G.L. Prasad*, Bobbette A. Jones, Peter Chen and Evan O. Gregg

A cross-sectional study of biomarkers of exposure and effect in smokers and moist snuff consumers

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Abstract

Background: Cigarette smoking is a major risk factor for several chronic diseases. Epidemiological data indicate the use of smokeless tobacco (ST) is associated with significantly lower risk for smoking-related diseases compared to cigarettes. Several biomarkers of exposure (BioExp) and effect (BioEff) associated with smoking and use of moist snuff (ST) were evaluated.

Methods: A single site, cross-sectional clinical study enrolled three groups of generally healthy male smokers (SMK), moist snuff consumers (MSC), and non-tobacco consumers (NTC), and several BioExp and BioEff were evaluated.

Results: Blood and urinary BioExp, including total nicotine equivalents and tobacco-specific nitrosamines, were higher in MSC compared to SMK. Biomarkers of combustion-related toxicants and cadmium were elevated in SMK. Elevated levels of some BioEff associated with oxidative stress (urinary isoprostanes and leukotriene E4), inflammation (white blood cell count), platelet activation (thromboxane metabolites), and lipid metabolism (apolipoprotein B100 and oxidized low-density lipoprotein) were observed in SMK relative to NTC and MSC (all $p<0.05$). The non-smoking groups (MSC and NTC) showed similar levels of combustion-related BioExp and BioEff.

Conclusions: Higher levels of exposure to nicotine and some N′-nitrosamines may be observed in MSC, and SMK are exposed to higher levels of combustion-related toxicants. Changes in BioEff consistent with some aspects of inflammation, oxidative stress, and altered lipid metabolism were detected in SMK compared to the non-smoking groups. The biomarker data further improve our understanding of pathophysiological changes and the risk continuum associated with various tobacco products, and could be useful components of future assessments of tobacco products.

Keywords: biological effect; biomarkers; exposure; moist snuff; smoking.

Introduction

Cigarette smoke is a complex aerosol containing more than 8000 different constituents [1], of which about 69 are known or probable human carcinogens [2, 3]. This dynamic, reactive mixture consists of gas-vapor and particulate phases. The constituents of each phase contribute to the diverse biological properties of cigarette smoke [3]. Cigarette smoke may exert direct effects at local areas of exposure (e.g. within the oral cavity and lungs), as well as eliciting systemic responses, and consequently could impact diverse physiological processes [4, 5].

Smokeless tobacco (ST) is non-combustible tobacco that comes in many forms and is consumed globally. Fermented moist snuff, or dipping tobacco, is the traditional form and the most widely consumed ST product category in the USA [6]. It is made primarily from dark air- and fire-cured tobaccos with moisture content that is typically near 50% of the product weight [6].

The long-term risks of smoking have been thoroughly documented, particularly with regard to the development of cancer, respiratory diseases such as chronic obstructive pulmonary disease (COPD), and cardiovascular disease (CVD) [3, 7, 8]. Existing epidemiological data show that ST consumption is associated with significantly less risk of adverse health effects than smoking [9]. For example, the incidence rates of lung cancer, COPD, CVD, and oral cancers are significantly reduced in ST users relative to cigarette smokers [10–16]. However, the mortality rates of some diseases, such as CVD, are elevated in ST users, relative to non-tobacco users [17], and ST users may be at a somewhat higher risk for fatal myocardial infarction and fatal stroke [18]. It has been suggested that consumers of various tobacco products can be placed on a risk continuum,

***Corresponding author: G.L. Prasad,** R.J. Reynolds Tobacco Company, P.O. Box 1487, Winston-Salem, NC 27102, USA, Phone: +1 336 741 7562, Fax: +1 336 741 2841, E-mail: prasadg@RJRT.com
Bobbette A. Jones and Peter Chen: R.J. Reynolds Tobacco Company, Winston-Salem, NC, USA
Evan O. Gregg: ENI Limited, Towcester, UK

with cigarette smokers at the upper end (i.e. higher risk for the lung cancer, COPD, and CVD) and ST consumers lower on this risk continuum [9, 16].

Biomarkers of effect (BioEff) offer the prospect of measuring biological change following exposure, and these effects may also be used to differentiate between smoking, ST consumption, and tobacco abstinence; several such BioEff have been suggested [3, 11, 19].

Several biomarkers of exposure (BioExp) such as cotinine and other nicotine metabolites, N′-nitrosamines and their metabolites, and the mercapturic acid metabolites of several gas-vapor-phase carbonyl compounds have been used to assess exposure to cigarette smoke [20–23]. Further, long-term cigarette smoking may affect multiple aspects of physiology [3]. Thus, a diverse array of BioEff, which measures physiological changes following tobacco-product use, has been proposed to assess the short-term effects of smoking and for the comparative evaluation of tobacco products [21, 22, 24–27].

