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Assessment of biomarkers of exposure and potential harm, and physiological and subjective health measures in exclusive users of nicotine pouches and current, former and never smokers

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ABSTRACT

Background: Oral nicotine pouches (NPs) are smokeless, tobacco-free products that have a potential role in tobacco harm reduction strategies.

Methods: In a cross-sectional study in Sweden/Denmark, several recognised biomarkers of potential harm (BoPHs) linked to smoking-related diseases/their initiating biological processes, and biomarkers of exposure (BoEs) to tobacco/tobacco smoke toxicants were compared among exclusive adult users of Velo NPs and current/former/never smokers. Over 24 h, participants used their usual product (Velo NP or cigarette) as normal, and BoEs/BoPHs were assessed via blood/24-h urine/exhaled breath/physiological assessments.

Results: Among the primary endpoints, total NNAL (16.9 ± 29.47 vs 187.4 ± 228.93 pg/24 h), white blood cell count (5.59 ± 1.223 vs 6.90 ± 1.758 × 10^9/L), and COHb (4.36 ± 0.525 vs 8.03 ± 2.173% saturation) were significantly lower among Velo users than among smokers (91%, 19% and 46% lower, respectively, all $P < 0.0001$), while fractional exhaled NO, previously shown to be lower in smokers, was significantly higher (23.18 ± 17.909 vs 11.20 ± 6.980 ppb) among Velo users (107% higher, $P < 0.0001$). Furthermore, sICAM-1 tended to be lower (185.9 ± 42.88 vs 204.5 ± 64.85 ng/mL) among Velo users than smokers (9% lower). Several secondary endpoints, including six BoEs (3-HPMA (246.7 ± 91.07 vs 1165.7 ± 718.35 µg/24 h), 3-OH-B[*a*]P (82.4 ± 217.58 vs 258.3 ± 190.20 pg/24 h), HMPMA (135.1 ± 77.85 vs 368.8 ± 183.15 µg/24 h), MHBMA (0.22 ± 0.166 vs 3.39 ± 2.943 µg/24 h), S-PMA (0.10 ± 0.059 vs 3.53 ± 2.736 µg/24 h) and total NNN (7.5 ± 24.84 vs 9.7 ± 5.93 ng/24 h)), were significantly lower among Velo users (78.8%, 68.1%, 63.4%, 93.5%, 97.2% and 22.7% lower, respectively, $P < 0.0001$–0.0011), while total nicotine equivalents was significantly higher among Velo users (22.6 ± 12.69 vs 12.1 ± 7.92 mg/24 h, $P < 0.0001$), although Velo user levels are comparable to those previously reported among oral tobacco users, and Velo user and smoker mean levels were similar in Denmark.

Conclusion: As compared with smokers, exclusive users of Velo NPs have significantly less exposure to tobacco toxicants and more favourable BoPHs associated with initiating biological processes of smoking-related diseases.

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Biomarkers of exposure; biomarkers of potential harm; nicotine pouches; tobacco harm reduction; cross-sectional clinical study

Introduction

Smoking-related diseases such as lung cancer and cardiovascular disease (CVD) are due to the long-term inhalation of tobacco smoke (US Department of Health and Human Services (US DHHS) 2014, Doll *et al*. 1994), which contains multiple harmful and potentially harmful constituents (HPHCs) (US Food and Drug Administration (FDA) 2012a, Rodgman & Perfetti 2013). These diseases develop gradually over time through pathological mechanisms involving DNA damage and oxidative stress, among other factors (Fearon *et al*. 2011, US DHHS 2010). Because many smokers will not otherwise quit (Chaiton *et al*. 2016), many health bodies now support an approach of tobacco harm reduction (THR) (Royal College of Physicians (RCP) 2016, Royal College of General Practitioners (RCGP) 2017, Public Health England (PHE) 2019), in which smokers are encouraged to switch tobacco cigarettes for an alternative nicotine product with potentially fewer health risks than continued smoking (Institute of Medicine (IOM) 2001).

Tobacco harm reduction is supported by data from Sweden, where overall tobacco product use is similar to that of other European countries (Clarke *et al*. 2019), but the incidence of smoking-related mortality is much lower (Swedish Match 2020, Ferlay *et al*. 2013). This is due to the historical displacement of cigarette smoking by use of snus

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(Clarke *et al*. 2019, World Health Organization (WHO) 2019), a moist tobacco product that is placed next to the gum, enabling the nicotine to be absorbed through the oral mucosa. The absence of exposure to tobacco smoke is the main factor leading to the reduced health risks of snus as compared to conventional cigarettes, which has been demonstrated in extensive epidemiological and prevalence studies (Gartner *et al*. 2007, Lee 2013, Clarke *et al*. 2019). Consistent with this, snus has been recognised as a nicotine product that can reduce the risk of developing smoking-related diseases as compared to cigarette smoking by the FDA, who granted 'Modified Risk' status to eight snus products (General, Swedish Match) (FDA 2019), permitting labelling of these products in the US as reduced risk.

Nicotine pouches (NPs), a recent category of nicotine product that has similar potential to reduce the health risks of smoking, have been commercially available since the mid-2010s in countries such as the US, UK, Sweden and Denmark. Similar to snus, these oral products are placed between the gum and top lip, where nicotine and flavourings are gradually released and the nicotine is then absorbed through the oral mucosa (Plurphanswat *et al*. 2020, Lunell *et al*. 2020, Azzopardi *et al*. 2022a). Tobacco is known to contain many toxicants (Rodgman & Perfetti 2013). Because NPs contain pharmaceutical-grade nicotine added to a simple cellulose-based matrix without tobacco, they are likely to present similar or fewer health risks than snus. Indeed, recent studies have demonstrated that NPs contain significantly fewer toxicants (Azzopardi *et al*. 2022a, Jablonski *et al*. 2022) and produce greatly reduced toxicological effects (Bishop *et al*. 2020, Yu *et al*. 2022) as compared with snus and cigarette smoke.

While their reduced HPHC content and absence of combustion support the use of NPs in a THR approach, at present there are no data on users' actual exposure to toxicants from these relatively new products. In this regard, clinical studies have begun to measure levels of biomarkers in biological samples from users of other modern nicotine and tobacco products in order to determine internal dose of HPHCs. Several studies have shown that users of nicotine products such as vapour products (e.g. e-cigarettes) and tobacco heating products (THPs) have considerably lower levels of several biomarkers of exposure (BoEs) to tobacco smoke HPHCs as compared with current smokers (e.g. Round *et al*. 2019, Hatsukami *et al*. 2020, Oliveri *et al*. 2020, Gale *et al*. 2021, McEwan *et al*. 2021). Similarly, biomarkers of potential harm (BoPHs) can give an indication of changes in a user's wider biological system due to mechanistic and physiological effects caused by toxicant exposure (IOM 2001, Chang *et al*. 2019). For example, BoPHs related to the development of CVD and respiratory disorders, and pathological mechanisms such as oxidation and inflammation have also been shown to be lower among e-cigarette and THP users than among smokers (Oliveri *et al*. 2020, Gale *et al*. 2021).

