.9$  for SMK and  $7.94 \pm 72.9$  for NTC, units in picogram per milligram CRE). Biomarkers for the combustion compounds benzene (S-PMA), acrolein (3-HPMA), pyrene (1-OHP-T), 1,3-butadiene (MHBMA), crotonaldehyde (HMPMA), aromatic amines (*o*-T, 2-AN, 4-ABP and 3-ABP), acrylamide (AAMA and GAMA) and carbon monoxide (%COHb) were statistically significantly (Tables 6 and 7) greater in SMK compared to both MSC and NTC. Levels of these compounds were not statistically significantly different between MSC and NTC.

### Discussion

A continuum of risk exists among tobacco products, with combustible products associated with the greatest risk of

Table 4. Demographic summary statistics by age stratum, SMK, MSC and NTC.

Age (years) <i>n</i>	SMK			MSC			NTC					
	26-31 15	32-37 15	38-43 15	44-49 15	26-31 14	32-37 12	38-43 15	44-49 7	26-31 15	32-37 15	38-43 15	44-49 15
Race												
African-American	1	0	0	1	1	0	0	0	2	1	2	1
Caucasian	14	14	15	14	13	12	15	7	7	12	12	14
Hispanic	0	0	0	0	0	0	0	0	4	1	0	0
Other <sup>a</sup>	0	1	0	0	0	0	0	0	2	1	1	0
Age												
Mean (SD)	28 (1.4)	34.3 (1.9)	40.7 (1.5)	45.7 (1.4)	28.7 (1.8)	35 (1.6)	40.7 (1.7)	45.9 (2.2)	27.6 (1.4)	34.3 (1.7)	39.5 (1.5)	46.1 (1.3)
Min, max	26, 31	32, 37	38, 43	44, 48	26, 31	32, 37	38, 43	44, 49	26, 31	32, 37	38, 42	44, 48
Weight (lbs)												
Mean (SD)	197.2 (32.0)	196.7 (26.1)	205.7 (44.8)	188.4 (39.5)	214.0 (39.7)	217.7 (34.0)	216.5 (43.3)	203.2 (30.2)	187.2 (31.4)	200.2 (35.2)	191.7 (14.8)	200.1 (30.4)
Min, max	136.8, 251.6	158.0, 259.2	124.0, 269.0	127.4, 300.4	149.0, 273.2	158.0, 259.8	152.0, 333.6	153.8, 244.2	138.6, 232.8	156.4, 284.6	163.8, 225.2	155.2, 252.4
Height (inch)												
Mean (SD)	70.4 (2.3)	71.2 (2.4)	69.9 (2.3)	71.1 (2.4)	70.8 (2.0)	69.8 (2.7)	71.1 (2.4)	70.9 (1.6)	70.6 (2.4)	70.2 (2.3)	70.6 (2.0)	70.0 (2.6)
Min, max	66.5, 73.3	68.0, 76.8	65.5, 74.0	68.5, 77.0	67.5, 73.8	64.5, 73.3	67.8, 75.0	69.0, 73.0	67.0, 75.0	65.8, 74.8	67.5, 73.8	66.0, 73.8
BMI (kg/m <sup>2</sup> )												
Mean (SD)	28.0 (4.9)	27.4 (4.3)	29.6 (6.1)	26.1 (4.2)	29.9 (4.4)	31.2 (3.4)	30.3 (6.8)	28.4 (3.8)	26.4 (4.1)	28.8 (6.3)	27.1 (2.5)	28.7 (4.1)
Min, max	19.9, 39.1	22.6, 38.3	19.7, 39.3	19.1, 35.7	23.0, 35.7	24.5, 35.3	22.5, 49.3	21.5, 32.2	19.9, 33.0	22.3, 46.3	22.7, 31.1	22.2, 36.8
COPD status ( <i>n</i> )												
FEV1/FVC <70	1	1	4	2	0	0	1	0	0	0	2	2
FEV1/FVC ≥70	14	14	11	13	14	12	14	7	15	15	13	13

<sup>a</sup>American Indian/Alaskan Native, Asian/Pacific Islander and European/Middle Eastern. SD, standard deviation.

Table 5. Summary of tobacco product use, SMK and MSC.

