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Biomarkers of Exposure and Potential Harm in Exclusive Users of Electronic Cigarettes and Current, Former and Never-Smokers: A Cross-Sectional Study Protocol

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Abstract: Despite public health efforts to reduce the health burden of cigarettes by encouraging smoking cessation, a proportion of smokers remain unwilling to quit. A shift from smoking cessation to tobacco harm reduction, based on smokers switching completely to potentially less harmful products such as electronic cigarettes (ECs), has been proposed as an alternative strategy. This is a single-centre, cross-sectional confinement study, involving healthy exclusive Vuse EC users and current, former, or never-smokers. Exclusive EC use and smoking status will be confirmed by urinary cotinine and exhaled carbon monoxide levels. Participants will be confined for 24 hours, during which they will use their usual product (EC or cigarette) as normal. Biomarkers of exposure and potential harm will be analysed in 24-hour urine and blood and compliance will be measured using N-(2-cyanoethyl)valine. The primary objective is to quantitatively assess differences between EC users and current smokers in urinary total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and 8-epi-prostaglandin $F_{2\alpha}$ Type III, exhaled nitric oxide, and carboxyhaemoglobin, white blood cell count, soluble intercellular adhesion molecule-1, and high-density lipoprotein. Secondary objectives are to quantitatively assess differences between EC users and current smokers in selected urinary biomarkers of tobacco exposure, 11-dehydrothromboxane B2, forced expiratory volume in 1 second as a percentage of predicted, carotid intima-media thickness and a quality of life questionnaire. Endpoints will also be compared between EC users and former and never-smokers. The results of this study are anticipated to add to the current knowledge about the role of ECs in tobacco harm reduction.

Keywords: Biomarkers of Exposures, Biomarkers of Potential Harm, Electronic Cigarettes, Tobacco Harm Reduction

1. Introduction

Smoking is a known cause of cardiovascular disease, chronic obstructive pulmonary disease, and lung cancer [1]. Risks correlate with smoking duration and daily cigarette consumption, and are principally due to inhalation of toxicants transferred into cigarette smoke during the combustion of tobacco [1-7]. Quitting smoking greatly reduces the risk of disease [1], which has led to the public health priority of reducing the health burden of cigarette smoking by encouraging smoking abstinence [8]. Despite these efforts, smoking rates in adult populations worldwide remains around 20%, although numbers are declining slowly in many countries [9].

Of the more than 6,500 identified chemical constituents of combustible cigarette smoke [10], 158 are established as toxicants [11], long-term exposure to which can lead to smoking-related disease. DNA damage and oxidative stress have been identified as key disease mechanisms in this process [12, 13], but the precise mechanisms are not yet fully understood [2]. Compared to cigarettes, novel tobacco and nicotine products, including electronic cigarettes (ECs) and tobacco heated products (THPs), are thought to reduce the exposure of users to harmful and potentially harmful

constituents (HPHCs) [14-16] and thereby may reduce the health risks associated with combustible cigarettes for smokers who switch completely to such products. In particular ECs contain no tobacco, instead the e-liquid, usually containing water, nicotine and/or flavours in a base of glycerol and propylene glycol [17, 18], is heated without combustion to form an aerosol vapour for inhalation. Even for ECs with nicotine-containing e-liquids, the aerosol contains significantly fewer HPHCs than cigarette smoke, and the toxicants that are present are generally at much lower levels [19-23]. Public Health England [24] have recently stated that ECs could pose 95% less risk than combustible cigarettes and the UK Royal College of Physicians have supported the use of ECs as replacement products for smokers [14]. As a result, use of ECs has been proposed as a tobacco harm reduction strategy to help smokers switch away from smoking [14, 24, 25], an approach that is supported by data from large cross-sectional and longitudinal surveys in the UK [26-29].

An assessment framework for evaluating the potential risk reduction effect of EC has been proposed by Murphy et al [30]. In this framework clinical studies measuring both biomarkers of exposure (BoE) and biomarkers of potential harm (BoPH) are key to evaluating the potential individual risk reduction of EC use compared to continuing to smoke. In the context of tobacco harm reduction BoEs reflect either a chemical or its metabolite that indicate the internal dose or exposure to tobacco constituents [2]. BoPH, also called biomarkers of biological effect, reflect changes in the broader biological system resulting from exposure to harmful substances [2]. The aim of this study will be to compare selected BoEs, BoPHs and physiological measures (Table 1) between adults who use Vuse (formerly branded Vype) ePod and/or ePen3 (now marketed as ePen) ECs with those in current, former and never smokers.

