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Original Investigation

Evaluation of biomarkers of exposure in adult cigarette smokers using Marlboro Snus

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Abstract

Introduction: It has been reported that adult smokers (AS) may be considering smokeless tobacco products as an alternative to smoking. The objective of this study was to evaluate the change in exposure in AS using Marlboro snus (MSNUS) (a tobacco pouch product in test market in June 2007).

Methods: AS were randomized into the following groups—CS: subjects ($n = 30$) continue smoking their own brand; DU: subjects ($n = 60$) reduced their daily cigarette consumption by ≥50% and were allowed to use MSNUS; SN: subjects ($n = 15$) stopped smoking their cigarettes but were allowed to use MSNUS; NT: subjects ($n = 15$) were not allowed to use any tobacco products for the entire duration of the 8-day study. Biomarkers of smoke exposure (BOE) measured at baseline and postbaseline were 24-hr urinary excretion of metabolites of *N*-nitrosamines, nicotine (urine and plasma), aromatic amines, benzene, and polycyclic aromatic hydrocarbon; urine mutagenicity; and carboxyhemoglobin at various timepoints.

Results: Statistically significant ($p < .05$) reductions in all the urinary BOE were observed in the DU group compared with the CS group. After correcting for the residual effect, a proportionate reduction (~50%) in most of the biomarkers was observed. Even larger reductions, similar to the NT group, were observed in the SN group.

Discussion: The proportionate reduction in exposure when reducing the number of cigarettes by 50% and using MSNUS, under the consumption patterns observed, suggest that the AS did not appear to alter their smoking behavior. The added exposure from MSNUS usage in this group was minimal. The AS sustained substantial reductions in exposure when using MSNUS exclusively.

Introduction

The predominant form of tobacco use in the United States is cigarette smoking. Other tobacco products, such as cigars, pipe tobacco, chewing tobacco, and snuff, are used by a smaller number of the adult U.S. population. The concurrent use of smokeless tobacco (ST) and cigarettes is increasingly becoming frequent among adult cigarette smokers (Backinger et al., 2008; Levy et al., 2005). There are a number of ST products commercially available in North America and in Scandinavian countries ranging from moist snuff, chewing tobacco, lozenges, and tobacco pouch products (prevalent in Sweden). Some of the ST products used in other parts of the world—for example, betel nut (India; Wasnik, Ughade, Zodpey, & Ingole, 1998) and toombak (Sudan; Idris et al., 1998)—appear to be associated with significant health risks (Boffetta, Hecht, Gray, Gupta, & Straif, 2008; Wasnik et al.). Recently, significant attention has been focused on the tobacco pouch products, similar to those available in Sweden (Daniel, Roth, & Liu, 2005; Levy et al., 2004). The health risks associated with the use of these ST products are substantially lower than those associated with smoking cigarettes (Levy et al., 2004, 2005). It has been reported that "the consumption of non-combustible tobacco is of the order of 10–1,000 times less hazardous than smoking, depending on the product" (Tobacco Advisory Group of the Royal College of Physicians, 2007).

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Despite the considerable lower tobacco-related disease risk in these ST product users, questions are often raised regarding the impact of dual usage of ST and cigarettes on the disease risks (Bombard, Pederson, Nelson, & Malarcher, 2007; Wetter et al., 2002). Several studies (Hatsukami et al., 2004; Hecht et al., 2007, 2008; Idris et al., 1992; Kotlyar et al., 2007; Post et al., 2005) have investigated the changes in exposure when adult smokers (AS) *exclusively* use ST products. However, no reports exist of exposure measurements from dual usage of both ST products and cigarettes. There are a number of biomarkers of exposure (Roethig et al., 2007; Sarkar et al., 2008) that provide an estimate of smoke exposure through selected particulate and gas vapor phases of the complex aerosol generated from cigarette smoke. These biomarkers not only provide an estimate of exposure but also may provide insight regarding changes in smoking behavior (Sarkar et al.). The primary purpose of this study was to evaluate exposure to selected tobacco and smoke constituents in AS who reduce their number of cigarettes by at least 50% and were allowed to use a pouch tobacco product, compared with those who continue to smoke the conventional cigarettes.

Methods

This study used a randomized, controlled, open-label, parallel group, short-term study design and was conducted at a single clinical center between July and August 2007. The study was approved by the MDS Pharma Services Institutional Review Board and was conducted in accordance with Good Clinical Practice and the Declaration of Helsinki criteria (Rickham, 1964). All subjects signed informed consent prior to study participation and were paid for their participation.

Subjects

Self-affirmed male and female AS with a daily cigarette consumption of 10–40 cigarettes (nonmenthol, any tar yield) for at least the preceding 12 months were recruited through a local advertisement. Subjects did not use any nicotine-containing product other than manufactured cigarettes for at least the 3 months prior to the start of the study. Main exclusion criteria included oral lesions; renal, liver, metabolic, cardiac, and pulmonary diseases; and illicit drug uses. Women who were pregnant, lactating, or intending to get pregnant during the course of the study were also excluded.

Test products

The ST products used in this study were unidentified Marlboro Snus Mild and Marlboro Snus Mint, both products manufactured by Philip Morris (Richmond, VA) for test marketing during July 2007. Since both products were identical except for the flavor components, they will be abbreviated as MSNUS throughout the body of text. Each pouch contained approximately 0.3 g of filler, consisting of pasteurized tobacco and added ingredients. The analytic characteristics of the products (shown as range for the two products used) are as shown below. Marlboro Snus moisture content was 9.58%–13.22%, nicotine 1.52%–2.9%, pH 6.80–7.19, 4-(methylnitrosamino)-1-(3-pyridyl)-butanone (NNK) 0.094–0.224 ppm, *N*′-nitrosonornicotine (NNN) 0.682–1.117 ppm, total tobacco-specific *N*′-nitrosamines (TSNA) 1.375–2.019 ppm, benzo[*a*]pyrene 0.37–0.67 ng/g, and the estimated % unprotonated nicotine values as determined by the Henderson–Hasselbach equation were ~9%. These product characteristics are within the range of other commercial tobacco pouch products and are much lower than those reported for other categories of ST products, such as moist snuff and chewing tobacco (Rickert et al., 2009; Stepanov, Hecht, Ramakrishnan, & Gupta, 2005; Stepanov, Jensen, Hatsukami, & Hecht, 2008). The cigarettes smoked during the study were subjects' own usual brand and were brought by the subjects when they checked into the clinic.

Prescreening product trial usage

During the first visit, after signing the informed consent, AS were given 12 snus samples each from two different flavors (unidentified MSNUS product) to test their willingness to use this product for the duration of the study and their flavor preference. This trial period lasted for 2 weeks, in order to provide enough time for the subjects to acclimatize to this new ritual. There were three AS who decided not to participate in the study based on their prescreening MSNUS trial experience. When checking into the clinic, the subjects reported their preference for the MSNUS product and, if randomized to an MSNUS usage group, were allowed to use only the selected MSNUS product during the study. On average, about 60% chose the Mint product and the remaining 40% chose the Mild product.