	SMK				MSC			
	26–31	32–37	38–43	44–49	26–31	32–37	38–43	44–49
Age (years)								
<i>n</i>	15	15	15	15	14	12	15	7
Years of product use								
Mean (SD)	10.9 (2.1)	17.5 (3.8)	21.1 (3.9)	27.0 (5.1)	12.0 (3.2)	18.3 (5.8)	24.3 (2.9)	26.3 (3.3)
Min, max	7, 15	10, 24	14, 27	11, 33	7, 18	4, 28	18, 29	20, 29
Product use <sup>a</sup>								
Mean (SD)	20.5 (5.7)	19.3 (4.8)	19.2 (4.2)	24.3 (8.4)	4.6 (1.9)	4.4 (1.7)	3.8 (1.2)	3.9 (2.0)
Min, max	15, 40	10.5, 30	15, 30	15, 40	2, 7	2, 7	2, 7	2, 7
Product use, last 24 h <sup>b</sup>								
Mean (SD)	18.0 (3.2)	15.1 (5.4)	19.8 (5.5)	21.9 (9.1)	1.0 (0.3)	1.0 (0.1)	1.1 (0.3)	0.9 (0.2)
Min, max	11.5, 24	2, 21	10, 30	10, 40	0.5, 2	0.5, 1	0.5, 2	0.5, 1
FTND <sup>c</sup> score								
Mean (SD)	4.5 (1.8)	5.3 (1.6)	4.7 (1.6)	5.9 (2.1)	5.4 (2.0)	5.8 (1.9)	5.3 (2.2)	5.6 (1.2)
Min, max	2, 8	2, 8	2, 7	3, 10	2, 9	3, 9	2, 9	4, 7

<sup>a</sup>Cigarettes per day for SMK, tins per week for MSC.

<sup>b</sup>Number of cigarettes for SMK and number of tins for MSC reported on 1-day diary.

<sup>c</sup>SMK completed the Fagerström test for nicotine dependence, MSC completed the Fagerström test for nicotine dependence-ST. SD, standard deviation.

Table 6. Summary of urine BoE comparisons.

Chemical constituent	Biomarker	Age (years)	LS means $\pm$ SE <sup>a</sup>			Group comparison <i>p</i> value <sup>b</sup>		
			SMK	MSC	NTC	SMK versus MSC	SMK versus NTC	MSC versus NTC
Nicotine	NicEq ( $\mu\text{g mg}^{-1}$ CRE) <sup>c</sup>	26–31	8.22 $\pm$ 1.27	7.92 $\pm$ 1.36	0.06 $\pm$ 1.27	1.0000	<0.0001	<0.0001
		32–37	8.03 $\pm$ 1.27	11.6 $\pm$ 1.42	0.04 $\pm$ 1.31	0.1908	<0.0001	<0.0001
		38–43	7.73 $\pm$ 1.27	10.49 $\pm$ 1.31	0.06 $\pm$ 1.27	0.3957	<0.0001	<0.0001
		44–49	9.53 $\pm$ 1.31	18.74 $\pm$ 1.86	0.04 $\pm$ 1.27	0.0003	<0.0001	<0.0001
NNK	NNAL-T ( $\text{pg mg}^{-1}$ CRE)		656 $\pm$ 72.9	1594 $\pm$ 85.6	7.94 $\pm$ 72.9	<0.0001	<0.0001	<0.0001
Benzene	5-PMA ( $\text{ng mg}^{-1}$ CRE)		2.63 $\pm$ 0.19	0.51 $\pm$ 0.23	0.46 $\pm$ 0.19	<0.0001	<0.0001	1.0000
Acrolein	3-HPMA ( $\text{ng mg}^{-1}$ CRE)		1824 $\pm$ 73.7	160 $\pm$ 85.7	145 $\pm$ 74.3	<0.0001	<0.0001	1.0000
1-Hydroxypyrene	1-OHP-T ( $\text{ng mg}^{-1}$ CRE)		0.22 $\pm$ 0.01	0.08 $\pm$ 0.02	0.05 $\pm$ 0.01	<0.0001	<0.0001	0.5439
1,3-Butadiene	MHBMA ( $\text{ng mg}^{-1}$ CRE)		4.37 $\pm$ 0.29	0.45 $\pm$ 0.34	0.72 $\pm$ 0.29	<0.0001	<0.0001	1.0000
Crotonaldehyde	HMPMA ( $\text{ng mg}^{-1}$ CRE)		7229 $\pm$ 263	598 $\pm$ 309	660 $\pm$ 263	<0.0001	<0.0001	0.8787
<i>o</i> -Toluidine	<i>o</i> -T ( $\text{pg mg}^{-1}$ CRE)		193 $\pm$ 7.15	43.3 $\pm$ 8.84	41.2 $\pm$ 7.16	<0.0001	<0.0001	1.0000
2-Aminonaphthalene	2-AN ( $\text{pg mg}^{-1}$ CRE)		31.2 $\pm$ 1.16	1.99 $\pm$ 1.44	1.23 $\pm$ 1.16	<0.0001	<0.0001	0.6813
4-Aminobiphenyl	4-ABP ( $\text{pg mg}^{-1}$ CRE)		21.4 $\pm$ 0.93	2.74 $\pm$ 1.15	2.43 $\pm$ 0.93	<0.0001	<0.0001	0.8307
3-Aminobiphenyl	3-ABP ( $\text{pg mg}^{-1}$ CRE)		11.1 $\pm$ 0.50	0.6 $\pm$ 0.62	0.66 $\pm$ 0.50	<0.0001	<0.0001	0.9412
Acrylamide	AAMA ( $\text{ng mg}^{-1}$ CRE)		221 $\pm$ 8.65	106 $\pm$ 10.1	78.0 $\pm$ 8.73	<0.0001	<0.0001	0.1199
Acrylamide	GAMA ( $\text{ng mg}^{-1}$ CRE)		35.1 $\pm$ 1.08	16.1 $\pm$ 1.26	15.8 $\pm$ 1.09	<0.0001	<0.0001	1.0000