Table 1. *Biomarkers of exposure and potential harm assessed in the study.*

Biomarker of exposure	Associated toxicant	Matrix	Method	References
Primary endpoints				
Total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (Total NNAL)	Nicotine-derived nitrosamine ketone (NNK)	24-hour urine	LC-MS/MS	[65-67]
Secondary endpoints				
Total nicotine equivalents (TNeq)[1]	Nicotine	24-hour urine	LC-MS/MS	[67]
Monohydroxybutenylmercapturic acid (MHBMA)	1,3-Butadiene	24-hour urine	LC-MS/MS	[67]
3-Hydroxy-1-methylpropylmercapuric acid (HMPMA)	Crotonaldehyde	24-hour urine	LC-MS/MS	[67]
3-Hydroxypropylmercapturic acid (3-HPMA)	Acrolein	24-hour urine	LC-MS/MS	[67]
Total N-nitrosonornicotine (Total NNN)	NNN	24-hour urine	LC-MS/MS	[67]
3-Hydroxybenzo[a]pyrene (3-OH-B[a]P)	Benzo[a]pyrene (B[a]P)	24-hour urine	LC-MS/MS	[68]
S-Phenylmercapturic acid (S-PMA)	Benzene	24-hour urine	LC-MS/MS	[67]

Biomarker of potential harm	Associated biological process	Matrix	Method	References
Primary endpoints				
Nitric oxide (FeNO)	Airway inflammation	Exhaled breath	Chemical field-effect transistor	[61, 62, 82]
8-Epi-prostaglandin $F_{2\alpha}$ Type III (8-Epi-$PGF_{2\alpha}$ Type III)	Oxidative stress	24-hour urine	LC-MS/MS	[62, 63, 69]
Carboxyhaemoglobin (COHb)	Cardiovascular disease	Blood	HS GC-MS	[42-44, 70]
Total white blood cell count (WBC)	Inflammation	Blood	Flow cytometry	[49, 51, 53, 83]
Soluble intercellular adhesion molecule-1 (s-ICAM1)	Cardiovascular disease	Serum	ELISA	[36, 39, 40, 80]
High-density lipoprotein (HDL)	Cardiovascular disease	Blood	Enzyme colorimetric	[33-35, 80]
Secondary endpoints				
11-Dehydrothromboxane B2 (11-dTX B2)	Cardiovascular disease	24-hour urine	LC-MS/MS	[46, 47, 69]
Forced expiratory volume in 1 second as% of predicted (FEV_1%pred)	Chronic obstructive pulmonary disease	Physiological measurement	Spirometry	[71, 84]
Carotid intima-media thickness (CIMT)	Cardiovascular disease	Physiological measurement	Ultrasound	[58-60, 85]

[1] Nicotine, cotinine, 3-hydroxycotinine and their glucuronide conjugates.

2. Materials and Methods

2.1. Study Design

This single-centre, cross-sectional confinement study will be conducted with EC users and current, former and never smokers attending a single study site operated by Richmond Pharmacology Limited (RPL), London, UK, between September 2020 and August 2021. All participants will be asked to provide written informed consent, which must be obtained prior to their participation in the study and before undergoing any study procedures, including screening assessments. Favourable opinion (equivalent to Institutional Review Board approval) for this research was given by the Research Ethics Committee of the NHS Health Research Authority, South Central – Berkshire B, UK Research Ethics Committee (reference number 21/SC/0005). This study will be conducted in accordance with the relevant articles of the Declaration of Helsinki and reported in accordance with the International Council on Harmonisation guidelines. The trial was prospectively registered on the International Standard Registered Clinical/Social Study Number Registry (ISRCTN58921739).