Study design and conduct

Eligible subjects were admitted to the clinic unit and confined for the 10-day study. A total of 137 subjects were enrolled, 120 subjects (87 males and 33 females) were randomized into four groups and 115 subjects completed the study (see Figure 1 for subject disposition). The randomization was stratified by number of cigarettes per day (CPD) and gender to ensure equal distribution across all study groups. During the first 2 days (baseline, Days −2 and −1), subjects continued to smoke their own brand of cigarettes and daily cigarette consumption (CPD) was determined. Beginning on the morning of Day 1, 120 subjects were randomized into one of the following four groups:

- Continue smoking group (CS, $n = 30$): Subjects were allowed to continue to smoke their own brand of cigarettes approximately every 32 min between 7:00 and 23:00 each day up to a maximum of 30 CPD.
- Dual usage group (DU, $n = 60$): Subjects reduced their daily number of cigarettes to 50% or less of the number of cigarettes smoked at baseline and were allowed to use MSNUS. Smoking was allowed only at restricted time periods between 7:00 and 9:07, 11:48 and 13:56, and 17:08 and 19:15; MSNUS usage was allowed every 32 min between 7:00 and 23:00. The subjects were not allowed to use both cigarettes and MSNUS simultaneously.
- Exclusive MSNUS group (SN, $n = 15$): Subjects stopped smoking and were allowed to use MSNUS every 32 min between 7:00 and 23:00.
- Stop tobacco use group (NT, $n = 15$): Subjects abstained from all types of tobacco use.

Subjects were never forced to smoke or to use MSNUS by the investigators. They could reduce their numbers of cigarettes or even quit smoking. Smoking and tobacco usage cessation counseling was provided to the subjects at the beginning and at the end of the study. The AS were allowed to smoke under controlled conditions, that is, smoking opportunities were limited to every

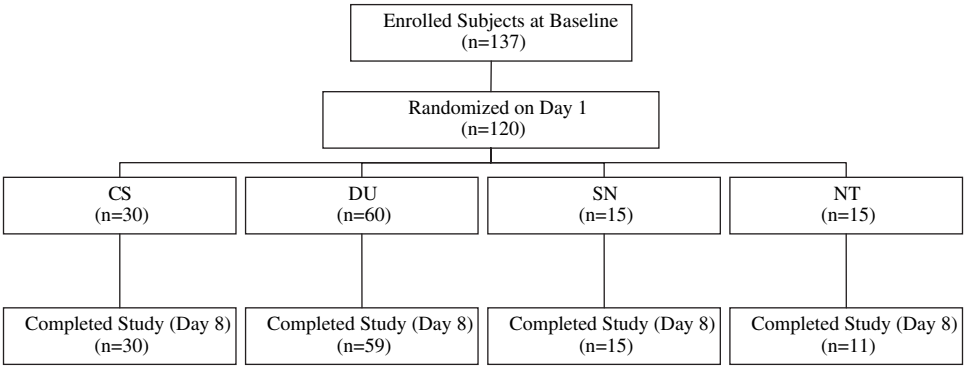

Figure 1. Study design: At baseline (Day −2, Day −1), all subjects smoked their own cigarettes *ad libitum*. Starting from Day 1, subjects were randomized into CS (continue smoking), DU (dual cigarette and MSNUS), SN (MSNUS only), and NT (no tobacco) groups. In the DU group, subjects reduced to 50% or less of their daily cigarette consumption at baseline and were allowed to use MSNUS *ad libitum*. Subjects in SN group stopped smoking and were allowed to use MSNUS *ad libitum*. Subjects in the NT group were not allowed to use any type of tobacco products.

32 min. This time period was calculated based on smoking being permitted between 7 a.m. and 11 p.m. and on the maximum number of cigarettes allowed being 30. The standardized experimental conditions ensured minimal variability between subjects, particularly as it relates to the nicotine in plasma, which has a relatively short half-life. The study staff monitored the 32-min interval for each subject, documented all cigarettes smoked by each subject, and verified that each cigarette smoked came from the appropriate subject-specific bar-coded package. The cigarette was lit by the study staff using a standardized blue flame lighter. No smoking was allowed during meals or between 11 p.m. and 7 a.m. For study integrity, all butts from smoked cigarettes were collected in a container to ensure that subjects did not have access to partially smoked cigarettes at any time during the study. Subjects were given the option of choosing to quit smoking at any time during the study. All smoking took place in a designated outdoor smoking area, and the groups were physically separated to avoid any potential smoke-related exposure for the subjects in the no-smoking groups (SN and NT).

Blood sample collection

At Days −2, −1, 7, and 8, blood samples for nicotine, cotinine, and *trans*-3′-hydroxycotinine measurements were collected at 7:00, 9:07, 11:48, 13:55, 17:08, and 19:15 hr (no blood draws were carried out for the NT group on Days 7 and 8). An additional blood sample was collected at 19:00 for the measurement of carboxyhemoglobin (COHb).

Urine sample collection

All urine voided by each participant was collected over a 24-hr period from 7 a.m. to 7 a.m. the next morning from baseline through Day 8 and refrigerated at 2–8 °C. The urine volume collected during the 24-hr interval was recorded for each subject, and aliquots for biomarker analysis were stored at −20 or −70 °C, as appropriate for the biomarker to be analyzed.

Biomarkers of exposure to selected smoke constituents

All urinary biomarkers were measured by validated methods (Food and Drug Administration, 2001). The interday and intraday variability for all the analytes was within the acceptability limits (≤15%).

Nicotine exposure. Nicotine exposure was determined by measuring nicotine and five of its major metabolites, nicotine *N*-glucuronide, cotinine and cotinine-*N*-glucuronide, *trans*-3′-hydroxycotinine, and *trans*-3′-hydroxycotinine glucuronide by liquid chromatography–tandem mass spectrometry (LC-MS/MS; Sarkar et al., 2008). The 24-hr excretion of nicotine, nicotine-*N*-glucuronide, total cotinine, and total *trans*-3′-hydroxycotinine was calculated from the measured concentration and 24-hr urine volume, converted into moles excreted in 24 hr, added to yield moles of nicotine equivalents (NE), and multiplied by the molecular weight of nicotine to yield NE in milligrams (Roethig et al., 2007). The lower limit of quantitation was 10 ng/ml for all the six analytes.