<sup>a</sup>Least squares means  $\pm$  SE.

<sup>b</sup>Bonferroni-adjusted. *p* values that exceeded 1.000 after adjustment are reported as 1.000.

<sup>c</sup>Significant group-by-age interaction (*p* < 0.05).

Table 7. Summary of blood BoE comparisons.

Chemical constituent	Biomarker	Age (years)	LS Means $\pm$ SE <sup>a</sup>			Group comparison <i>p</i> value <sup>b</sup>		
			SMK	MSC	NTC	SMK versus MSC	SMK versus NTC	MSC versus NTC
Carbon monoxide	COHb (% saturated)		7.88 $\pm$ 0.17	1.12 $\pm$ 0.20	1.06 $\pm$ 0.17	<0.0001	<0.0001	0.7960
Nicotine	NIC-U ( $\text{ng mL}^{-1}$ )		31.5 $\pm$ 0.99	28.7 $\pm$ 1.15	0.27 $\pm$ 0.99	0.0709	<0.0001	<0.0001
Nicotine	COT-U ( $\text{ng mL}^{-1}$ ) <sup>c</sup>	26–31	323 $\pm$ 45.3	466 $\pm$ 46.9	1.20 $\pm$ 45.3	0.0870	<0.0001	<0.0001
		32–37	352 $\pm$ 45.3	466 $\pm$ 50.6	0.11 $\pm$ 45.3	0.2826	<0.0001	<0.0001
		38–43	363 $\pm$ 45.3	554 $\pm$ 45.3	0.25 $\pm$ 45.3	0.0102	<0.0001	<0.0001
		44–49	339 $\pm$ 45.3	773 $\pm$ 66.3	0.11 $\pm$ 45.3	<0.0001	<0.0001	<0.0001

<sup>a</sup>Least squares means  $\pm$  SE.

<sup>b</sup>Bonferroni-adjusted. *p* values that exceeded 1.000 after adjustment are reported as 1.000.

<sup>c</sup>Significant group-by-age interaction (*p* < 0.05).

disease (Nutt et al., 2014; Zeller, 2009). Reducing the risk of diseases attributed to conventional cigarette smoking may be possible with the reduction in exposure to harmful or potentially harmful tobacco and tobacco smoke constituents [Institute of Medicine (IOM), 2011; Stratton et al., 2001]. BoE are useful for the determination of actual consumer exposures to constituents when using one tobacco product versus another (Gregg et al., 2013; Hatsukami et al., 2006; IOM, 2011; Stratton et al., 2001).