Such studies can help to build an overall picture of comparative health risks posed by novel nicotine and tobacco products as compared to cigarette smoking (IOM 2012) and thereby inform regulatory processes (IOM 2012, FDA 2012b). The aim of the present study was therefore to assess whether the lower toxicants found in NPs relative to tobacco smoke translate to lower levels of selected BoEs, and favourable changes in selected BoPHs, physiological and subjective measures of health by comparing the results between adults who exclusively use NPs and adult current smokers. We hypothesised that there would be reductions in the levels of BoEs (except for total nicotine equivalents (TN_{eq})), and favourable changes in BoPHs, physiological and subjective measures of health in users of the NPs, and former and never smokers compared with current smokers.

Methods

Study design

The present cross-sectional study was conducted among exclusive users of Velo NPs and current, former and never smokers attending one of two centres in Herlev, Denmark, and Uppsala, Sweden, between March 2021 and January 2022. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice after obtaining ethical approval from the Scientific Ethics committees centre for Regional Development Health Research and innovation, Denmark, and the Ethical Review Authority, Sweden. All participants provided written informed consent prior to undergoing any screening procedures. The study design and protocol has been described in full elsewhere (Azzopardi *et al*. 2022b) and is reported in accordance with International Council for Harmonisation guidelines. The study was registered on the ISRCTN registry (ISRCTN16988167).

Objectives

The primary objective was to assess quantitative differences in the levels of key BoPHs (fractional exhaled nitric oxide (FeNO), 8-epi-prostaglandin $F_{2α}$ Type III (8-epi-PGF$_{2α}$ Type III), carboxyhaemoglobin (COHb), white blood cell count (WBC), soluble intercellular adhesion molecule-1 (sICAM-1) and high-density lipoprotein (HDL)) between Velo users and current smokers. In addition, differences in the level of total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), both a BoE for NNK exposure and a BoPH linked to the development of lung cancer in smokers (Church *et al*. 2009, Hecht *et al*. 2013, Xue *et al*. 2014), was also quantitatively assessed between Velo users and current smokers.

The secondary objectives were to assess quantitative differences in the levels of seven BoEs (TN_{eq}, monohydroxybutenylmercapturic acid (MHBMA), 3-hydroxy-1-methylpropylmercapuric acid (HMPMA), 3-hydroxypr opylmercapturic acid (3-HPMA), total *N*-nitrosonornicotine (NNN), *S*-phenylmercapturic acid (S-PMA) and 3-hydroxyben zo(a)pyrene (3-OH-B[*a*]P)), one BoPH (11-dehydrothromboxane B2 (11-dTX B2)), two physiological measures (forced expiratory volume in one second as a percentage of the predicted value (FEV_1%pred) and carotid intima-media thickness (CIMT)), quality of life (QOL) and oral health scores between Velo users and current smokers. In addition, all endpoints were qualitatively compared among the four study groups (NP users, smokers, former smokers and never smokers).

Furthermore, the amount of nicotine transferred from NPs during use was also assessed.

Study participants

Potential participants in Sweden and Denmark were identified from a database of individuals registered for clinical trials at each participating clinic, or through study-specific advertising (e.g. social media) and/or external recruitment agencies. Potential participants were invited to attend the clinic for a screening session. Eligible participants were healthy, adult (age 19–55 years inclusive), exclusive users of NPs, and current (≥10 cigarettes/day), former and never smokers. The aim was to recruit an equal number of participants from Sweden and Denmark for each group.

The full inclusion and exclusion criteria are given in Azzopardi *et al*. 2022b. The main inclusion criteria were general good health and no clinically relevant abnormal findings on physical examination, vital signs assessment, electrocardiogram, clinical laboratory evaluations or lung function tests, or on medical history. The main exclusion criteria were pregnant/breastfeeding (females); blood donation (≥400 mL) within 90 days of screening; and acute illness requiring treatment within 28 days of screening. In addition, participants were asked to avoid (1) alcohol completely for a period of 24 h before the study; (2) eating food containing poppy seeds for three days; and (3) eating or cooking cruciferous vegetables or grilled/fried food for 48 h prior to attending the clinic.

Participants in the Velo group were self-reported users of at least three Velo NPs (marketed as Lyft at the time of the study in Sweden and Denmark; British American Tobacco) per day for a minimum of six months prior to screening (urinary cotinine ≥200 ng/mL and exhaled breath carbon monoxide (eCO) <7 ppm at screening and at admission). Participants in the current smoker group were self-reported solus smokers of at least 10 combustible cigarettes per day for at least one year prior to screening (cotinine ≥200 ng/mL and eCO ≥7 ppm). Participants in the former smoker group were self-reported ex-smokers who stopped at least six months before screening (cotinine <200 ng/mL and eCO <7 ppm). Participants in the never smoker group self-reported having never smoked (<100 cigarettes smoked in their lifetime and none within the past six months; cotinine <200 ng/mL and eCO <7 ppm).

Compliance with long-term smoking abstinence and short-term smokeless tobacco abstinence in the Velo and former smoker groups was verified by analysis of *N*-(2-cyanoethyl)valine (CEVal) in erythrocytes (Gale *et al*. 2021), and urinary levels of anabasine (AB) and anatabine (AT), respectively (Jacob *et al*. 1999, Jacob *et al*. 2002). CEVal has been demonstrated as a measure of compliance with non-smoking among THP users (Gale *et al*. 2021), while urinary levels of two tobacco alkaloids, AB and AT, have been shown to correlate with self-reported tobacco use among smokers and smokeless tobacco users (Jacob *et al*. 2002) and have been reported as biomarkers of tobacco use among smokers (von Weymarn *et al*. 2016). Despite snus being recognised as a reduced risk product (FDA 2019), it generally contains higher numbers and levels of HPHCs compared with NPs (Azzopardi *et al*. 2022a). Therefore, snus use could potentially affect BoEs and BoPHs if participants in the NP group used snus but failed to report this, which is possible given the similarities in use and physical characteristics of NPs and snus. Hence, a further compliance assessment of AB and AT was included as CEVal, a biomarker for acrylonitrile exposure, cannot detect snus use.