Plasma nicotine, cotinine, and *trans*-3′-hydroxycotinine. Plasma levels of nicotine, cotinine, and *trans*-3′-hydroxycotinine were measured by LC-MS/MS following solid-phase extraction (SPE)–based sample clean-up. The samples were extracted on a Waters Oasis HLB μElution plate and were diluted with an acetonitrile:water solution. Two Phenomenex Chromolith columns were used in series along with a mobile phase consisting of methanol and ammonium formate buffer to achieve separation. An AB½MDS Sciex API 5000 detected positive ions in the multiple reaction monitoring (MRM) mode. The lower limit of quantitation was 0.2 ng/ml for nicotine and 1.0 ng/ml for the two metabolites. Study samples were analyzed in batches that included appropriate quality control and calibration samples. Interbatch precision (% coefficient of variability [CV]) of low, medium, and high levels of quality control samples was less than or equal to 9.3% for nicotine, 2.4% for cotinine, and 3.2% for *trans*-3′-hydroxycotinine. Interbatch accuracy (% relative error) of low, medium, and high levels of quality control samples was less than or equal to 4.7% for nicotine, 2.1% for cotinine, and 1.3% for *trans*-3′-hydroxycotinine. Assay selectivity was demonstrated by quantitation of standard spikes into six separate lots of blank human plasma (ethylenediaminetetraacetic acid). No significant matrix effect was observed at or near the lower limit of quantitation concentration or at the high-quality control sample concentration for nicotine, cotinine, or *trans*-3′-hydroxycotinine.

Nitrosamine exposure: NNK. Nitrosamine exposure was determined by measuring NNK metabolites 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its *O*- and

N-glucuronide conjugates. The sum of the free and conjugated metabolites was represented as total NNAL. Total NNAL levels in the urine were determined after cleavage of the glucuronide conjugates (*O*- and *N*-glucuronides) and analysis by LC-MS/MS as reported previously (Sarkar et al., 2008). The LLOQ was 5.0 pg/ml.

Nitrosamine exposure: NNN. Nitrosamine exposure was also determined from urine total NNN excretion measured by an LC-MS/MS method. Further details of this analytic method and validation will be published separately; however, the following description should be sufficient to allow replication of the results. Since background levels of NNN in the majority of human urine sources were greater than the LLOQ of the method (0.747 pg/ml), a substitute urine matrix, Urisub, free of NNN, was used to prepare calibration standards. The detailed method procedure and validation results will be published separately. Briefly, a 3-ml aliquot of urine was acidified with 0.2 ml of sodium acetate buffer (pH 5.0), and 50 µl of d_4-NNN at 2 ng/ml was added as an internal standard. The sample was incubated with 1 ml of β-glucuronidase from *Helix pomatia* (2,150 units/ml in water) at 37 °C for 16–18 hr for the enzymatic hydrolysis. To the sample was added 0.4 ml of concentrated NH_4OH and the sample was then subjected to SPE on Oasis MCX Vac RC (60 mg × 20 ml) preconditioned with 2 ml of 2% NH_4OH in acetonitrile. The SPE cartridge was washed with 4 ml of 5% NH_4OH in water, 3 ml of 50 mM $HCOONH_4$ buffer (pH 2.8), 3 ml of methanol, and 1 ml of acetonitrile, sequentially. The sample was eluted with 1 ml of 0.5% NH_4OH in acetonitrile. After the addition of 5 µl of dimethyl sulfoxide (DMSO), the sample was evaporated under nitrogen at 45 °C and reconstituted with 0.25 ml of acetonitrile:10 mM ammonium acetate:formic acid (10:90:0.04). Fifty microliters of the sample was injected into an LC-MS/MS system (AB/Sciex API 5000) equipped with a guard column (Phenomenex Synergi Polar-RP, 50 × 4.6 mm, 4 µm) and analytic column (Phenomenex Synergi Polar-RP, 150 × 4.6 mm, 4 µm). The elution was performed isothermally at 40 °C with a gradient consisting of 25:75 acetic acid (0.1%) in acetonitrile:10 mM ammonium acetate (A) and 30:70 formic acid (0.1%) in acetonitrile:10 mM ammonium acetate (B) at a flow rate of 1.0 ml/min. The following gradient was used: 0.0–2.5 min, 100% A; 2.5–5.5 min, 100%–20% A; and 5.5–6.5 min, 100% A. The MS/MS was operated in positive electrospray ionization (ESI) mode, and multiple reaction monitoring was applied. The ion transitions were *m/z* 178.1 → 148.1 and 182.1 → 152.1 for the analyte and the internal standard, respectively. Retention times for NNN and d_4-NNN were 5.64 and 5.58 min, respectively. The linear range was 0.75–99 pg/ml, and the R^2 was >0.99. The interday precision ranged from 1.7% to 9.5%, and the interday accuracy (% deviation from the theoretical value) ranged from −2.0% to 0% at six different quality control concentration levels.

Polycyclic aromatic hydrocarbon exposure: 3-Hydroxybenzo[*a*]pyrene. Levels of 3-hydroxybenzo[*a*]pyrene (3-OHBaP) in human urine were measured using an LC-MS/MS method. Further details of this analytic method and validation will be published separately; however, the following description should be sufficient to allow replication of the results. Human urine pools containing basal levels of 3-OHBaP (present as 3-OHBaP-glucuronide) and basal pools fortified with additional amounts of 3-OHBaP were used as quality control samples. Since background levels of 3-OHBaP in the majority of human urine sources were greater than the lower limit of quantitation (50 fg/ml), canine urine, free from 3-OHBaP, was used to prepare calibration standards. Two hundred microliters of 1 M sodium acetate buffer (pH 5.0), 50 µl of internal standard (d_5-3-OHBaP at 500 pg/ml), and 0.1 ml of β-glucuronidase from *H. pomatia* (14,350 units/ml in 20 mM of acetate buffer) were added to a 3-ml urine sample. The enzymatic hydrolysis of the sample was completed after 16–18 hr of incubation at 37 °C. The sample was then subjected to SPE on a Bond Elut-LMS cartridge (100 mg × 3 ml) preconditioned with 3 ml of methylene chloride and 2 ml of methanol sequentially. The sample was washed sequentially three times with methanol:water (5:95, 50:50, and 100:0, respectively) and once with 50:50 methanol:acetonitrile and eluted with 2 × 1 ml of methylene chloride. The sample was evaporated under nitrogen at 40 °C after the addition of 20 µl of DMSO. The analytes were then derivatized with 0.25 ml of 2-fluoro-1-methylpyridinium-*p*-toluenesulfonate (0.5 mg/ml in acetonitrile solution) and 50 µl of 0.2% dimethylethylamine at room temperature for 15 min. The sample was evaporated under nitrogen at 45 °C, and the residue was reconstituted in 0.25 ml of methanol:water:formic acid (50:49:1). The sample was centrifuged at 4,000 rpm for 10 min at 5 °C, and 50 µl of the sample was injected into an LC-MS/MS system (Acquity UPLC coupled with AB/Sciex API 5000). A Waters ethylene bridged hybrid C18 column (50 × 2.1 mm, 1.7 µm) with a frit filter (2.1 mm, 0.2 µM) was used. The elution was performed isothermally at 50 °C with a gradient consisting of 0.5% formic acid in water (A) and 0.5% formic acid in acetonitrile (B) at a flow rate of 0.7 ml/min. The following gradient was used: 0.0 min, 5% B, and 1.0–7.0 min, 20%–31% B, and kept at 31% B for 2.4 min; 9.4–9.9 min, 31%–90% B and then returned to 5% B. The MS/MS was operated in positive ESI mode, and the MRM was applied. The ion transitions were *m/z* 360.2 → 251.1 and 365.1 → 256.2 for the analyte and the internal standard, respectively. Retention times for the derivatized 3-OHBaP and d_5-3-OHBaP were 8.28 and 8.32 min, respectively. The linear range was 50–2,000 fg/ml, and the R^2 was >0.99. The interday precision ranged from 3.6% to 10.9%, and the interday accuracy (% deviation from the theoretical value) ranged from -3.6% to 0.2% at five different quality control concentration levels.