2.2. Objectives and Endpoints

2.2.1. Primary Objective

To quantitatively assess differences between EC users and current smokers in the BoEs total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and the BoPHs fractional exhaled nitric oxide (FeNO), 8-epi-prostaglandin $F_{2\alpha}$ Type III (8-Epi-$PGF_{2\alpha}$ Type III), carboxyhaemoglobin (COHb), white blood cell count (WBC), soluble intercellular adhesion molecule-1 (s-ICAM1), and high-density lipoprotein (HDL).

2.2.2. Secondary Objectives

To quantitatively assess differences between EC users and current smokers in the BoEs total nicotine equivalents (nicotine, cotinine, 3-hydroxycotinine and their glucuronide conjugates) [TNeq], monohydroxybutenylmercapturic acid (MHBMA), 3-hydroxy-1-methylpropylmercapuric acid (HMPMA), 3-hydroxypropylmercapturic acid (3-HPMA), total N-nitrosonornicotine (NNN), 3-hydroxybenzo[a]pyrene (3-OH-B[a]P), and S-phenylmercapturic acid (S-PMA), the BoPHs 11-dehydrothromboxane B2 (11-dTX B2), the physiological measures forced expiratory volume in 1 second as % of predicted (FEV_1%pred), and carotid intima-media thickness (CIMT) and a quality of life questionnaire. Additionally, the differences in all study endpoints between EC users and former smokers and between EC users or former smokers and never-smokers will be assessed.

2.3. Biomarker Selection

The BoEs have been selected from the WHO Study Group on Tobacco Product Regulation (TobReg 9) initial list of priority toxicants [31]. For two of these toxicants (acetaldehyde and formaldehyde), there are no reliable BoEs at present; therefore, levels of crotonaldehyde will be assessed (via HMPMA) instead.

The BoPHs selected for this study are associated with smoking-related diseases such as cardiovascular disease and respiratory disease, and underlying disease processes such as oxidative stress: HDL [32-35], s-ICAM1 [36-40], COHb [41-44], 11-dTX B2 [45-47], WBC [48-57] and CIMT [58-60], FeNO [61, 62] and FEV_1%pred, 8-Epi-$PGF_{2\alpha}$ Type III [62, 63]. NNAL is a BoE for the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). It is also a carcinogen and urinary NNAL is associated with lung cancer risk [64], therefore is considered a BoPH for lung cancer [65, 66].

2.4. Study Participants

In the first instance, participants will be recruited from RPL's volunteer database, referral scheme and social media channels. To support recruitment, an external recruitment agency will assist in identifying potential EC user participants through database contact, advertising campaigns and social media channels and will refer them to RPL. No personal information will be released by the agency. Inclusion and exclusion criteria are listed in Table 2. Eligible participants will be healthy adult exclusive users of Vuse ePod or Vuse ePen3 ECs or current, former, or never-smokers and who are judged to be suitable by pre-study screening. Exclusive EC use will be defined as self-reporting daily use and will be confirmed by measurements of urinary cotinine (>200 ng/mL) and exhaled breath carbon monoxide (CO; <7 ppm). Current smoking status will be defined as self-reporting smoking of at least 10 cigarettes per day and will be confirmed by urinary cotinine (>200 ng/mL) and exhaled breath CO (≥7 ppm). Former smokers will be defined as self-reporting having quit smoking for at least 6 months, confirmed by urine cotinine (<200 ng/mL) and exhaled breath CO (<7 ppm). Never-smokers will be defined as self-reporting smoking of no more than 100 cigarettes during their lifetime and none within the 6 months prior to screening, confirmed by urinary cotinine (<200 ng/mL) and exhaled breath CO (<7 ppm). In EC users and former smokers, compliance with smoking abstinence in the previous 6 months will be assessed by measurement of N-(2-cyanoethyl)valine (CEVal) in erythrocytes. Potential participants invited for screening will be asked to avoid alcohol completely for a period of 24 hours before attending the clinic. Any deviation will be assessed on a case-by-case basis by the principal investigator (PI) and sponsor. Participation may be allowed at their discretion provided that the alcohol intake will not impact the participant's safety and or the objectives of the study. Participants will be advised not to eat food containing poppy seeds for 3 days before screening to avoid a positive opiate result in the drugs of abuse test, and to avoid eating or being in the presence of cooking of cruciferous vegetables (e.g., bok choy, broccoli, cabbage, cauliflower, kale, watercress, etc) and grilled, fried, or barbequed food for 48 hours prior to screening. Other advice will be to refrain from unusually intense or strenuous exercise during the study period.