Investigational products

No investigational products were provided during the study; instead, participants were asked to bring their own supply of NPs or cigarettes for use during the confinement period. All participants in the NP group were self-reported users of Velo-brand NPs and used only this brand during confinement. Participants in the current smoker group smoked their own brand of factory-made cigarette.

Study protocol

Potential participants attended a screening session, which included physical examination, routine clinical laboratory testing, alcohol and drug consumption testing, and pregnancy testing. Nicotine use and smoking status were assessed by CO and cotinine testing, and extent of tobacco and nicotine use was determined by questionnaire. Individuals who met the inclusion criteria were enrolled in the study.

Participants attended the clinic within seven days of screening, bringing with them sufficient NPs or cigarettes to last the confinement period. After eligibility was reconfirmed, participants were admitted to the study (Day 1) and immediately began the 24-h study period. During this time, they were asked to use their NPs or cigarettes as and when they normally would, and all urine voided was collected for analysis of biomarkers. In the NP user group, pouches were collected after use in addition to three unused pouches to measure nicotine content in order to estimate nicotine transfer to the user.

On days 1 or 2, prior to discharge, blood samples (maximum 100 mL) were obtained by direct venepuncture or a cannula in the forearm, and physiological assessments were carried out. Oral health and QOL were evaluated by the Oral Health Assessment Tool (OHAT) (Chalmers *et al*. 2004, NICE 2016) and RAND 36-Item Short Form Health Survey (RAND 2021), respectively (Supplementary Table S1). Participants were discharged after the completion of all study and safety assessments (Figure 1). Post-study follow-up was performed by telephone call within the following seven days.

Participants were able to withdraw from the study at any time and for any reason. The principal investigator could also withdraw participants from the study, for example, for protocol deviations or health reasons.

Study assessments

Compliance

Long-term smoking abstinence and short-term smokeless tobacco abstinence prior to the study was confirmed for

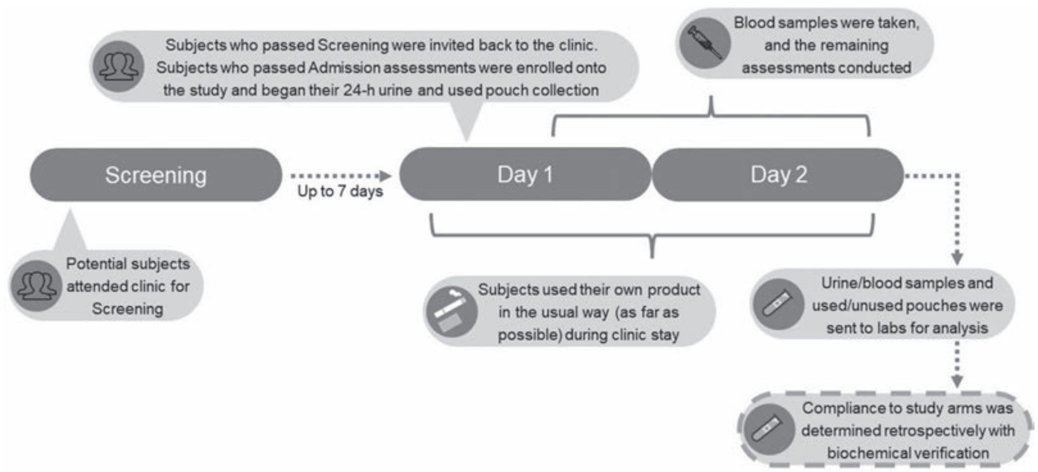

Figure 1. Schematic of the study protocol.

participants in the NP, and former smoker groups by measurement of CEVal in erythrocytes derived from 5 mL of whole blood (Scherer *et al*. 2014), and measurement of urinary AB/AT (Jacob *et al*. 1999, Jacob *et al*. 2002), respectively. Analyses were conducted at Analytisch-biologisches Forschungslabor GmbH (ABF), Munich, Germany. The compliance threshold for CEVal was based on a previous study (Gale *et al*. 2021); that for AB/AT was set as the limit of quantification of the analysis (0.5 ng/mL).

Biomarkers

The rationale for the biomarkers analysed in this study has been described in full elsewhere (Azzopardi *et al*. 2022b). In brief, eight BoEs to certain priority toxicants identified by the WHO Study Group on Tobacco Regulation (WHO 2015) and previously established as suitable for assessing exposure to tobacco HPHCs (Gregg *et al*. 2013, Gale *et al*. 2021), were analysed in urine. For each participant, all urine samples collected during the 24-h study period were pooled prior to analysis. The BoE analyses were carried out at ABF as previously described (Azzopardi *et al*. 2022b, Gale *et al*. 2017).

Seven BoPHs associated with the development of smoking-related diseases and with disease mechanisms that have been previously used to evaluate the effect of switching from cigarettes to alternative nicotine products (Haziza *et al*. 2020, Lüdicke *et al*. 2019, Gale *et al*. 2021) were assessed. 11-dTx B2, 8-epi-PGF$_{2\alpha}$ Type III and COHb were measured at ABF, sICAM-1 was measured at Celerion AG (Zurich, Switzerland), WBC was measured at Sanos Clinic (Herlev, Denmark) and the Clinical Chemistry and Pharmacology laboratory at Uppsala University Hospital (Uppsala, Sweden) and HDL was measured at Nordic Bioscience (Herlev, Denmark) and the Clinical Chemistry and Pharmacology laboratory at Uppsala University Hospital.

Regarding the two physical assessments, CIMT was measured on the distal portion of the common carotid artery by the Siemens Acuson P500 Ultrasound System (Siemens Healthcare GmbH, Erlangen, Germany); FEV$_1$%pred was measured as spirometry and values were standardised to predictive values of the Global Lungs Initiative (Quanjer *et al*. 2012); and FeNO was measured by a Vivatmo Pro device (Bosch Healthcare Solutions, Waiblingen, Germany). Participants were not allowed to smoke or use NPs for 1 h, or to eat for 2 h, before respiratory assessments.

Safety assessments

Participant safety was monitored by physical examination and clinical laboratory assessments throughout the study, and by telephone follow-up within seven days of discharge. All adverse events (AEs) were coded in accordance with the latest version of the Medical Dictionary for Regulatory Activities, and severity was recorded as mild, moderate or severe, as previously described (Azzopardi *et al*. 2022b). Participants who developed an AE were followed up until the event was no longer clinically significant.