Some BioEff that are related to platelet activation, endothelial function, blood coagulation pathways, and lipid metabolism have been found to differentiate smokers from non-smokers [21, 24–27]. In contrast, limited information exists on whether smokers and moist snuff consumers (MSC) would differ in terms of their overall biomarker profiles or whether MSC and non-tobacco consumers (NTC) exhibit different biomarker levels. Many of the putative BioEff offer the potential to be used in comparative studies of combustible cigarettes and ST use. Additional pathways, such as those of inflammation and oxidative stress, are also of interest [4, 26, 28, 29], and they might be useful in further differentiating cigarette smokers from ST users. In efforts to identify potential biomarkers, we have conducted several cross-sectional biomarker studies. In a recently published series of papers [30–32], we presented results from a cross-sectional study that was aimed at evaluating several markers of CVD. The biomarker data were obtained from adult male participants, stratified into four age groups from 26 to 49 years, who were cigarette smokers, consumers of moist snuff, and those who did not consume any tobacco products.

The purpose of this biomarker discovery study was to investigate the outcome of long-term smoking and moist snuff consumption using BioExp and BioEff. Such data will help in understanding the biological effects of long-term consumption of tobacco products and assist in placing them on a risk continuum. The study was performed using generally healthy subjects to minimize interference from pathophysiological pathways and therapeutic interventions on the biomarker responses.

Materials and methods

Study design

This was a single-site, cross-sectional study of male tobacco-product consumers and a non-tobacco-consuming control group, conducted at a clinical research unit (CRU), High Point Clinical Trials Center, High Point, NC, USA.

A total of 120 generally healthy male subjects, aged 35–60 years, were enrolled in parallel into one of three groups: 1) exclusive cigarette smokers (SMK) of any brand ≥6 mg "tar" (measured by the Cambridge Filter Pad method), who self-reported smoking at least 10 cigarettes/day for at least 3 years and had an expired carbon monoxide (ECO) level of 10–100 ppm; 2) exclusive MSC of any brand, who self-reported using ≥2 cans of moist snuff/week for at least 3 years and had an ECO of 0–5 ppm; 3) NTC, who self-reported not using any tobacco or nicotine-containing products for at least 5 years and had an ECO of 0–5 ppm. Female subjects were not recruited because of the low rate of ST use among women in the USA.

Ethical conduct of the study

The study was performed in compliance with the US Code of Federal Regulations (CFR) governing Protection of Human Subjects (21 CFR Part 50), Financial Disclosure by Clinical Investigator (21 CFR Part 54), and Institutional Review Board (IRB) (21 CFR Part 56). In addition to these federal regulations, this study followed the 1996 guidelines of the International Conference on Harmonisation, commonly known as Good Clinical Practice (GCP), which are consistent with the Declaration of Helsinki as adopted in 2008. The study was registered with the Clinical Trials Registry (ClinicalTrials.gov) with the identifier: NCT01923402. The study was conducted under the approval of a central IRB, Independent Investigational Review Board, Inc. (Plantation, FL, USA).

Biomarkers

The biomarkers used in this study were separated into two major categories, BioExp and BioEff, and they were measured in whole blood, serum, plasma, or urine. The BioEff were chosen based on functional pathways previously reported to be altered in smokers [3]. Only those biomarkers for which differences were statistically significant ($p \leq 0.05$) are discussed in this report. Total nicotine exposure was calculated from urinary levels of nicotine and its nine metabolites, which account for approximately 95% of nicotine exposure, as described earlier [30]. Bioanalysis was conducted using a fit-for-purpose paradigm [33, 34], under good laboratory practices (GLP) or GLP-like conditions (21 CFR Part 58), and method details are presented as Supplemental Data (see Tables S1a–S1d).

A 24-h urine sample was collected after acceptance into the study but before entering the CRU, under subjects' typical daily routines, and was brought to the CRU by the subjects on study Day –1. Trace metals and some BioEff were analyzed from the first morning void on Day 1, which was collected in acid-washed, metal-free tubes. When biomarkers were measured in urine samples other than 24-h

collections, they were expressed as a creatinine-normalized value. Blood biomarkers were analyzed from the blood collected on the morning of Day 1, after overnight fasting from food and tobacco consumption.

Statistical analysis

Mean and standard deviation of the biomarkers were calculated for each group. A formal outlier test was not performed. However, one data point, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), was beyond the group mean plus or minus five times the standard deviation, and it was excluded. To compare the differences of groups, one-way analysis of variance with Tukey-Kramer HSD test was conducted to determine the statistical significance ($p \leq 0.05$). This statistic gives a connecting letter report, which is shown on Figures 1 and 2 above each group. Groups that are not connected by the same letter are significantly different ($p \leq 0.05$). JMP 10 (SAS Institute, Cary, NC, USA) was used for data analysis.

Results

The subject recruitment, a disposition summary, the group demographics and tobacco product use characteristics are summarized in Table 1. Overall, the groups were well balanced, with no statistically significant differences between group mean ages or body mass index (BMI), across groups. Duration of tobacco consumption was similar between MSC and SMK, with self-reported group means being approximately 25 and 20 years of exposure to cigarettes and moist snuff, respectively.