Benzene exposure. Exposure to benzene was measured by the 24-hr urinary excretion of the mercapturic acid metabolite, *S*-phenyl mercapturic acid (*S*-PMA), measured by an LC-MS/MS reported previously (Sarkar et al., 2008). The LLOQ was 60 pg/ml.

Exposure to aromatic amines. Urinary excretion of the aromatic amines o-toluidine, 2-aminonaphthalene, and 4-aminobiphenyl was measured based on a method reported previously (Riedel, Scherer, Engl, Hagedorn, & Tricker, 2006). The method used acid hydrolysis of the arylamine conjugates in urine, extraction with *n*-hexane, derivatization with pentafluoropropionic anhydride, and subsequent analysis with gas chromatography combined with MS using negative ion chemical ionization. The limits of detection were 4 ng/L for o-toluidine and 1 ng/L for 2-aminonaphthalene and 4-aminobiphenyl.

Urine mutagenicity. Urine mutagenicity (revertants per 24 hr) was assessed (Labstat International, Ontario, Canada) in a modified quantitative *Salmonella typhimurium* reverse-mutation assay (Maron & Ames, 1983; Sarkar, Nseyo, & Zhong,

2002) using concentrated aliquots of 24-hr urine collected from each subject. Urine samples were concentrated 250-fold by using XAD-2 resin and then diluted with DMSO to yield different dose levels. The samples were incubated at 37 °C with 5% Arochlor-induced rat liver S9 (metabolic activating system) and YG1024 (tester strain), which is an *O*-acetyltransferase–overproducing derivative of strain TA98 and has a high sensitivity for detecting the mutagenicity of aromatic amine compounds.

Carbon monoxide exposure. Exposure to carbon monoxide was determined by measuring COHb levels in the blood, assayed spectrophotometrically with a CO oximeter (IL Multi-4; Instrumentation Laboratory, Lexington, MA) at Covance Central Clinical Laboratory (Indianapolis, IN; Sarkar et al., 2008).

Statistical analysis methods

The sample size was determined based on urinary total NNAL levels, since it had been previously reported (Hecht et al., 2004) that NNAL levels are reduced by ~30% when AS reduced their number of cigarettes by ≥50%. Since NNAL had the longest half-life, if the sample size was determined to distinguish changes in urinary excretion of NNAL and its metabolites, then this sample size was considered to be sufficient for the other biomarkers. The sample size used in the study was considered adequate to detect a 30% reduction in total NNAL between the DU and CS groups with at least 80% power at a 5% significance level and two-sided testing. All randomized subjects with baseline measurement and at least one postbaseline measurement were included in the statistical analysis. Although the groups were stratified based on number of cigarettes smoked and gender, a statistical analysis (using the Fisher's Exact test) was conducted to examine the comparability between the groups accounting for the apparent imbalance in gender. The result confirmed that the groups were not statistically different in the proportion of gender $p = .3126$ (two sided), therefore it was not considered as a covariate in the linear mixed effects model. Data from subjects who withdrew early during the study were included up to the time the subjects stopped participating in the study. Baseline was defined as the average of values on Days −2 and −1 provided no day effect was detected between Days −2 and −1. Day −1 values were used as baseline levels if there were statistically significant differences between Day −2 and −1. Pooled postbaseline was defined as the average of values on Days 7 and 8 if no day effect was detected. Otherwise, Day 8 values were used as postbaseline. Descriptive statistics were calculated for demographic characteristics and smoking history at baseline. Descriptive statistics were calculated for each biomarker, daily cigarettes, and MSNUS consumption by study group and study day. A linear mixed model for analysis of variance was used to perform the comparisons in original and percent change from baseline to postbaseline biomarkers among the four study groups, with study group, study day, and the interaction between study group and study day as the model terms. Body mass index was used as a covariate in the model. The interaction term was removed if it was not statistically significant ($p > .10$). PROC MIXED procedure of SAS (v. 8.2; SAS Institute, Inc., Cary, NC) was used to run the analysis. Log transformation of data was applied on some of the analysis variables to achieve normal distribution. Differences were considered statistically significant at $p < .05$. Regression analysis was performed to examine the relationship of daily NE and total NNAL with cigarettes and MSNUS consumptions on Day 8 in the DU group. SAS PROC REG procedure was used to run the analysis. The adjustment for residual effect (Roethig et al., 2007) was carried out as follows: the percent change from baseline adjusted for residual correction = (postbaseline_adj – baseline_adj)/baseline_adj × 100, where Postbaseline_adj = group mean value at postbaseline – residual (where residual = postbaseline mean value for the biomarker from the NT group); baseline_adj = group mean value at baseline – residual. Values for the NT group were considered to be −100%. The median values were used for urine mutagenicity calculations. For all statistical analyses, Type I error rate was controlled at the 0.05 level for two-sided test.

Results

A total of 137 subjects were enrolled, and 120 subjects were randomized to one of the four groups; 115 subjects completed the study. The demographic information about the subjects is presented in Table 1. There were no statistically significant ($p > .05$) differences in the baseline characteristics of the subjects between the groups. There were no clinically significant trends among all the groups in the clinical laboratory, vital sign, electrocardiogram, or spirometry findings during this study. Headache was the most commonly reported postbaseline adverse event.

Daily cigarette consumption and MSNUS usage during the study are presented in Table 1. At baseline, average CPD were similar in all the four groups, ranging from 16.7 to 18.5. The cigarettes smoked on Day 8 were 15.6 ± 4.6 for subjects in the CS group and 8.4 ± 1.9 in the DU group. The average daily MSNUS consumption on Day 1 was higher for both the DU (3.2 ± 2.3) and SN (4.5 ± 2.5) groups compared with that observed on Day 8 (2.2 ± 2.6 for DU and 3.5 ± 3.2 for SN). The number of subjects using MSNUS was also higher on Day 1 (54/60 for DU and 13/15 for SN) compared with Day 8 (38/59 in DU and 10/15 in SN).

The biomarkers of exposure at baseline and postbaseline and the percent changes from baseline are presented in Table 2. Significant ($p < .05$) reductions (relative to baseline) were observed in all the urinary excretions in the DU and SN groups compared with the CS group. Stop smoking (NT) resulted in even greater reductions from baseline ($p < .0001$). For tobacco-specific biomarkers, relative to baseline, the mean 24-hr NE decreased by 34% in the DU group, 71% in the SN group, and 99% in the NT group; the mean 24-hr total NNAL decreased by 30%, 62%, and 69% for the DU, SN, and NT groups, respectively; and the mean 24-hr total NNN decreased by 47%, 81%, and 94% for the DU, SN, and NT groups, respectively. Similar levels of reduction were observed for the non–tobacco-specific biomarkers of exposure (Table 2).