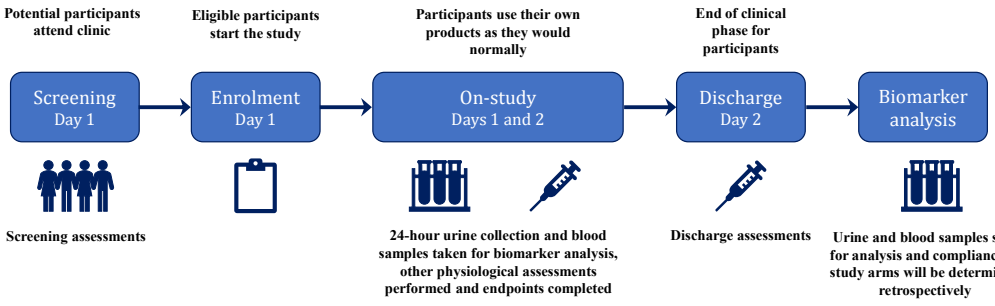

Figure 1. *Study schematic.*

Table 2. *Study Inclusion and exclusion criteria.*

Inclusion criteria	Exclusion criteria
Subjects will be: a) Males or females b) 19 to 55 years of age, inclusive	Female subjects who are pregnant or breastfeeding (will be confirmed at screening)
Subjects will have a: a) Body mass index (BMI) of 18.5 to 30.0 kg/m^2, inclusive b) Body weight exceeding 52 kg (males) or 45 kg (females)	Subjects who have donated: 1) ≥400 mL of blood within 90 days prior to screening 2) Plasma in the 7 days prior to screening 3) Platelets in the 6 weeks prior to screening
Subjects will be in good health, as judged by the PI or the appropriately qualified designee based on: a) Medical history b) Physical examination c) Vital signs assessment d) 12-lead ECG e) Clinical laboratory evaluations (to be reviewed post-enrolment, on the morning of Day 2; any subject with a result which, in the opinion of the PI or research physician, is clinically-significant, will be withdrawn) f) Lung function tests/spirometry	Subjects who have had an acute illness (e.g. upper respiratory tract infection, viral infection, etc) requiring treatment within 4 weeks prior to screening
Subjects will have given their written informed consent to participate in the study and will have agreed to abide by the study restrictions	Subjects who have a significant history of alcoholism or drug/chemical abuse (apart from known smoking/vaping history) within 24 months prior to screening, as determined by the PI or the appropriately qualified designee
Subjects must demonstrate the ability to comprehend the informed consent form (ICF), be able to communicate well with the PI or the appropriately qualified designee, understand and comply with the requirements of the study, and be judged suitable for the study in the opinion of the PI or the appropriately qualified designee	Subjects who have a positive urine drugs of abuse or breath alcohol screen (confirmed by repeat) at screening
Subjects will refrain from consuming alcohol for 24 hours prior to screening	Subjects who: 1) Have serum hepatitis/are carriers of the hepatitis B surface antigen (HBsAg) 2) Are carriers of the hepatitis C antibody 3) Have a positive result for the test for human immunodeficiency virus (HIV) antibodies 4) Have a positive result in the COVID-19 test at screening indicating current, active infection
Subjects will refrain from consuming cruciferous vegetables, and grilled, fried or barbequed food, and avoid being in the presence of the cooking of cruciferous vegetables, and grilled, fried or barbequed food for 48 hours prior to screening	Subjects who have used prescription or over-the-counter bronchodilator medication (e.g. inhaled or oral β-adrenergic agonists) to treat a chronic condition within the 12 months prior to screening
Arm A: exclusive EC users a) Subjects will be regular (daily) users of the Vuse ePen3 and/or Vuse ePod vaping devices b) Subjects will have used the Vuse ePen3 and/or Vuse ePod vaping devices for a minimum of 6 months prior to screening c) Subjects will have a urinary cotinine level >200 ng/mL and an exhaled breath CO level <7 ppm at screening	Subjects who have received any medications or substances (other than nicotine) which: 1) Interfere with the cyclooxygenase pathway (e.g. anti-inflammatory drugs including aspirin and ibuprofen) within 14 days prior to screening 2) Are known to be strong inducers or inhibitors of cytochrome P450 enzymes within 14 days or 5 half-lives of the drug (whichever is longer) prior to screening
Arm B: current smokers a) Subjects will be regular smokers of commercially manufactured filter cigarettes b) Subjects will have smoked for at least one year prior to screening c) Subjects will typically smoke at least 10 CPD and must have a urinary cotinine level >200ng/mL and an exhaled breath CO level ≥7 ppm at screening	Subjects who would need to take prescription medication during the period beginning with screening and ending with discharge (for female subjects, hormonal contraceptives are acceptable, and for all subjects, painkillers (e.g. paracetamol) are permitted)
Arm C: former smokers a) Subjects will be former smokers of commercially manufactured filter cigarettes who quit smoking at least 6 months prior to screening b) Subjects will have a urinary cotinine level <200 ng/mL and an exhaled breath CO level <7 ppm at screening	Subjects who are unwilling or unable to comply with the study requirements
Arm D: never smokers a) Subjects will have never smoked (<100 cigarettes in their life and none within the 6 months prior to screening) b) Subjects will have a urinary cotinine level <200 ng/mL and an exhaled breath CO level <7 ppm at screening	Employees and immediate relatives of the tobacco industry or the clinical site
	Subjects who have any clinically relevant abnormal findings on the physical examination, medical history, ECG, lung function tests or clinical laboratory panel, unless deemed not clinically significant by the PI or the appropriately qualified designee