Biomarkers of exposure

The tobacco BioExp, plasma nicotine and cotinine concentrations, were higher in both groups of tobacco consumers than the NTC group (Table 2). The biomarkers of combustion products, carboxyhemoglobin (COHb) and thiocyanate (SCN), were higher in the SMK, consistent with their smoking status. The group mean values for COHb and SCN were comparable between the MSC and NTC groups, indicating a lack of exposure to combustion-related toxicants.

The 24-h urine samples were analyzed for BioExp. Biomarkers that indicate nicotine and tobacco specific nitrosamines (TSNAs) exposures were higher in MSC relative to SMK. Levels of nicotine and its metabolites, as well as the total nicotine equivalents (NicEq-T), are shown in Table 2. Group mean NicEq-T were highest in MSC compared to SMK, while the NTC had background levels. The urinary excretion of nicotine and nine metabolites of nicotine over 24 h followed the pattern of the overall NicEq-T excretion. In each group, the urinary concentration of 3′-hydroxycotinine-O-glucuronide was the most abundant nicotine metabolite detected, and there was no gross difference in the ratios of metabolites across tobacco consumers. The TSNAs – NNAL (a biomarker of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, NNK), *N*-nitrosonornicotine (NNN), *N*-nitrosoanatabine (NAT), and *N*-nitrosoanabasine (NAB) – were also significantly higher in MSC followed by SMK and NTC.

The general pattern of SMK group means being higher than MSC and NTC group means, and no difference between the MSC and NTC group means, was also observed for urinary BioExp to most tobacco combustion products, covering the chemical classes of polycyclic aromatic hydrocarbons (PAHs) and aromatic amines. However, for two of the seven biomarkers of PAH exposure (2- and 3-hydroxyphenanthrene and 1-hydroxypyrene), a graded response of SMK>MSC>NTC was observed for the group means.

Many of the volatile constituents of tobacco smoke are metabolized through pathways that lead to at least partial

Table 1: Enrollment groups: disposition, demographics, and tobacco use.

	Group		
	SMK	**MSC**	**NTC**
Enrolled, n	41	41	40
Completed, n	40	40	40
Withdrawn from study, n	1[a]	1[a]	0
Race: Caucasian, Other, n	31, 10	35, 6	28, 12
Mean age, range, years	46.9, 35–59	45.0, 35–60	47.2, 35–60
Mean BMI, kg/m^2	28.4	29.7	29.4
Years of product use, mean±SD, (min, max)	25.1±9.6, (6, 45)	20.6±8.5, (5, 37)	0, (0, 0)
Product use in last month, mean±SD, (min, max)	21.5±5.3, (12, 30)[b]	6.3±3.5, (1.75, 14)[c]	0, (0, 0)

[a]Withdrawn after vasovagal reaction to blood withdrawal and data removed from other parameters in this Table. [b]Self-reported cigarettes per day. [c]Self-reported cans per week. BMI, body mass index; SMK, smokers; MSC, moist snuff consumers; NTC, non-tobacco consumers.

Table 2: Blood and urine biomarkers of exposure in smokers, moist snuff consumers, and non-tobacco consumers.