Nicotine content in NPs

All NPs used in the study were collected and stored at 2–8 °C until analysis of nicotine content. In addition, participants in the NP group supplied three unused NPs for analysis. The NPs were extracted and analysed as previously described (Azzopardi *et al*. 2022a).

Statistical analysis

Sample size calculation

Owing to a lack of biomarker data on NPs, the sample size was determined from differences in plasma sICAM-1 between current and former smokers (Haswell *et al*. 2014) using PROC POWER in SAS version 9.4 (SAS Institute, Cary, NC, USA). Based on an sICAM-1 ratio of 0.847 (Haswell *et al*. 2014), a β value of 0.2 and an α value of 0.05, it was estimated that 84–120 participants in total would be needed to demonstrate a significant difference between NP and smoker groups. To allow for attrition and potential non-compliance with non-smoking in the NP group, we aimed to recruit 140 participants across the NP and current smoker groups. For qualitative observations of biomarker levels in former and never smokers, we aimed to recruit approximately 40 participants to each group.

Data analysis

Data were captured on source paper documents and entered in the electronic data capture system with quality control checks. Datasets were generated from the final study database and analysed by using SAS version 9.4. Continuous variables are reported as means of descriptive statistics (*n*, mean, standard deviation, median, and minimum and maximum) and categorical variables by means of frequencies.

The group means of each primary endpoint were compared between the Velo group and current smoker group in both the per-protocol (PP) and CEVal/AB/AT-compliant populations by using a multiple linear regression model in which the respective biomarker was the dependent variable and participant group was the independent variable. Age, sex and site (Sweden/Denmark) were added to the model in a stepwise manner and retained in the final model if they showed significance at an α level of 0.05. Where the assumption of the model was not valid (normally distributed residual data), the biomarker data were log-transformed; if the residuals remained not normally distributed after log transformation of the data, the Mann–Whitney U test was used to test differences. To adjust for multiple testing for the seven primary endpoints, Bonferroni correction was applied and the α level was amended to 0.05/7 = 0.007143. The same approach was applied to the secondary endpoints but without Bonferroni correction.

Results

Study participants

The study aimed to include a total of 140 participants in the Velo and current smoker groups. Across the two study centres, 97 solus users of Velo NPs (Danish $n = 47$; Swedish, $n = 50$) were enrolled; however, only 30 smokers (Danish, $n = 11$; Swedish, $n = 18$; other, $n = 1$) could be recruited within the time frame allowed for the study, although the combined final number of Velo users and smokers satisfied the minimum requirement. In addition, 29 former smokers and 39 never smokers were recruited, giving a total of 195 participants. All 195 participants completed the study and formed the PP population.

Velo users were slightly younger than smokers (mean ± SD age, 25.6 ± 5.94 vs 29.7 ± 8.52 years), while the oldest group of participants were former smokers (32.5 ± 10.37 years). The proportion of females in the Velo and smoker groups was 45.4% and 60.0%, respectively. Weight and BMI were relatively similar among the four groups (Table 1).

Regarding differences between the Danish and Swedish populations, gender (M/F) ratio (52.3%/47.7% and 50%/50%, respectively), age (25.9 ± 7.10 and 29.8 ± 8.97 years, respectively) and BMI (23.62 ± 2.934 and 23.97 ± 2.779, respectively) were similar (data not shown).

Nicotine and tobacco use

Use of nicotine and tobacco products among the study participants was assessed by questionnaire before the study. Overall, the 97 solus Velo users reported mean use of 10.4 ± 4.67 pouches per day, which was similar between Danish (10.5 ± 4.26 pouches per day) and Swedish (10.3 ± 5.07 pouches per day) participants. Danish participants generally used higher nicotine strengths (93.6% using medium-high or high) as compared with Swedish participants (62.0% using medium-high or high) (Supplementary Table S2). The overall duration of product use was 2.8 ± 1.83 years for the Velo users compared with 11.4 ± 7.31 years among the 30 smokers. Overall daily cigarette consumption was 13.5 ± 3.53 cigarettes per day, although daily cigarette consumption was higher among the Danish smokers (15.3 ± 3.58 versus 11.7 ± 8.53 cigarettes per day). In the former smoker group, the overall duration of quitting smoking was 3.5 ± 4.22 years (median, 1.8; range, 0.6–18.1 years). (Supplementary Table S2). All of the former and never smokers reported no current use of any form of nicotine.

Compliance

Compliance with self-reported solus use of NPs was assessed by levels of CEVal and AB/AT. No previous studies among NP users have used AB/AT levels as a measure of compliance with non-tobacco product use; therefore, the threshold for compliance was set as 0.5 ng/mL, corresponding to the detection limit of the analytical technique. While a large proportion of the NP user group (77.3%) showed 'non-compliance' with non-tobacco product use based on all three measures, 100% (97/97) showed compliance based on CEVal (54 pmol/g Hb), 90.7% (88/97) based on AT (0.5 ng/mL), and only 22.7% (22/97) based on AB (0.5 ng/mL), CEVal measurements suggest that the NP users were compliant with non-smoking. Nevertheless, statistically significant differences between the Velo and smoker groups were similar for the PP set and CEVal/AB/AT-compliant set. Accordingly, only the results from the PP set are reported in detail in the following sections.

Biomarkers of tobacco exposure

The primary endpoint total NNAL was more than 10-fold lower in the Velo users than in smokers (16.9 ± 29.47 vs 187.4 ± 228.93 pg/24 h, $P < 0.0001$). Six of the secondary BoEs (3-HPMA (246.7 ± 91.07 vs 1165.7 ± 718.35 µg/24 h), 3-OH-B[*a*]P (82.4 ± 217.58 vs 258.3 ± 190.20 pg/24 h), HMPMA (135.1 ± 77.85 vs 368.8 ± 183.15 µg/24 h), MHBMA (0.22 ± 0.166 vs 3.39 ± 2.943 µg/24 h), S-PMA (0.10 ± 0.059 vs 3.53 ± 2.736 µg/24 h) and total NNN (7.5 ± 24.84 vs 9.7 ± 5.93 ng/24 h)) were also significantly lower among Velo users than among smokers ($P < 0.0001$–0.0011); reductions of 78.8%, 68.1%, 63.4%, 93.5%, 97.2% and 22.7%, respectively (Supplementary Table S3). These significant differences were observed in both the PP and CEVal/AB/AT-compliant populations. Notably TN_{eq} was significantly higher among NP users than smokers (22.6 ± 12.69 vs 12.1 ± 7.92, mg/24 h, $P < 0.0001$), however, Velo user and smoker TN_{eq} levels were much closer in the Danish participants (19.5 ± 11.20 vs 15.9 ± 8.96 mg/24 h, respectively, Supplementary Figure S1). Overall, similar differences were observed for the CEVal-compliant population and apart from TN_{eq}, levels of tobacco BoEs were similar in the Velo, former and never smoker groups (Figure 2, Supplementary Tables S3 and S8).