Inclusion criteria	Exclusion criteria
	Subjects who have been diagnosed with a significant history of urticaria or asthma (childhood asthma is acceptable) Subjects who have, or who have a history of any clinically significant neurological, gastrointestinal, renal (including urinary tract infection or nephrolithiasis), hepatic, cardiovascular, psychiatric, respiratory, metabolic, endocrine, haematological or other major disorder that, in the opinion of the PI or the appropriately qualified designee, would jeopardise the safety of the subject or impact on the validity of the study results Subjects who have previously been diagnosed with any form of malignancy Subjects who are currently participating in another clinical trial (including follow-up) Subjects who, in the opinion of the PI or the appropriately qualified designee, should not participate in this study Arm A: exclusive EC users 1) Subjects who have used any form of tobacco or nicotine-containing product, other than the Vuse ePen3 and Vuse ePod, within the 6 months prior to screening 2) Subjects who are self-reported non-inhalers (vapers/e-cigarette users who draw aerosol from their device into the mouth and throat but who do not inhale) Arm B: current smokers Subjects who are self-reported non-inhalers (smokers who draw smoke from the cigarette into the mouth and throat but who do not inhale) Arms C and D: former and never smokers Subjects who have used any form of tobacco or nicotine-containing product within the 6 months prior to screening

2.5. Study Procedures

Figure 1 provides an overview of the study procedures. Potential participants will attend the clinic for screening, enrolment, and an overnight confinement period. Each participant will receive verbal and written information and will be asked to sign the informed consent form prior to undergoing any screening procedures taking place.

Screening will include physical and vital signs examinations, routine clinical laboratory testing, alcohol and drug consumption testing and pregnancy testing; and in addition, nicotine use and smoking status will be determined. The extent of tobacco and nicotine use will be assessed via a questionnaire. All screening assessments will be performed on the morning of Day 1, and enrolment will take place on the same day. Each participant will be given a unique study enrolment number and the participants' data will be collected on case report forms (CRF). After enrolment, participants will begin a 24-hour urine collection period and will be confined overnight at the clinic. Participants will be discharged from the clinic on Day 2 after completion of their 24-hour urine collection period and discharge assessments.

Any participant will be able to withdraw from the study at any time and for any reason, or may be withdrawn at the discretion of the PI or the study sponsor for reasons including compromise of health or protocol deviations. Alternatively, the PI or designee may suspend or terminate the study for any reason (e.g. clinical, administrative, etc) after consultation with the sponsor. The sponsor may also suspend or terminate the study either in part or in its entirety. Reasons for study termination will be fully documented and provided to the ethics committee.