	Units	Group mean[a]±SD		
		SMK	MSC	NTC
Blood biomarkers				
Plasma nicotine, unconjugated	ng/mL	3.3±2.0	2.7±1.9	0.7±0.8
Plasma cotinine, unconjugated	ng/mL	247.7±119.9	309.7±166.2	0.5±0.8
COHb (CO exposure)	%	4.0±1.7	0.9±1.3	1.0±1.4
Plasma SCN (hydrogen cyanide exposure)	µmol/L	155.6±51.5	16.7±7.9	39.4±26.6
Urine nicotine and nine metabolites[b]				
NicEq-T	mg/24 h	18.5±7.6	29.4±20.9	0.1±0.1
Nicotine	µg/24 h	2719.6±1539.7	3219.6±2916.4	6.3±6.5
Nicotine-1′-oxide	µg/24 h	1040.6±409.4	1649.7±1086.4	1.9±3.0
Nicotine-N-glucuronide	µg/24 h	1344.5±1111.2	1688.1±1256.6	3.5±2.4
Norcotinine	µg/24 h	281.0±145.8	339.4±247.3	2.0±1.4
Nornicotine	µg/24 h	122.1±66.0	186.5±135.4	3.9±3.6
Cotinine	µg/24 h	2787.8±1484.7	3916.0±2678.4	6.5±5.4
Cotinine-N-glucuronide	µg/24 h	4312.2±2868.8	6535.0±4316.3	4.3±5.4
Cotinine-N-oxide	µg/24 h	855.2±498.1	1218.6±829.4	3.0±4.2
3′-Hydroxycotinine	µg/24 h	6141.2±4091.7	11,581.6±9106.9	8.9±12.1
3′-Hydroxycotinine-O-glucuronide	µg/24 h	7494.1±3871.2	13,526.5±18,557.9	123.3±137.4
Urine nitrosamines				
Total NNAL (NNK exposure)	ng/24 h	578.3±366.3	2310.8±2415.0	55.8±53.3
Total NNN	ng/24 h	17.2±14.2	48.9±34.7	2.1±1.9
Total NAB	ng/24 h	61.8±37.3	157.0±220.1	1.3±1.1
Total NAT	ng/24 h	320.2±201.0	1328.7±1438.8	2.0±1.7
Urine PAHs				
1- and 9-Hydroxyphenanthrene	ng/24 h	347.8±281.2	70.9±106.2	56.2±85.4
1-Hydroxypyrene	ng/24 h	369.3±345.2	181.4±238.0	113.4±113.8
1-Naphthol	µg/24 h	10.3±8.1	1.9±2.0	19.4±11.0
2- and 3-Hydroxyphenanthrene	ng/24 h	547.5±506.3	325.0±420.3	208.0±177.0
2-Hydroxyfluorene	ng/24 h	2208.3±1611.3	811±1185.3	560.9±623.3
2-Naphthol	µg/24 h	21.3±13.4	7.6±6.1	7.7±10.5
Urine aromatic amines				
2-Aminonaphthalene	ng/24 h	45.9±37.9	7.4±5.9	7±5.7
3-Aminobiphenyl	ng/24 h	11.09±9.05	1574.9±2305.3	1193.4±615.3
4-Aminobiphenyl	ng/24 h	23±11.3	4.6±2.4	5.5±2.8
o-Toluidine	ng/24 h	245.1±115.5	84.3±48.8	65.5±35.8
Urine mercapturic acid metabolites				
MHBMA (1,3 butadiene exposure)	ng/24 h	195.6±106.4	70.0±99.1	31.5±45.4
3-HPMA (acrolein exposure)	µg/24 h	3747.2±1663.9	746.0±648.1	632.5±436.9
HMPMA (crotanaldehyde exposure)	µg/24 h	1782.9±894.7	333.0±212.5	346.0±192.3
SPMA (benzene exposure)	ng/24 h	6043.2±4998.5	603.3±890.7	746.8±716.1
AAMA (acrylamide exposure)	µg/24 h	388.1±166.6	172.8±93.2	160.2±70.8
GAMA (acrylamide exposure)	µg/24 h	51.7±25.3	23.2±10.9	24.6±10.5
Trace metals[c]				
Cadmium	µg/g creatinine	0.5±0.4	0.2±0.1	0.3±0.2
Chromium	µg/g creatinine	0.2±0.3	0.2±0.2	0.1±0.1
Nickel	µg/g creatinine	2.6±1.3	2.3±1.0	2.2±0.8
Tin	µg/g creatinine	0.5±0.4	0.4±0.4	1.1±2.6
Selenium	µg/g creatinine	33.9±13.8	37.2±14.4	34.4±11.2

Analytes with statistically significant differences relative to the NTC are represented as shaded values. It should be noted that the statistical pair-wise comparison was conducted by Tukey-Kramer HSD test. [a]All values are rounded to one decimal place. [b]All measurements were made in 24-h urine collections and are expressed as mass per 24-h excretion. [c]Trace metals were measured in the first void and are corrected for creatinine excretion. AAMA, *N*-acetyl-*S*-(2-carbamoylethy)-cysteine; CO, carbon monoxide; COHb, carboxyhemoglobin; GAMA, *N*-acetyl-*S*-(2-carbamoyl-2-hydroxyethy)-cysteine; 3-HPMA, 3-hydroxypropyl-mercapturic acid; HMPMA, hydroxy-methyl-propyl-mercapturic acid; MHBMA, monohydroxy-butenyl-mercapturic acid; MSC, moist snuff consumers; NAB, N-nitrosoanabasine; NAT, N-nitrosoanatabine; NicEq-T, total nicotine equivalents; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, N-nitrosonornicotine; NTC, non-tobacco consumers; PAHs, polycyclic aromatic hydrocarbons; SCN, thiocyanate; SD, standard deviation; SMK, smokers; SPMA, *S*-phenyl-mercapturic acid.

elimination by conjugation with mercapturic acid derivatives, and they can be used as biomarkers for exposure to the parent compounds present in cigarette smoke [34]. The group mean 24-h urinary excretions of the following biomarkers were elevated in the SMK group compared to the MSC and NTC groups, which were indistinguishable from each other: *N*-acetyl-*S*-(2-carbamoylethy)-cysteine (AAMA) and *N*-acetyl-*S*-(2-carbamoyl-2-hydroxyethy)-cysteine (GAMA) (biomarkers of acrylamide); *S*-phenyl-mercapturic acid (SPMA, biomarker of benzene); monohydroxy-butenyl-mercapturic acid (MHBMA, biomarker of 1,3-butadiene); 3-hydroxypropyl-mercapturic acid (3-HPMA, biomarker of acrolein); and hydroxy-methyl-propyl-mercapturic acid (HMPMA, biomarker of crotonaldehyde) (Table 2).