Table 1. Clinico-demographic data of the study participants.

Characteristic	Velo users ($N=97$)	Smokers ($N=30$)	Former smoker ($N=29$)	Never smokers ($N=39$)	Total ($N=195$)
Sex					
Female	44 (45.4)	18 (60.0)	15 (51.7)	18 (46.2)	95 (48.7)
Male	53 (54.6)	12 (40.0)	14 (48.3)	21 (53.8)	100 (51.3)
Age (years)	25.6 ± 5.94	29.7 ± 8.52	32.5 ± 10.37	29.6 ± 9.97	28.1 ± 8.38
Nationality					
Danish	47 (48.5)	11 (36.7)	10 (34.5)	18 (46.2)	86 (44.1)
Swedish	50 (51.5)	18 (60.0)	19 (65.5)	21 (53.8)	108 (55.4)
Other (French)	0 (0.0)	1 (3.3)	0 (0.0)	0 (0.0)	1 (0.5)
Ethnicity					
Hispanic/Latino	4 (4.1)	1 (3.3)	2 (6.9)	0 (0.0)	7 (3.6)
Non-Hispanic/Latino	93 (95.9)	29 (96.7)	27 (93.1)	39 (100.0)	188 (96.4)
Race					
White	82 (84.5)	23 (76.7)	27 (93.1)	35 (89.7)	167 (85.6)
Black/African American	1 (1.0)	3 (10.0)	0 (0.0)	1 (2.6)	5 (2.6)
Asian	10 (10.3)	2 (6.7)	1 (3.4)	3 (7.7)	16 (8.2)
Other	4 (4.1)	2 (6.7)	1 (3.4)	0 (0.0)	7 (3.6)
Waist circumference (cm)	80.6 ± 8.58	84.1 ± 9.73	83.3 ± 10.76	82.6 ± 10.14	82.0 ± 9.45
Height (cm)	174.6 ± 8.31	173.9 ± 11.33	173.8 ± 9.04	174.7 ± 9.81	174.4 ± 9.17
Weight (kg)	72.3 ± 11.86	73.9 ± 10.48	73.6 ± 12.39	71.8 ± 12.50	72.7 ± 11.80
BMI (kg/m^2)	23.64 ± 2.795	24.48 ± 2.873	24.25 ± 3.067	23.41 ± 2.760	23.81 ± 2.840

Abbreviations: BMI, body mass index; SD, standard deviation. Values are given as number (percentage) or mean ± SD.

Biomarkers of potential harm

Among the primary BoPH endpoints, both COHb and were significantly lower in Velo users than in smokers (4.36 ± 0.525 vs 8.03 ± 2.173% saturation and 5.59 ± 1.223 vs 6.90 ± 1.758 × 10^9/L, respectively, both $P < 0.0001$, reductions of 46% and 19%). The primary endpoint FeNO, was significantly higher in Velo users than in smokers (23.18 ± 17.909 vs 11.20 ± 6.980 ppb, $P < 0.0001$, 107% higher).

Among the other three primary BoPH endpoints, sICAM-1 was 9% lower in Velo users than in smokers (185.9 ± 42.88 vs 204.5 ± 64.85 ng/mL) and trended towards significance ($P = 0.0124$, Figure 3 and Supplementary Table S4). Levels of 8-epi-PGF$_{2\alpha}$ Type III were highest in smokers (351.3 ± 210.59 ng/24 h) as compared with the other three groups, but although the level was 10% lower in the Velo group (315.2 ± 128.34 ng/24 h), the difference between Velo users and smokers was not significant (Figure 3, Supplementary Tables S4 and S8). Levels of the primary endpoint HDL were highly variable among all groups, ranging from 0.9 to 2.7 mmol/L in NP users, 1.1 to 2.7 mmol/L in smokers, 0.8 to 2.8 mmol/L in former smokers and 0.9 to 2.2 mmol/L in never smokers. The mean value was marginally lower in the Velo group compared with the smokers (1.55 ± 0.383 vs 1.59 ± 0.371 mmol/L, 2.5% lower), however, the difference was not significant ($P = 0.9972$) and in general, mean values were similar across all four groups (Figure 3, Supplementary Tables S4 and S8.

The secondary endpoint 11-dTX B2 was significantly lower among Velo users than among smokers (302.9 ± 112.19 vs 370.3 ± 150.01 ng/24 h, $P = 0.0085$, Figure 3 and Supplementary Table S4). Overall, similar differences were observed for the CEVal-compliant population and levels of BoPHs were generally similar in the Velo, former and never smoker groups (Figure 3, Supplementary Tables S4 and S8).

Physiological measures, quality of life and oral health

There were no statistically significant differences between Velo users and smokers in the levels of the secondary CIMT and FEV$_1$%pred endpoints (Supplementary Table S5).

The RAND-36 secondary endpoint evaluated QOL in eight domains (physical functioning, role limitations due to physical functioning, pain, general health, energy fatigue, social functioning, role limitations due to emotional problems and emotional wellbeing). Velo users scored significantly higher for physical functioning (PP set mean score: 97.2 vs 94.4; $P < 0.0003$) and for energy/fatigue (PP set mean score: 68.3 vs 63.2; $P < 0.0413$). No significant differences were observed between Velo users and smokers in the remaining six domains (Supplementary Table S6).

The oral health secondary endpoint assessment included assessments of lips, tongue, gums and tissues, saliva, health of natural teeth, dentures, oral cleanliness, and dental pain. There was no statistically significant difference between Velo users and smokers in any item or in overall oral health (Supplementary Table S7).

NP use and nicotine transfer

During the study, the length of time that the pouch was held in the mouth by the 97 participants varied widely from 6.7 min to 145.7 min (mean ± SD: 33.3 ± 24.60 min). Overall, the durations were longer for lower nicotine strength NPs, and shortest for high nicotine strength NPs. In addition, Swedish users held NPs in their mouth for considerably longer than Danish users (mean ± SD: 46.6 ± 26.22 vs 19.1 ± 11.50 min) (Supplementary Table S9).