The percent change from baseline for the biomarkers of exposure, after correcting for the residual levels observed in the AS in the NT group, are illustrated in Figure 2. For total NNAL, NNN, and NE, 36%–59% reductions were seen in the DU group and 78%–89% reductions were seen in the SN group. For the other biomarkers, approximately 50% reduction was observed in most of the biomarkers (except for o-toluidine ~24%, blood COHb ~29%, and 3-OHBaP ~71%) in the DU group, and 60%–99% reduction was seen in the SN group.

Table 1. Baseline characteristics and daily cigarette and/or MSNUS consumption at baseline and postbaseline

	Groups			
Trait	CS ($n = 30$)	DU ($n = 60$)	SN ($n = 15$)	NT ($n = 15$)
Age (years)	35.3 ± 8.1 (22–52)	32.6 ± 9.8 (21–61)	35.7 ± 8.3 (23–53)	34.6 ± 11.1 (21–52)
Sex (m/f, %)	21/9 (70/30)	46/14 (77/23)	12/3 (80/20)	8/7 (53/47)
BMI (kg/m^2)	25.9 ± 4.7 (19.5–37.4)	26.3 ± 4.3 (19.8–39.5)	29.9 ± 4.6 (22.0–39.5)	26.6 ± 4.3 (21.7–37.8)
Smoking years				
≤9	7	17	3	8
10–20	14	31	9	1
>20	9	12	3	6
Number of cigarettes				
Baseline	16.7 ± 3.8 (10–25)	17.6 ± 4.2 (10–29)	17.8 ± 5.3 (10–30)	18.5 ± 5.8 (10–29)
Postbaseline	15.6 ± 4.6 (0–22)	8.4 ± 1.9 (5–12)	NA	NA
MSNUS Usage				
n[a]	NA	38	10	NA
SPD	NA	2.2 ± 2.6 (0–14)	3.5 ± 3.2 (0–10)	NA
Duration (min)[b]	NA	61.3 ± 31.0 (4.15–135.42)	53.9 ± 32.2 (11.92–121.65)	NA

Note. $M \pm SD$ (range). BMI = body mass index; CS = continue smoking; DU = dual cigarette and MSNUS; NA = not applicable; NT = no tobacco; SN = MSNUS only; SPD = MSNUS per day.

[a]Number of subjects who used MSNUS.

[b]Duration of MSNUS in mouth.

The Day 8 plasma nicotine levels measured at 7:00, 9:07, 11:48, 13:55, 17:08, and 19:15 for the CS, DU, and SN groups are presented in Figure 3. No statistically significant differences ($p > .05$) in the plasma nicotine concentrations were observed between the DU and CS groups at the 9:07-, 13:55-, and 19:15-hr timepoints. The plasma nicotine concentrations were significantly lower when the subjects were allowed to use only MSNUS (at 11:48 and 17:08) in the DU group. In addition, the average plasma nicotine concentrations were substantially lower ($p < .001$) in the SN group at each timepoint of measurements as compared with either the CS or the DU group. The postbaseline plasma cotinine and *trans*-3′-hydroxy cotinine levels (Figure 4) were observed to be significantly different ($p < .05$) between the three groups (except for plasma cotinine at 19:15, $p = .0714$).

Regression analysis for the relationship between tobacco-specific biomarkers of exposure and daily tobacco consumption indicated that both CPD and MSNUS per day (SPD) had significant ($p < .001$) effects on NE levels ($R^2 = 0.64$). However, total NNAL excretion ($R^2 = 0.18$) was affected significantly only by CPD ($p = .0025$), but not by MSNUS consumption (SPD, $p = .7924$).

Discussion

The results from this study demonstrate that under the conditions of the study, when AS reduced their number of cigarettes by 50% or more and used MSNUS, there was a corresponding 50% reduction in most of the biomarkers of exposure to cigarette smoke. These results suggest that dual usage of cigarettes and MSNUS does not result in compensatory changes in the way each cigarette was smoked. The exposure to tobacco-specific constituents and other smoke constituents in the AS who stopped smoking cigarettes and switched to exclusive MSNUS usage was minimal compared with when they were smoking.

To date, there are *no* reports in literature where a systematic assessment of cigarette smoke exposure has been carried out in AS reducing their number of cigarettes *and* using tobacco pouch products like MSNUS. A significant reduction in smoke exposure was observed, evident from the reductions in urinary (NNAL, NNN, NE, o-toluidine, 2-aminonaphthalene, 4-aminobiphenyl, *S*-PMA, 3-OHBaP, and urine mutagenicity) and blood biomarkers of exposure (COHb) in the DU group compared with the CS group. These observations are applicable to the product investigated in this study and may not be generalized for all snus-like tobacco pouch products. Total NNAL levels in the DU group were reduced by 30%, similar to that observed in AS *only* reducing the numbers of cigarettes by 50%, measured over a 4-week period (Hatsukami et al., 2006). The levels of the other nitrosamine, total NNN, which has a relatively shorter half-life (Adams et al., 1985), were reduced proportionately (54%) to the 50% reduction in cigarette consumption in the DU group, suggesting very little exposure from MSNUS usage.

Additionally, plasma nicotine, cotinine, and *trans*-3′-hydroxycotinine were statistically significantly lower in the DU group compared with those who continued to smoke (CS group), during the periods when smoking was not allowed. It was observed that the number of cigarettes smoked during the restricted smoking periods in the DU group was similar to that smoked by the subjects in the CS group (approximately two to three cigarettes over the 2-hr interval). Therefore, it is not surprising that plasma nicotine levels at the end of the smoking period, which resulted from smoking similar numbers of cigarettes, were not significantly different, due primarily to the short half-life of nicotine (~200 min; Benowitz, Jacob, Denaro, & Jenkins, 1991). It has been suggested in literature (Rosa et al., 1992) that AS strive to maintain a steady-state nicotine level. The peaks and troughs in the plasma nicotine levels suggest that

Table 2. Biomarker of exposure at baseline and postbaseline and % change from baseline