Among the five metals measured in the first void urine, only cadmium concentration was found to be different between the groups. The SMK group had a higher creatinine-normalized mean concentration of urinary cadmium than both the MSC and NTC groups (Table 2). Chromium, nickel, selenium, and tin excretions were not different across all groups.

Biomarkers of effect

Cigarette smoking is associated with oxidative stress and a chronic inflammatory state [4, 5, 35]. Therefore, many candidate BioEff spanning several physiological

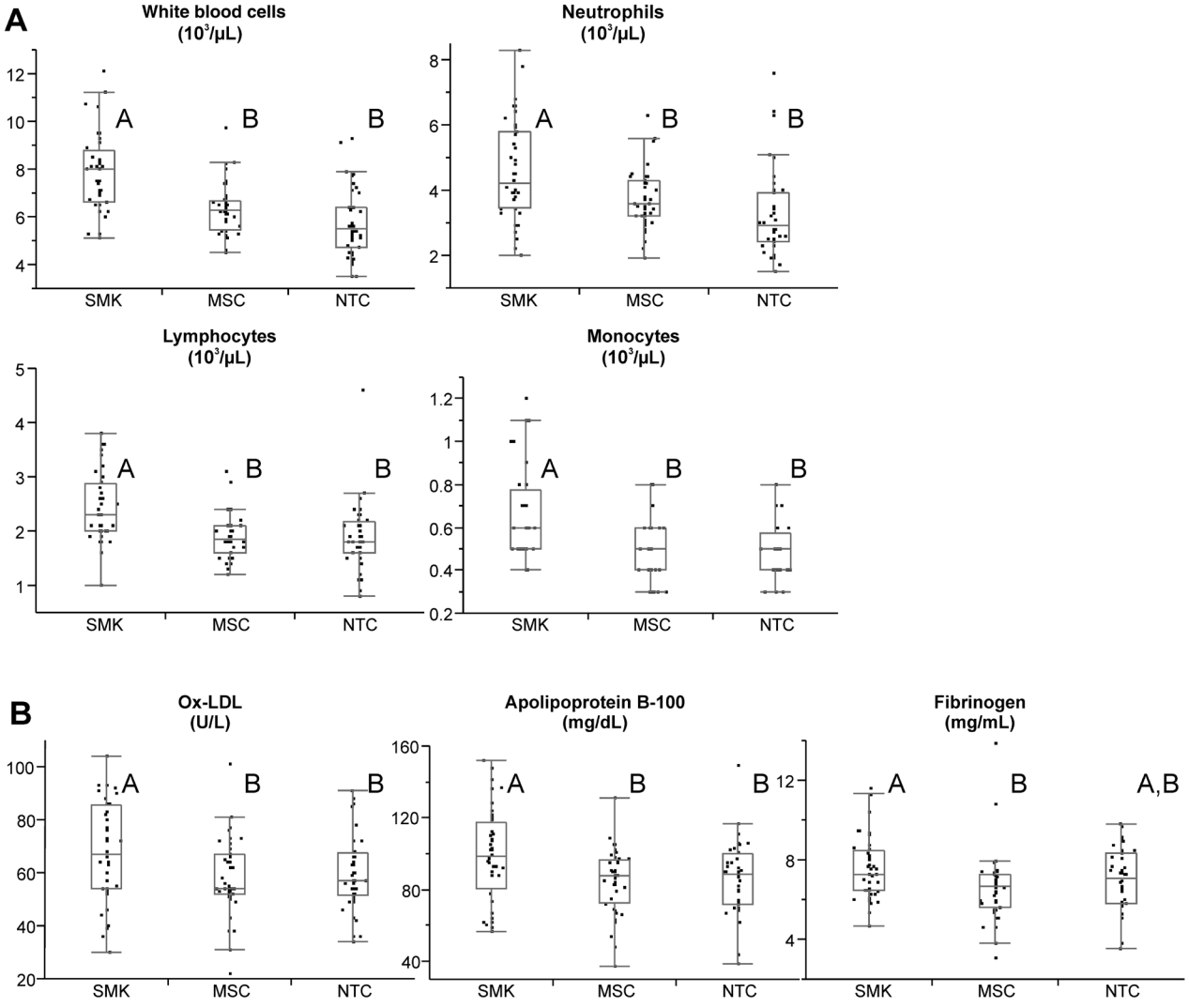

Figure 1: Box and Whisker plots for (A) total white blood cells and the major leukocyte subsets and (B) blood lipids and fibrinogen.
The Boxes represent the upper and lower quartiles with the median shown as a solid line. Whiskers connect the 90th percentile data point and 10th percentile data points within median ±1.5 times the inter-quartile range. The Tukey-Kramer HSD statistic gives a connecting letter report. Groups not connected by the same letter are significantly different ($p \leq 0.05$).

pathways were assessed in this study (see Supplemental Data, Tables S2 and S3). Only BioEff that were statistically different for at least one group are discussed.