The residual nicotine content in used pouches was measured for 96 participants. Based on the residual nicotine, the mean transfer of nicotine to Velo users in the study was 33.3%. Consistent with the longer in-mouth duration noted above, transfer of nicotine from the pouches was considerably greater for Swedish Velo users (40.9%) than for Danish Velo users (25.4%) (Supplementary Table S10) and percentage transfer of nicotine decreased as the nicotine strength of the NPs increased as follows: 71.1% transfer for low-nicotine NPs; 45.6% transfer for medium-low nicotine NPs; 30.8% transfer for medium-high nicotine NPs; and 25.3% transfer for high-nicotine NPs (Supplementary Table S11).

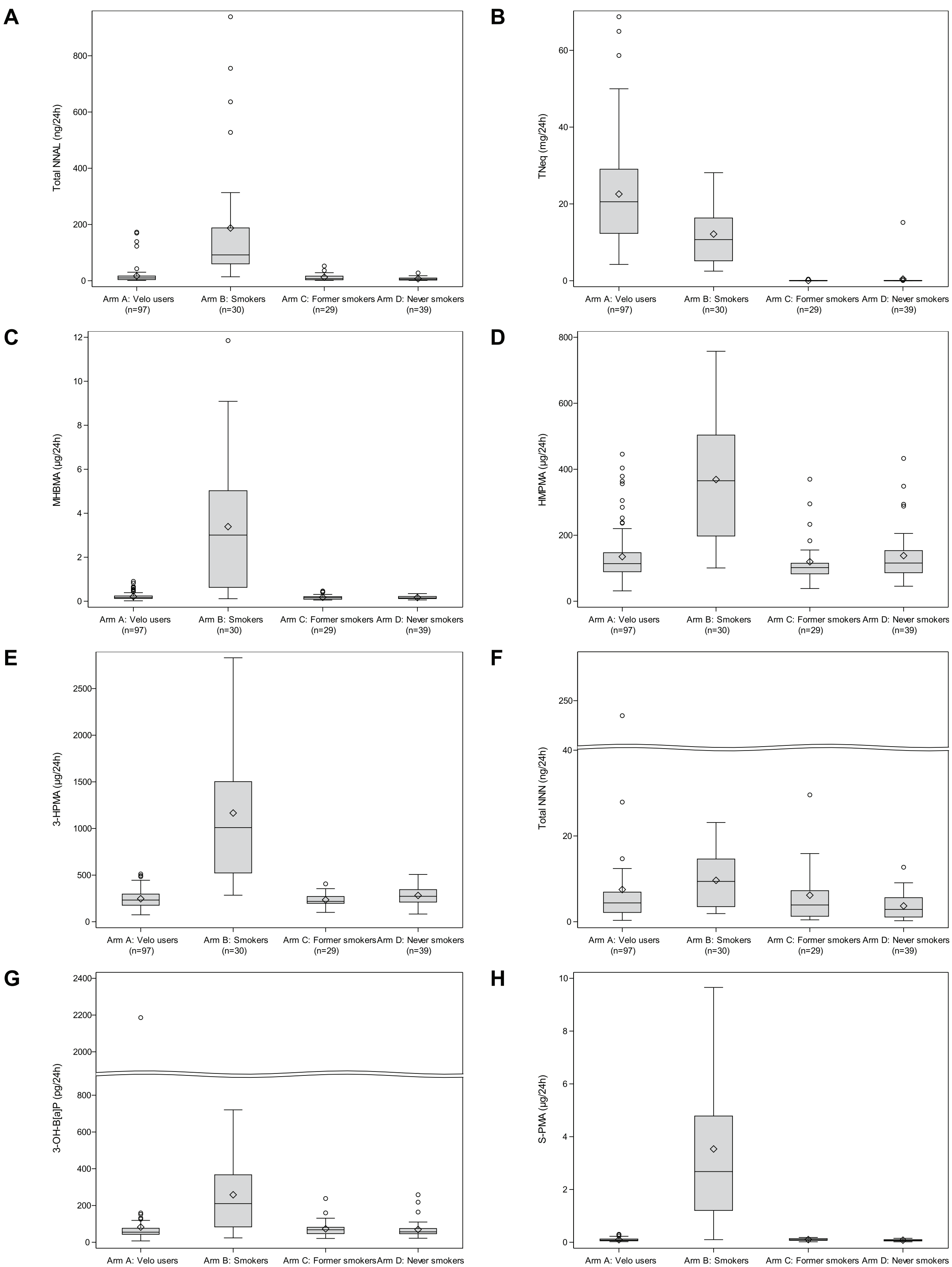

Figure 2. Box plots of BoEs among Velo users, smokers, formers smokers, and never smokers (PP set). A, Total NNAL; B, TN_{eq}; C, MHBMA; D, HMPMA; E, 3-HPMA; F, total NNN; G, 3-OH-B[a]P; H, S-PMA.