More than 90 constituents found in tobacco and tobacco smoke have been identified as harmful or potentially harmful [Food and Drug Administration (FDA), 2012]. Specific to cardiovascular toxicity, acrolein, benzene and certain polycyclic aromatic hydrocarbons have been identified (FDA, 2012). In addition, carbon monoxide and 1,3-butadiene have been suggested to be potential contributors to the mechanisms for increased risk of CVD in cigarette smokers (SMK) (Benowitz, 2003; USDHHS, 2010). Nicotine has also been proposed as a contributor, although this potential relationship may not be supported based on the lack of evidence of increased risk for CVD among smokers who used NRT (Benowitz, 2003; USDHHS, 2010) and users of Swedish snus (ST) (Hansson et al., 2012, 2014).

Results from the study reported here generally indicated a reduction in exposure to tobacco and tobacco smoke BoE among MSC compared with SMK, with levels in MSC similar to those among NTC. Findings from previous studies have also indicated statistically significantly higher tobacco and tobacco smoke BoE in SMK compared with non-smokers (Calapai et al., 2009; Heudorf & Angerer, 2001; Lowe et al., 2009; Nan et al., 2001; Naufal et al., 2011; Roethig et al., 2009; Scherer, 2005; Vesper et al., 2010). In addition, based on data from the National Health and Nutrition Examination Survey (NHANES), few statistically significant differences in tobacco and tobacco smoke BoE were reported among ST consumers compared with consumers not using tobacco (Naufal et al., 2011).

Specific to constituents potentially associated with cardiovascular toxicity, biomarkers of acrolein, benzene, pyrene, carbon monoxide and 1,3-butadiene were similarly all statistically significantly decreased in MSC versus SMK and were not statistically significantly different in comparisons of MSC and NTC. For nicotine, a significant age-by-cohort interaction was detected for both urinary NicEq and serum COT-U. No statistically significant age-related differences in nicotine systemic exposure (*p* values not reported) were observed in SMK and NTC; however, the statistically significant increases in serum COT-U in MSC compared with SMK in the oldest two age groups are consistent with findings from previous studies (Hecht et al., 2007; Naufal et al., 2011) and is possibly due to route of exposure differences (i.e. oral versus inhalation exposure).

In contrast to the majority of BoE reductions, the urinary NNAL level in MSC was statistically significantly greater compared to SMK overall. These results are consistent with previous findings, which have indicated that NNK is metabolized to NNAL to a greater extent (~3- to 4-fold) in consumers of ST compared to SMK (Hecht et al., 2007, 2008; Stepanov & Hecht, 2005). Additionally, data from NHANES (2007–2008) indicated that NNAL concentrations

were 4.7- to 6.0-fold higher in ST consumers than in SMK (Naufal et al., 2011). Results from the current study showed that urinary creatinine-adjusted NNAL levels were ~2.5-fold higher in MSC than SMK, similar to the ~2-fold higher levels of NNAL in ST consumers versus SMK reported by Hecht et al. (2007). The increased systemic exposure to the carcinogen NNK [International Agency for Research on Cancer (IARC), 2007] among ST users is not consistent with epidemiological findings, which do not show a statistically significant increase in the risk of cancer among ST users in studies that appropriately controlled for confounding factors (Lee & Hamling, 2009).

A limitation of this study is the exclusion of female subjects. Females were excluded due to the low potential recruitment pool for the MSC group, and because of the complications in data interpretation related to between-gender and within-gender differences (especially during menstrual cycles) in biomarkers of inflammation such as the interleukins and C-reactive protein (Jilma et al., 1997). Strengths of this study include the relative exclusivity of the SMK, MSC and NTC groups and the extensive number of BoE evaluated. BoE results distinguish between SMK, MSC and NTC based on product use, and quantify the differences in exposures to individual tobacco and tobacco smoke constituents. The observed patterns of systemic exposure to many harmful and potentially harmful constituents, including constituents that may be relevant to the development of CVD, are consistent with the different risks for CVD associated with the use of combusted and non-combusted tobacco product categories observed in epidemiological studies (Henley et al., 2005; Thun et al., 2000). Ultimately, these findings support variations in systemic exposure with the use of different tobacco products and are relevant to the understanding of a tobacco product risk continuum among combustible and non-combustible tobacco products.

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## Declaration of interest

All authors are current or former employees of R.J. Reynolds Tobacco Company and RAI Services Company and report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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