Among the blood BioEff, leukocyte counts, including total white blood cells (WBC), lymphocytes, monocytes, and neutrophil counts, were significantly higher in the SMK group, relative to MSC and NTC (Figure 1A). The MSC group did not differ from the NTC group in any of these parameters. For plasma biomarkers related to the lipid metabolism pathways (oxidized low-density lipoprotein and apolipoprotein B-100), the pattern of the data showed that the SMK group mean was higher than the MSC and NTC group means, and no difference was observed between the MSC and NTC groups (Figure 1B). The biomarker related to blood coagulation pathway, fibrinogen, was significantly lower in MSC compared to SMK, although the levels in SMK and NTC were not significantly different. Overall, for these blood and plasma BioEff, the pattern observed was the same as for the BioExp to the tobacco combustion products COHb and SCN (i.e. SMK>MSC=NTC).

Group mean values for some urinary BioEff, related to leukocyte and platelet function and thromboxane turnover (leukotriene E4 and 11-dehydrothromboxane B_2), also followed a pattern of SMK>MSC=NTC (Figure 2A). This pattern of group mean values was also observed for other prostanoid metabolites detected in urine ($iPF_{2\alpha}$-III and 8,12-$iPF_{2\alpha}$-VI) (Figure 2B); however, the mean values of $iPF_{2\alpha}$-VI gave a different pattern, with SMK=MSC>NTC. These patterns for prostanoid metabolites are summarized in a radar plot (Figure 3), in which the NTC group mean is set at a value of 1.0 and other group means are multiples of the NTC mean value. This plot emphasizes the overall similarity between NTC and MSC mean values for these and related prostanoid metabolites and highlights that the only point of similarity between MSC and SMK groups, within these metabolites, is for $iPF_{2\alpha}$-VI.

Discussion

This report presents detailed comparative evaluations of BioExp and BioEff in smokers and consumers of ST, particularly moist snuff. The key findings of this work are as follows: 1) the exposure to tobacco combustion-related toxicants in MSC is significantly reduced, while the exposures to nicotine (and its metabolites) and the four TSNAs are higher than, but comparable to, levels found in SMK; 2) the MSC and NTC groups exhibit similar levels of almost all BioEff that are indicative of oxidative stress, inflammation, platelet activation, and lipid metabolism; 3) most BioEff levels in SMK are higher than in MSC and NTC,