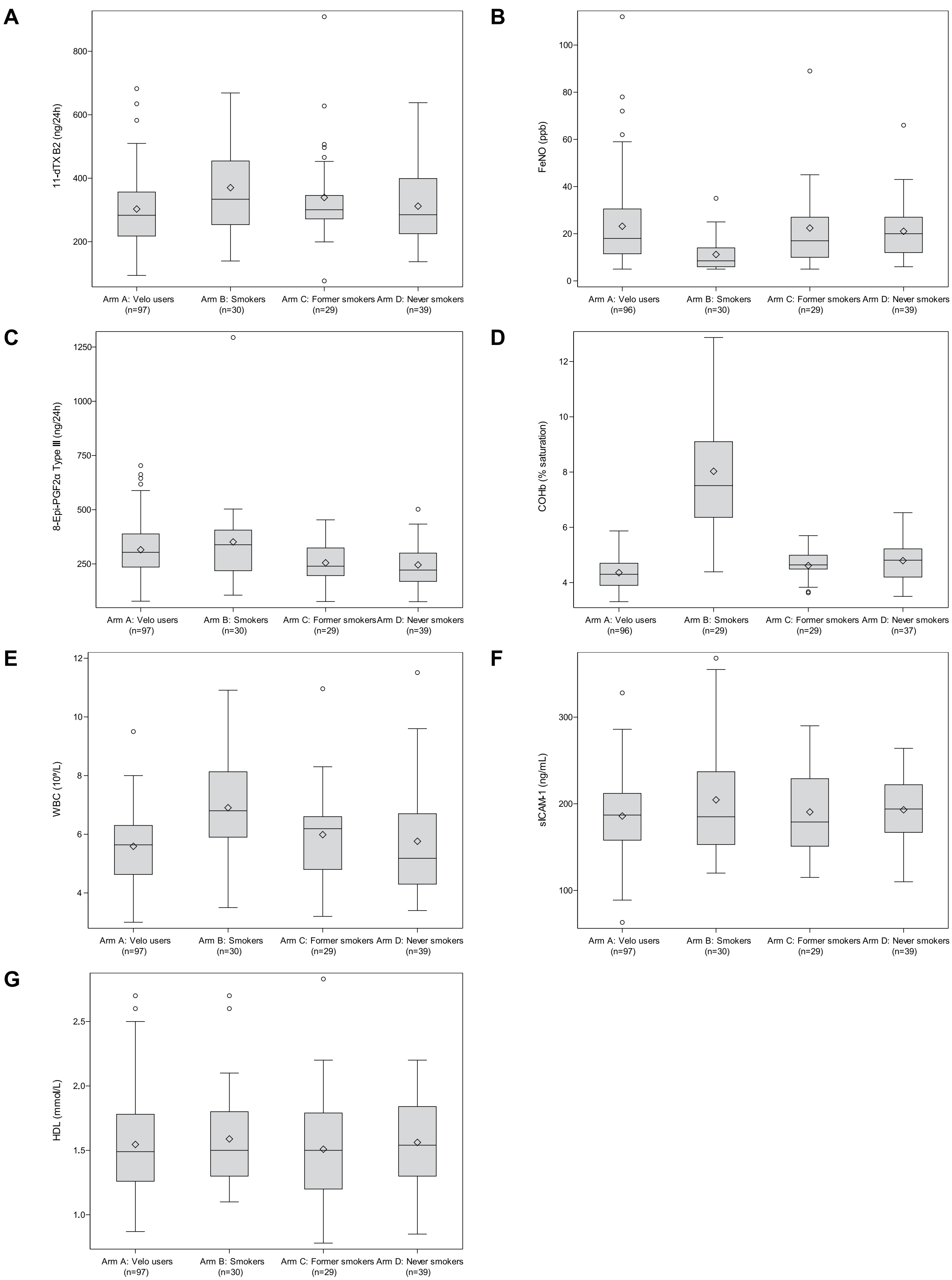

Figure 3. Box plots of BoPHs among Velo users, smokers, formers smokers, and never smokers (PP set). A, 11-dTX B2; B, FeNO; C, 8-Epi-PGF$_{2\alpha}$ Type III; D, COHb; E, WBC; F, sICAM-1; G, HDL.

Adverse events

A total of 14 AEs were reported by 14 participants (7.2% of total population). Eight AEs were reported from participants in the Velo group and were assessed as 'unlikely related' to the participant's own nicotine product. The remaining AEs were reported from the former ($n = 5$) and never smoker ($n = 1$) groups. All AEs were mild in terms of severity, with nasopharyngitis, headache and dysmenorrhoea being the most frequently reported.

Discussion

Although the global prevalence of smoking is falling, it currently is at approximately 17.5%, and is as high as 40% in some countries (WHO 2021). Given that many smokers will continue smoking without a satisfactory substitute product (Chaiton *et al*. 2016), many public health organisations now support a THR approach that encourages smokers to switch their tobacco cigarettes for a nicotine product with potentially fewer health risks (IOM 2001, RCP 2016, RCGP 2017, PHE 2019). The reduced toxicant content (Azzopardi *et al*. 2022a, Jablonski *et al*. 2022) and limited *in vitro* toxicological effects (Bishop *et al*. 2020, Yu *et al*. 2022) of NPs as compared with conventional cigarettes suggest that these products have the potential for reduced risk relative to smoking; however, it remains essential to determine whether this potential translates to the user. Recent studies of THPs have shown that levels of BoEs and BoPHs are reduced when smokers switch from cigarettes to THPs (Gale *et al*. 2017, Gale *et al*. 2021); therefore, the present study evaluated a number of BoPHs and BoEs among self-reported solus users of NPs.

Overall, levels of several biomarkers differed significantly between NP users and smokers, including four of the seven primary biomarkers. Notably, the primary endpoints total NNAL, a BoE for NNK and BoPH linked to the development of lung cancer in smokers, and COHb, a biomarker linked to the development of CVD, were significantly lower among NP users than in smokers (both $P < 0.0001$). As well as BoPHs linked to specific diseases, the study measured levels of biomarkers known to be raised during disease development. The primary endpoint WBC, which is linked to inflammation and has been shown to be increased among smokers (Higuchi *et al*. 2016), was significantly higher among smokers than among NP users ($P < 0.0001$). In addition, the primary endpoint FeNO, indicative of airway inflammation, and previously established as being lower in smokers (Nadif *et al*. 2010), was significantly higher in NP users than in smokers ($P < 0.0001$), and comparable to former and never smoker levels.

By contrast, levels of the primary endpoint 8-Epi-$PGF_{2\alpha}$ Type III, a biomarker of oxidative damage linked to oxidative stress that has been shown to be raised in smokers (Morrow *et al*. 1995), were not significantly lower in the NP users compared to smokers, although levels were similar among all four study groups. The level of the primary endpoint sICAM-1, an inflammation marker linked to development of CVD, was lower among NP users, the difference was not significant ($P < 0.0124$). In addition, no difference in the remaining primary endpoint HDL, a biomarker related to CVD risk, was observed between NP users and smokers. It should be noted that in addition to smoking, levels of HDL and sICAM-1 are known to be affected by numerous and varied parameters such as lifestyle and genetics (Lamon-Fava 2002, Chang *et al*. 2019).

In terms of the secondary endpoints, the BoPH 11-dTX B2, a biomarker of CVD, levels were significantly lower for NP users compared with smokers ($P < 0.0085$). Few significant differences were observed between NP users and smokers in the subjective and physiological secondary endpoints. No differences were observed between NP users and smokers in overall oral health. CIMT, linked to CVD risk, and FEV_1%pred, a measure of lung function for which abnormally low values are associated with chronic obstructive pulmonary disease, were similar and within normal ranges across all groups. As discussed previously for HDL and sICAM-1, CIMT is associated not only with smoking but with several other risk factors for CVD, including age, systolic blood pressure and waist circumference among others (Qu and Qu 2015). The population of smokers in the present study was relatively young (mean age, 28.1 years) with a mean smoking duration of 11.4 years, and it may take a longer period of time before differences between smokers and NP users in CIMT and other BoPHs/physiological measures such as FEV_1 and oral health due to smoking are manifested.

Interestingly, statistically significant differences were observed in the some QOL responses. NP users scored higher for the physical functioning and energy/fatigue domains ($P < 0.0003$ and $P < 0.0413$, respectively), which although perhaps surprising given the relatively young age of the participants, suggests a negative subjective health impact of smoking that is not observed in NP users.