	Groups							
	CS		DU		SN		NT	
Biomarkers	Baseline	Postbaseline	Baseline	Postbaseline	Baseline	Postbaseline	Baseline	Postbaseline
Total NNAL (ng/24 hr)	693.24 ± 339.86	599.95 ± 294.56	548.35 ± 258.27	370.58 ± 171.86	752.85 ± 401.77	278.25 ± 156.99	683.61 ± 598.03	198.25 ± 167.16
% Change		-13.86 ± 15.73		-30.36 ± 14.77[a]		-61.52 ± 12.96[a,b]		-69.41 ± 7.46[a]
Total NNN (ng/24 hr)	18.92 ± 11.74	15.22 ± 11.53	18.44 ± 13.24	9.39 ± 7.62	26.61 ± 17.76	4.52 ± 7.14	28.20 ± 33.71	0.83 ± 0.42
% Change		-24.26 ± 25.13		-47.16 ± 24.58[a]		-81.20 ± 20.72[a,b]		-93.87 ± 6.73[a]
NE (mg/24 hr)	20.76 ± 6.79	17.77 ± 6.56	17.79 ± 6.50	11.30 ± 5.06	21.47 ± 8.35	5.53 ± 5.21	18.00 ± 9.33	0.08 ± 0.04
% Change		-14.01 ± 24.84		-34.33 ± 25.36		-71.19 ± 25.87[a,b]		-99.48 ± 0.16[a]
o-Toluidine (ng/24 hr)	266.43 ± 188.71	278.26 ± 222.95	274.07 ± 257.35	220.59 ± 251.75	329.51 ± 351.72	162.82 ± 330.65	192.22 ± 75.52	54.38 ± 16.31
% Change		10.98 ± 67.49		-21.42 ± 51.31[a]		-60.81 ± 27.39[a]		-64.99 ± 24.01[a]
2-Aminonaphthalene (ng/24 hr)	37.76 ± 14..67	31.85 ± 12.38	30.70 ± 12.25	15.55 ± 6.31	38.61 ± 17.21	2.74 ± 1.08	33.50 ± 17.65	3.81 ± 4.74
% Change		-13.88 ± 24.53		-48.10 ± 11.49[a]		-91.71 ± 4.68[a]		-81.42 ± 36.83[a]
4-Aminobiphenyl (ng/24 hr)	22.13 ± 7.77	19.22 ± 7.29	18.72 ± 7.55	9.88 ± 3.79	23.04 ± 10.43	2.91 ± 0.92	19.61 ± 10.67	2.25 ± 0.88
% Change		-13.81 ± 22.18		-44.86 ± 16.58[a]		-85.92 ± 5.19[a]		-87.24 ± 4.52[a]
S-PMA (μg/24 hr)	1.24 ± 0.56	1.03 ± 0.48	0.98 ± 0.51	0.58 ± 0.26	1.06 ± 0.46	0.24 ± 0.05	0.99 ± 0.31	0.23 ± 0.11
% Change		-12.71 ± 28.56		-37.10 ± 18.72[a]		-73.18 ± 12.92[a]		-76.30 ± 9.89[a]
3-OHBaP (pg per 24 hr)	193.10 ± 100.63	155.03 ± 91.03	132.95 ± 72.22	78.01 ± 45.21	192.27 ± 128.35	78.89 ± 51.15	162.70 ± 126.33	55.93 ± 21.71
% Change		-15.36 ± 31.48		-36.75 ± 23.04[a]		-44.56 ± 36.84[a]		-55.70 ± 21.74[a]
COHb (% sat)	6.96 ± 2.30	6.37 ± 2.07	6.13 ± 1.78	4.74 ± 1.30	6.99 ± 2.45	1.53 ± 0.19	5.62 ± 1.89	1.35 ± 0.44
% Change		-6.45 ± 19.84		-21.03 ± 15.90[a]		-75.19 ± 10.16[a]		-74.64 ± 7.98[a]
Plasma nicotine (AUC)[c], ng/ml×hr	195.67 ± 73.05	170.34 ± 83.54	202.02 ± 71.41	132.94 ± 48.28	225.55 ± 90.10	40.31 ± 49.29	201.78 ± 87.04	–
% Change		-14.03 ± 23.06		-32.51 ± 17.48		-75.69 ± 32.49[a]		–
Plasma cotinine (AUC)[c], ng/ml×hr	3566.71 ± 1415.98	3139.96 ± 1441.78	3142.33 ± 911.08	2008.00 ± 798.18	3617.91 ± 1546.29	781.87 ± 822.12	3287.14 ± 1134.86	–
% Change		-12.19 ± 26.15		-34.32 ± 29.08		-72.17 ± 31.82[a]		–
Plasma *trans*-3-OH-cot (AUC), ng/ml×hr	1374.52 ± 595.65	1273.29 ± 555.34	1173.17 ± 539.42	827.61 ± 448.43	1187.42 ± 587.60	286.76 ± 322.41	1362.71 ± 746.22	–
% Change		-4.57 ± 30.66		-25.38 ± 39.14		-66.17 ± 40.04[a]		–
Mutagenicity (revertants per 24 hr)[d]	25,929	23,810	19,863	11,416	36,946	1,192	32,118	1,249
% Change		9.89		-50.61[a]		-97.34[a]		-96.67[a]

Note. Data are presented as $M \pm SD$. BMI = body mass index; COHb = carboxyhemoglobin; CS = continue smoking; DU = dual cigarette and MSNUS; NE = nicotine equivalent; NT = no tobacco; NNAL = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNN = *N*′-nitrosonornicotine; SN = MSNUS only; SPD = MSNUS per day; *S*-PMA = *S*-phenyl mercapturic acid; 3-OHBaP, 3-hydroxybenzo[*a*]pyrene.

[a] $p < .05$ for comparison with values in the CS group.

[b] $p < .05$ for comparison with values in the NT group.

[c] Area under the curve ($AUC t_0 - t_1$) values (ng/ml×hr), where t_0 is at 7:00 and t_1 19:15.

[d] Data are shown as median.

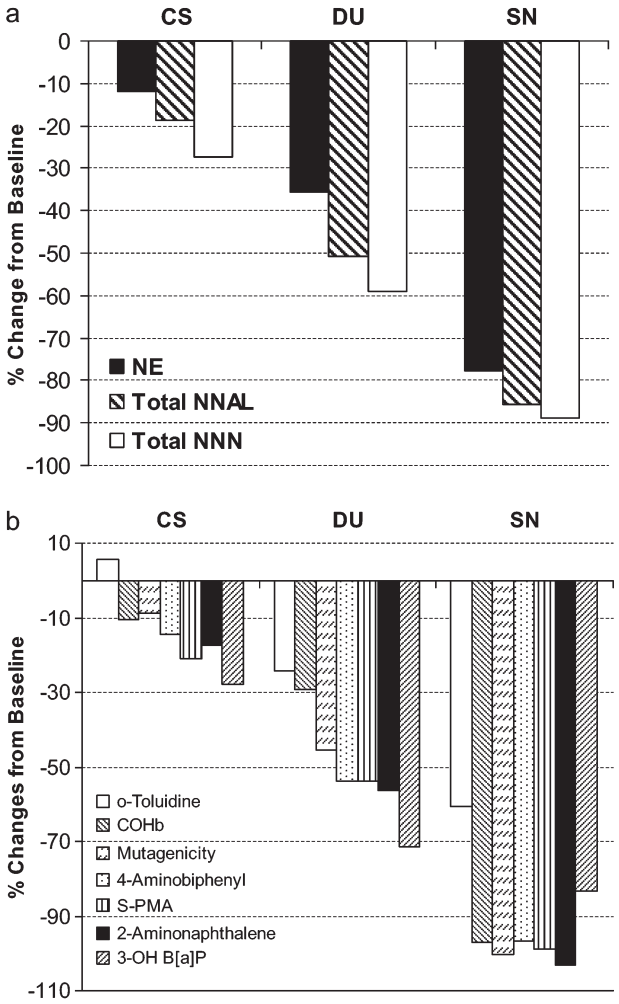

Figure 2. Percent changes of tobacco-specific (a) and non–tobacco-specific (b) biomarkers of exposure from baseline adjusted for residual values. Data shown are adjusted for the residual effect based on the postbaseline values from the no-tobacco group. CS = continue smoking group; DU = dual cigarette and MSNUS group; SN = exclusive MSNUS group.