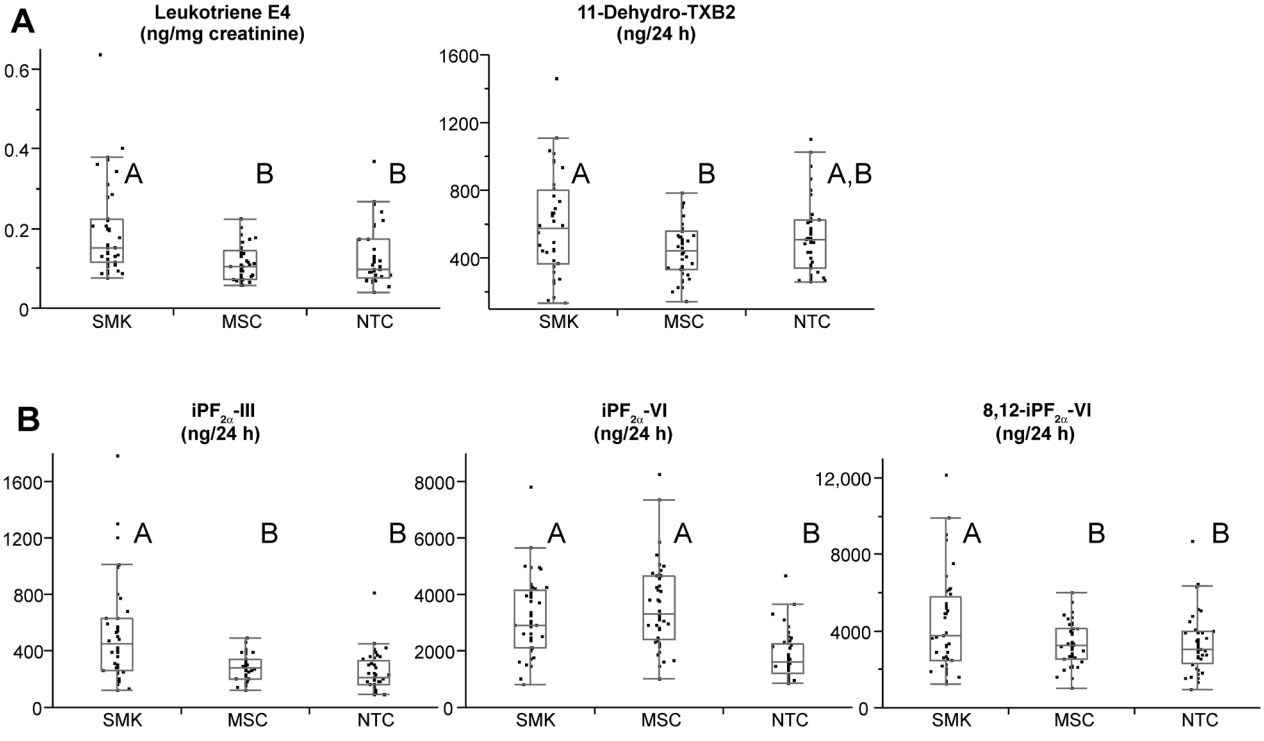

Figure 2: Box and Whisker plots for (A) leukotrienes, thromboxane, and (B) isoprostane metabolites for which at least one group difference was measured. The Box and Whiskers and the Tukey-Kramer HSD statistic convention is as given on Figure 1.

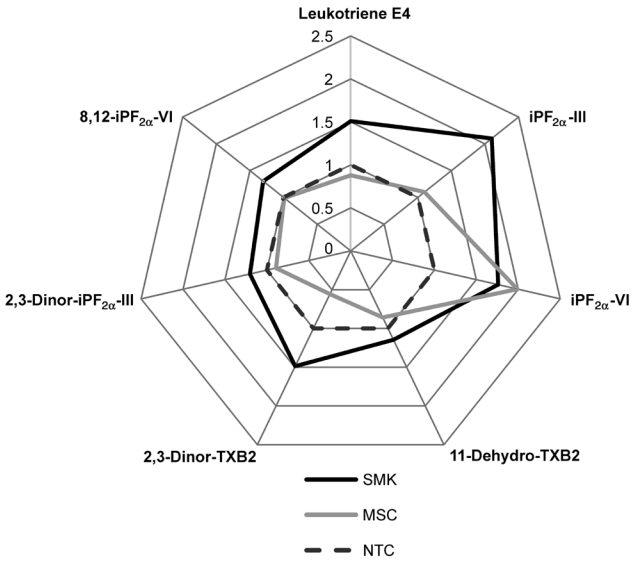

Figure 3: Radar plot summarizing relative similarities and differences between leukotrienes, thromboxane, and isoprostane metabolites mean values across SMK, MSC, and NTC groups. NTC mean was set as 1.0, and relative amounts of each metabolite are plotted and connected by a line.

although all measurements remain within normal physiological ranges.

The duration of tobacco-product use in SMK and MSC was comparable for subjects in this cross-sectional study (Table 1). The biomarker levels observed in this study are consistent with the published literature comparing smokers with non-smokers [21, 24–27, 34]. The BioExp and BioEff are able to distinguish the study groups. In addition to distinguishing the smoking (SMK) and non-smoking (MSC and NTC) groups, the biomarkers further discriminate the MSC and NTC groups (e.g. the difference between MSC and NTC in $iPF_{2\alpha}$-VI, which is a measure of platelet activation). The combustible tobacco smoke constituents (COHb and SCN) serve as BioExp between the SMK and MSC groups. Further, for these biomarkers, the MSC group could not be distinguished from NTC. These patterns were consistent across all groups of combustible smoke constituents that were measured (gases, volatile carbonyl compounds, aromatic amines, PAHs, and other organic chemicals), but two biomarkers of PAH exposure (1-hydroxypyrene and 2- and 3-hydroxyphenanthrene) showed a gradation in the order SMK>MSC>NTC. The apparent increase in these two PAHs in MSC could be attributed to the confounding effect of environmental and dietary exposures to PAH [36, 37] in the days leading up to the subjects' 24-h urine collections for measuring these biomarkers. Published data from the National Health and Nutrition Examination Survey also revealed that 1-hydroxypyrene and some halogenated PAHs are higher in ST consumers, relative to non-tobacco consumers [38]. Further, for nicotine, NNK and NNN, which are not formed during combustion and pyrolysis of tobacco [39], a different exposure pattern was observed. For these tobacco components, the MSC group had higher levels of systemic exposure than SMK, and both groups had greater systemic exposure than NTC.

Trace metals can be detected in tobacco, depending on the local crop growing conditions [40–42] but, with the exception of cadmium, no statistically significant differences were seen across all groups in this study for these trace metals, which is consistent with other published reports [38, 41]. Since urinary cadmium concentrations reflect long-term accumulation of this metal [43], the MSC were not exposed to cadmium at levels different from the NTC. Taken together with the published NHANES analyses [38, 41], the BioExp data generally indicate that the exposure of MSC to the combustion related toxicants is significantly less than SMK, and is comparable to NTC.