Apart from TN_{eq}, the levels of all BoEs were significantly lower ($P < 0.0001$–0.0011) among NP users than among smokers, and the levels of BoEs and BoPHs among NP users were similar to those among never smokers. Note that an outlier of 247 ng/24 h from one Velo user, most likely caused by exogenous formation (Pluym *et al*. 2022), elevated the NNN mean from ~5 to 7.5 ng/24 h. Perhaps unexpectedly, TN_{eq} was almost twice as high among NP users (22.6 mg/24 h) than among smokers (12.1 mg/24 h; $P < 0.0001$). However, higher daily uptake of nicotine (~25 mg) has been reported among oral pouch (snus) users with an ADC of 16 pouches (Andersson *et al*. 1997). Sweden is a unique oral tobacco market with a long tradition of snus use which appears to skew the overall TN_{eq} result. Indeed, the TN_{eq} results for the Danish NP users and smokers are similar and possibly these data are more representative of countries which do not have extensive smokeless tobacco use history. Furthermore, the convenience of NP use relative to cigarette smoking combined with the clinic setting may have contributed to an over-estimation of daily nicotine exposure; self-reported ADC values were slightly higher in this study (10.4 pouches/day) compared to a previously reported value in Sweden of 8.6 pouches/day (Azzopardi *et al*. 2022a). The reported level of nicotine uptake indicates that NPs are likely to provide sufficient delivery of nicotine to assist smokers switching to NPs as part of a THR approach.

In this study, participants were asked to use the NPs in their usual way, allowing us to obtain use behaviour data for this relatively new type of nicotine product. The mean in-mouth time was 33.3 min, which is considerably shorter than the fixed duration of 60 min that has been used in several pharmacokinetic (PK) studies of NPs (Lunell *et al*. 2020, McEwan *et al*. 2022, Azzopardi *et al*. 2022c). This suggests that the PK parameters (e.g. C_{max}, AUC) observed in those studies are likely to represent the higher end of nicotine exposure experienced by NP users. In addition, the usage times from this study are considerably lower than the 60–70 min previously reported for snus (Digard *et al*. 2013). The usage times recorded in the present study might be used to inform future PK studies to obtain more 'real-world' data. Interestingly, the Swedish participants used the NPs for much longer than the Danish participants (46.6 vs 19.1 min), which is likely to be the main driver for the higher TN_{eq} results in the Swedish NP users compared with Danish NP users. This disparity in usage times may be influenced by the overall use of lower nicotine strengths by the Swedish participants as compared with the Danish participants. Alternatively, it may reflect the long tradition of snus use that is unique to Sweden.

Consistent with the in-mouth durations, percentage transfer of nicotine was higher for the Swedish (40.9%) participants than for the Danish (25.4%) participants, and it decreased as the nicotine strength of the NPs increased (from 71.1% transfer for low-nicotine strength NPs to 25.3% transfer for high-nicotine strength NPs). The mean level of nicotine extraction from the NPs (33%) is generally consistent with those reported in PK studies (56% and 59% (Lunell *et al*. 2020); 62% (Azzopardi *et al*. 2022c)) when the reduced usage duration is considered (33.3 min in this study vs 60 min in PK studies).

In this study, we examined compliance with non-smoking and non-smokeless tobacco (e.g. snus) use among self-reported solus NP users based on three measures. CEVal has been demonstrated as a measure of compliance with non-smoking among THP users (Gale *et al*. 2021), while urinary levels of two tobacco alkaloids, AB and AT, have been shown to correlate with self-reported tobacco use among smokers and smokeless tobacco users (Jacob *et al*. 2002). While a large proportion of the NP user group (77.3%) showed 'non-compliance' with non-tobacco product use based on all three measures, 100% (97/97) showed compliance based on CEVal (54 pmol/g Hb), 90.7% (88/97) based on AT (0.5 ng/mL), and only 22.7% (22/97) based on AB (0.5 ng/mL), CEVal measurements suggest that the NP users were compliant with non-smoking. A previous study used a threshold of 2 ng/mL AT/AB to check for non-compliance with smokeless tobacco use among individuals switching to nicotine replacement therapy (Jacob *et al*. 2002); therefore, we tested whether this higher threshold would reduce the 'non-compliant' population observed here. At a threshold of 2 ng/mL, the compliant population increased to 99.0% for AT, but was still only 54.6% for AB; thus, it seems that AB may not be a suitable marker to evaluate compliance with non-tobacco product use among NP users. Further studies will be needed to explore urinary AB/AT as an indicator of non-smokeless tobacco compliance among users of NPs.

The study has a number of strengths, including the first comprehensive analysis of eight tobacco and tobacco smoke BoEs (total NNAL, MHBMA, HMPMA, 3-HPMA, NNN, 3-OH-B[a]P, S-PMA and TN_{eq}), seven BoPHs (CoHb, sICAM-1, 8-epi-$PGF_{2\alpha}$ Type III, WBC, FeNO, HDL, 11-dTX B2) and two physiological markers (FEV_1%pred and CIMT), many of which revealed large and significant differences between solus NP users and smokers. In addition, the cross-sectional design enabled us to gain insight into NP use behaviour for this relatively new nicotine product, including ADC, usage times and nicotine transfer. Last, the cross-sectional nature of the study means biomarker data may be more reflective of real-world usage than a longitudinal switching study.

The study also has some limitations. The cross-sectional nature of the study provides only a snapshot at a single point in time. Longitudinal studies among smokers will be needed to determine whether BoE and BoPH levels improve further if smokers switch from smoking combustible cigarettes to using NPs. In addition, the AB/AT measures of compliance may not have been appropriate for the study population, as discussed above, although this ultimately had minimal impact on biomarker levels between the Velo and smoker groups when the CEVal/AB/AT population was compared to the per protocol population.

In conclusion, this is the first study to evaluate levels of specific BoEs and BoPHs related to tobacco toxicants and to initiating biological processes involved in smoking-related diseases, respectively, in exclusive users of NPs alongside cigarette smokers. These data show that levels of these BoEs, with the exception of TN_{eq}, are substantially reduced in NP users compared with smokers, and most BoPHs show favourable statistically significant changes and/or are at similar levels to former and never smokers. These data provide further weight of evidence that Velo NPs are a potentially reduced risk alternative for smokers who completely switch from smoking cigarettes.

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Data availability

BAT is committed to the responsible sharing of data with the wider research community. Data access is administered for this study through an internal Data Sharing Committee on reasonable request following completion of a data sharing request form and if applicable, a Data Access Agreement. Requests for data sharing in the first instance should be emailed to the corresponding author.

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Disclosure statement

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