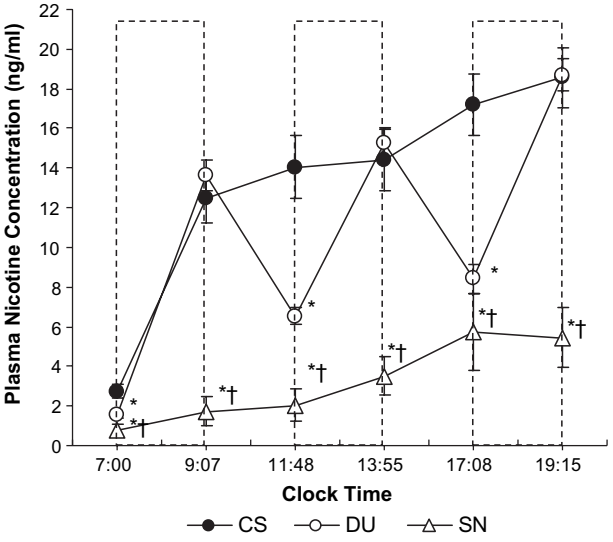

Figure 3. Plasma nicotine concentrations in adult smokers measured at different time intervals on Day 8. CS = continue smoking; DU = dual cigarette and MSNUS; SN = MSNUS only. Data shown as mean ± *SE*. Numbers of cigarettes smoked during restricted smoking period (dashed-line box) were similar between the CS and DU groups. *$p < .05$ compared with the CS group; †$p < .05$ compared with the DU group.

the AS in the DU group, despite having the opportunities to supplement their tobacco use with MSNUS, did not appear to be reaching out to maintain steady-state nicotine blood levels. These peaks and troughs in plasma nicotine levels observed in the DU group disappear (Figure 4) when the plasma levels of the two metabolites with longer half-lives (10–16 hr; Benowitz & Jacob, 2001; Curvall, Elwin, Kazemi-Vala, Warholm, & Enzell, 1990) are compared. In order to gain understanding of usage behavior of oral tobacco use in future clinical studies, plasma nicotine levels might be more relevant than urinary NE. However 24-hr urinary NE might still be appropriate when assessing overall daily exposure (Sarkar et al., 2008) related to cigarette smoking.

It should be noted that biomarkers of exposure to selected smoke constituents (for example, benzene, benzo[*a*]pyrene) provide a measure of cigarette smoke as well as environmental sources of exposure—for example, charbroiled meat for the benzo[*a*]pyrene (Strickland & Kang, 1999) or diesel exhaust for benzene (Dor, Dab, Empereur-Bissonnet, & Zmirou, 1999). Since these biomarkers have a relatively short half-life (Feng et al., 2006; Sarkar et al., 2008), the residual levels observed toward the end of the study can be assumed to be primarily from the non–cigarette-related exposure (Sarkar et al.). Therefore, the biomarkers' levels were adjusted based on the residual levels observed in the NT group. This residual adjustment also takes into consideration biomarkers of tobacco-specific constituents that have a relatively long half-life—for example, NNAL and its glucuronide conjugates (Hecht et al., 1993). After correcting for the residual effect, a ≥50% reduction in daily cigarette consumption and dual MSNUS usage resulted in a corresponding ~50% reduction in most of the biomarkers (except for urinary NE ~36%, o-toluidine ~24%, and blood COHb ~29%). These results suggest that there was minimal contribution to the exposure from MSNUS usage in the DU group and the smokers did not change the way they smoked each cigarette to compensate for the reduction in number of cigarettes. It is not surprising that the NE levels did not reduce by 50%, probably because of the additional nicotine uptake from MSNUS usage, as observed from the exclusive MSNUS users. The 50% reduction in the TSNA levels suggests that there was minimal contribution to the TSNA exposure from MSNUS usage. These conclusions are further corroborated by the linear regression analysis between NE, total NNAL, and the number of cigarettes and MSNUS, where a significant correlation was observed with only the cigarettes (CPD) but not MSNUS (SPD). Furthermore, it highlights the fact that the uptake of tobacco constituents from MSNUS into the body is relatively small, which must be taken into consideration when interpreting the levels of these constituents in the original product, reported recently (Stepanov et al., 2008).

Of the biomarkers of exposure to aromatic amines, 4-aminobiphenyl and 2-aminonapthalene were reduced by ~50%

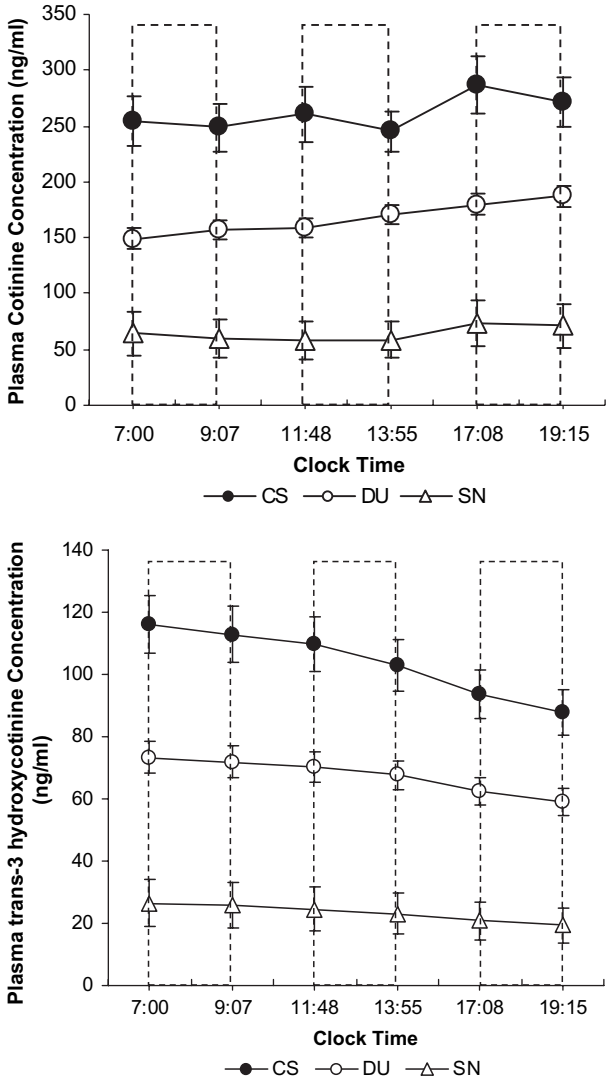

Figure 4. Plasma (a) cotinine and (b) *trans*-3′-hydroxycotinine concentrations in adult smokers measured at different time intervals on Day 8. CS = continue smoking; DU = dual cigarette and MSNUS; SN = MSNUS only. Data are shown as mean ± *SE*. Numbers of cigarettes smoked during restricted smoking period (dashed-line box) were similar between the CS and DU groups.