Although appreciable information on the pathophysiological mechanisms of smoking-associated diseases exists [3, 44], relatively little information is available on how chronic exposure to moist snuff alters biological pathways. Further, prospective data from epidemiological studies will accumulate slowly and, therefore, the current study sought objective biomarker measurements to differentiate further possible physiological differences between SMK, MSC, and NTC.

Our findings have reproduced and are supported by other reports of differences between smokers and non-smokers in many of these pathways and extended these reports by providing objective data on MSC in comparison to these other groups. For example, chronic smoking is associated with inflammation, as evidenced by increased WBC counts [24, 26, 27]. Consistently, data presented herein confirm that finding, showing lower WBC counts in MSC and NTC relative to SMK. Using these parameters, the similarity between the MSC and NTC groups in WBC, lymphocyte, neutrophil and monocyte counts suggests that systemic inflammation in MSC is lower than in SMK. Of the blood coagulation factors measured, consistent with published findings [5, 26], fibrinogen showed the expected difference between smokers and non-tobacco consumers, but additionally from this study, MSC were indistinguishable from NTC.

Several of the BioEff evaluated in this study measure different aspects of the arachidonic acid – prostaglandin – thromboxane – leukotriene pathway. This pathway is involved in many physiological responses, such as those related to inflammation, platelet adhesiveness,

endothelial function, and regulation of small capillary blood flow [45–49]. In this study, some biomarkers distinguished the study groups. The mean total 24-h excretion of the urinary biomarker of platelet activation, 11-dehydrothromboxane B_2, was lower for MSC than both the SMK and NTC groups.

The urinary isoprostanes are generated by free radical interaction with metabolites from the arachidonic acid metabolism pathway, and they are used as biomarkers of oxidative stress [28, 45, 48]. In this study, $iPF_{2\alpha}$-III and 8,12-$iPF_{2\alpha}$-VI showed higher mean 24-h urinary excretions in the SMK group compared to the NTC, consistent with previous reports [21, 26]. That $iPF_{2\alpha}$-III levels were lower in MSC and were comparable to those detected in NTC points to its potential value as a biomarker to distinguish SMK from MSC. Notably, the biomarker $iPF_{2\alpha}$-VI was the only one out of approximately 40 biomarkers related to inflammation and oxidative stress that showed statistically comparable levels in SMK and MSC. All other biomarkers, such as plasma C-reactive protein, β_2-microglobulin, or glutathione, showed no difference between the MSC and NTC groups (data not shown). Overall, these perturbations in the arachidonic acid – prostaglandin – thromboxane – leukotriene pathway could be of interest in distinguishing between groups of smokers and non-smokers (e.g. MSC and NTC), and they merit further investigation as putative biomarkers.

Higher concentrations in the lipid biomarkers, oxidized low-density lipoprotein and apolipoprotein B100 were observed in the SMK compared to the NTC group have also been reported by others [50]. Once again, the MSC were statistically indistinguishable from NTC for these biomarkers associated with elevated CVD risk.

Biomarker data from the CVD study [30, 32], generated from an independent study population, were also in general agreement with the overall results of this current study that the smokers exhibit distinct BioExp and BioEff patterns relative to the moist snuff and non-tobacco consumers. Notwithstanding the differences in study population characteristics between the CVD study [30] and this biomarker discovery study, $iPF_{2\alpha}$-III and 11-dehydrothromboxane B_2 levels were lower in moist snuff consumers, suggesting differences in arachidonic acid metabolism between smokers and non-smokers. Further, metabolomic profiles matching plasma, urine, and saliva collected from subjects in this biomarker study show that SMK profiles differ from those of non-smoking cohorts, with MSC and NTC resembling each other more closely than the SMK group [51].

Normal and pathological clinical ranges for most of the BioEff investigated herein have not been well-established, and this study enrolled generally healthy subjects. Therefore, most measurements for all groups were within the normal physiological ranges provided by the laboratories analyzing the samples and from literature sources. Cross-sectional observations on BioEff offer important insights into the comparative biological changes due to consumption of moist snuff and potentially other non-combustible tobacco-product use. Given that the duration of the product use and demographics among the tobacco consumers (SMK and MSC) are comparable, the observed differences in the BioEff among the study groups are possibly due to the category of tobacco product used. Additional longitudinal studies are required to qualify these and other biomarkers against disease outcomes.

Accumulating epidemiological data indicate that ST consumers are at much lower risk of developing smoking-associated diseases than smokers of combustible tobacco products [9, 10, 12–15]. Other studies show that the mortality due to some diseases is higher in ST consumers than in NTC [17]. Collectively, the biomarker changes discussed herein, metabolomic profiles [51], and the biomarker data from a previous CVD study [30, 32] suggest that over a wide range of biological pathways, MSC are much closer in profile to NTC than to SMK. The emerging biomarker data should further define the risk continuum associated with the use of different tobacco-product categories, and improve understanding of the pathophysiological changes due to tobacco consumption. Further, the emerging knowledge on the biological effects of chronic tobacco use contributes to reducing the harm from cigarette smoking.

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