compared with baseline after residual correction, whereas the reduction in o-toluidine was not comparable (~24%) despite the residual adjustment. The reason for this apparent discrepancy could possibly be related to large intra- and interindividual variations in the urinary excretion of o-toluidine as reported in the literature (el Bayoumy, Donahue, Hecht, & Hoffmann, 1986) and seen in this study. The coefficient of variability, in the percent change from baseline values, was much larger for o-toluidine (%CV = 239.5%) relative to that observed for 2-aminonapthalene (%CV = 23.9%) and 4-aminobiphenyl (%CV = 36.9%). These observations suggest that o-toluidine might not be an appropriate biomarker to estimate aromatic amine exposure, particularly since the other biomarkers, such as 4-aminobiphenyl and 2-aminonapthalene, appear to be far more reliable and robust. Similar variability in the COHb levels (%CV = 76%) and differences in baseline levels between the groups might explain why a proportionate reduction in the DU group was not observed in COHb despite residual correction.

AS in the SN group, who exclusively used MSNUS over the 8-day period, manifested an even larger reduction in all the urinary and blood biomarkers of exposure when compared with those in the DU group. The percent change from baseline in the biomarker levels for subjects in the SN group ranged from −45% (3-OHBaP) to −97% (urine mutagenicity). These reductions were not statistically different ($p > .05$) from that observed in the NT group for all the biomarkers except urinary NNN, NNAL, and NE. The reason for the tobacco-specific biomarker levels being slightly higher than the levels observed in the NT group could be the relatively small contribution from MSNUS usage. Nevertheless, these reductions are still relatively large when compared to the CC group. The percent reduction from baseline in the SN group was 62% versus 69% for the NT group for NNAL, suggesting that despite the relatively small uptake of nitrosamines from the tobacco pouches, there were large reductions in nitrosamine exposure relative to cigarettes. The large reductions in exposure observed in the AS switching to exclusive MSNUS usage, if sustained for a long enough period, may eventually manifest into clinically favorable reductions, particularly since it has been reported that the mortality of ST users is not significantly greater than that of non–tobacco users and is appreciably less than that of cigarette smokers (Hergens, Ahlbom, Andersson, & Pershagen, 2005; Huhtasaari, Asplund, Lundberg, Stegmayr, & Wester, 1992; Huhtasaari, Lundberg, Eliasson, Janlert, & Asplund, 1999).

AS in the SN group sustained significantly lower plasma nicotine levels compared with the CS group at postbaseline. The reduction in plasma nicotine was probably due to the lower tobacco consumption at postbaseline (~4 pouches per day) relative to baseline (~18 CPD). The lower plasma nicotine levels also suggest that the nicotine uptake from the pouches was relatively low. Indeed, it would be difficult to achieve the same nicotine level as that from smoking with the current MSNUS product. The important observation in this study was that, despite having opportunities to increase the frequency of using MSNUS products, the smokers chose to use fewer products and used them for a long time period (in both the DU and SN groups). Although the reasons are not clearly established, some of the AS using this version of MSNUS *may* be obtaining a satisfying tobacco experience at the usage level observed in this study by using for a relatively long time (up to 2 hr). This is the first report in which the actual residence time for pouch usage was accurately determined. Surprisingly, the overall average residence time per pouch for the MSNUS was ~54 min in the SN group and ~61 min in the DU group, lasting much longer than the time to smoke a cigarette (~5–7 min), which might also be the reason why the AS consumed only a relatively small amount of MSNUS units per day. It needs to be determined in an ambulatory setting whether using this product might actually delay the urge for a cigarette.

The variable consumption pattern observed in our study may be indicative of personal preferences of the AS to adopt a change in their tobacco usage behavior. It was observed that some smokers readily adopted this ritual change, while others never even attempted to try this change. About 31% of the AS in the DU group did not use any pouches on the postbaseline Days 7 and 8, whereas ~14% of the subjects in this group used 5 or

more pouches on both postbaseline Days 7 and 8, some using up to 10 pouches per day in the SN group. The variability in the consumption behavior did not appear to impact the reduction in biomarkers of exposure. For example, the median percent change from baseline in plasma nicotine AUC in the DU group, for those who did not use any pouches on Day 8, was −38% compared with −32% in those who used one or more pouches. It was also observed that in the SN group, all but one of the subjects tried MSNUS over the 8-day period, the usage varying from 1 to 10 MSNUS per day. This large variability in MSNUS consumption was surprising considering that during the prescreening product trial usage, all the subjects recruited in the study had an opportunity to try this product over a 2-week period and had expressed a willingness to use this product during the study. The actual usage behavior will need to be confirmed in an ambulatory setting. There is some evidence providing indications in this direction; for example, substantial initial interest for newly introduced snus products in a test market was observed among male smokers (Biener & Bogen, 2009). The impact of promotional mailings on this initial interest could not be clearly distinguished.

Overall, under the conditions of this study, AS when reducing the number of cigarettes by 50% and using MSNUS exhibited a corresponding 50% reduction in biomarkers of smoke exposure. It is important to note that while these reductions were observed in our study specifically with the MSNUS product investigated, these observations may not be generalized to other types of ST, such as chewing tobacco or moist snuff. In addition, further studies may be needed to determine whether similar reductions would be observed with other brands of snus-like tobacco pouch products. One of the limitations of this study was that it was conducted in a clinical setting under restricted smoking conditions, and extrapolation of these results to an ambulatory setting should be carried out with caution. Nevertheless, it has been observed in ambulatory settings by others that dual tobacco usage may either lead to significantly fewer cigarettes smoked per day compared with exclusive cigarette smokers (Furberg, Lichtenstein, Pedersen, Bulik, & Sullivan, 2006; Hellqvist, Rolandsson, Birkhed, & Hugoson, 2009) or eventually lead to smoking cessation (Furberg et al., 2005, 2008). Therefore, dual use of smoking and MSNUS (in the current version) might present an opportunity for overall smoke exposure reduction in AS; however, the specific outcome remains to be fully investigated in the actual usage settings. If the number of cigarettes smoked is reduced by a large enough number and sustained for a long enough period, then a favorable impact on smoking-related disease risks may be anticipated. Indeed, Godtfredsen, Prescott, and Osler (2005) have demonstrated that among individuals who smoke 15 or more CPD, smoking reduction by 50% significantly reduced the risk for lung cancer. Furthermore, since no added disease risk in smokers using tobacco pouch products has been reported (Luo et al., 2007), at least no negative impact should be expected from dual usage.

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Declaration of Interests

None declared.

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