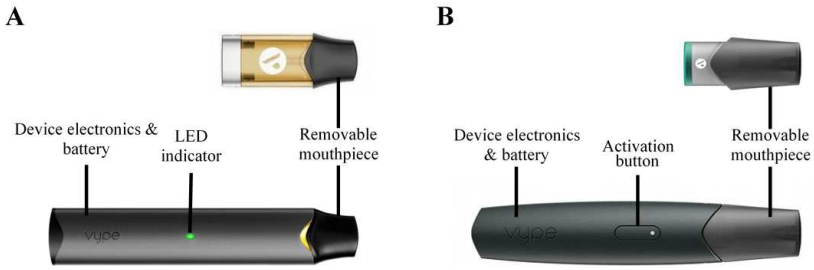

Figure 2. *Schematic representations of the Vuse ePod (A) and Vuse ePen3 (B) e-cigarettes.*

2.6. Investigational Products

No investigational products will be provided for use during the study. Smokers of any brand of cigarette will be recruited; however, EC users will be exclusive users of Vuse ePod or ePen3. These two products are commercially available (at the time of the study) closed-system ECs manufactured by British American Tobacco (Figure 2). ePod consists of a reusable section containing a 350 mAh rechargeable battery; and disposable cartridges containing 1.9 mL of e-liquid, a

heating element, and a heated ceramic wick that atomises the e-liquid. ePen3 is also a closed-system EC, but it has a more powerful 650 mAh battery and a larger 2.0 mL e-liquid cartridge. Both ECs may be used only with the specifically designed cartridges.

EC users and smokers will be instructed to bring a supply of their usual device and cartridges or cigarettes to the clinic, sufficient to cover their typical usage for the duration of the screening and confinement period. During screening and confinement, EC users and smokers will be free to use their products in a location suitable for this purpose, as and when they would normally, apart from at times where this will interfere with specific screening or on-study assessments. This approach will allow for measurement of short-term BoEs while being reflective of subjects' product use outside the clinic.

2.7. Study Assessments

The study schedule and assessments are shown in Table 3. Routine clinical laboratory tests will be performed at screening to exclude significant medical conditions and at discharge to monitor participant health and safety. During the clinic confinement period, participants will be asked to undertake a 24-hour urine collection and give blood samples for biomarker analysis. Urine collection will begin on Day 1, immediately after enrolment, and the remaining assessments will be performed on Day 2 prior to discharge.

Table 3. *Schedule of study assessments.*

	Screening (Day 1)	On-study (Days 1 and 2)	Discharge (Day 2)
Subject free to vape/smoke as usual[1]	X	X	X
Informed consent	X		
Inclusion/exclusion criteria	X		
Socio-demographic data	X		
Medical history	X		
Concomitant medications	X	X	X
Tobacco and nicotine use history questionnaire	X		
Pregnancy test (urine and serum)[2]	X		
COVID-19 test	X		X
Height, weight, BMI, waist circumference	X		
Vital signs[3]	X		X
Single 12-lead electrocardiogram	X		
Physical examination	X		X[4]
Urinary cotinine screen	X		
Urine drugs of abuse and alcohol screen[5]	X		
Serum biochemistry and haematology	X		X
Urinalysis	X		
Virology (hepatitis B and C, HIV)	X		
Exhaled CO measurement[6]	X		
Spirometry (without bronchodilator)[7]	X		
Exhaled nitric oxide measurement[8]		X	
24-hour urine collection		X	
Blood sampling for biomarker analysis		X	
Carotid intima-media thickness assessment		X	
Quality of life questionnaire		X	
Adverse event recording	X	X	X

1. At all times apart from when this would interfere with specific study assessments
2. Females only
3. Including pulse rate, systolic and diastolic blood pressure, respiratory rate and tympanic temperature
4. Symptom-driven physical examination will be performed if deemed necessary
5. Breath test
6. To be taken as final screening assessment; no food, smoking or vaping will be allowed within 30 minutes prior to assessment
7. No food allowed within 2 hours and no smoking or vaping within 1 hour prior to assessment
8. No food or drink allowed within the 1 hour and no smoking or vaping within 30 minutes prior to assessment.

2.7.1. Sample Collection

Urine samples will be collected over a 24-hour time period. The urine samples collected during a single 24-hour interval will be pooled together at the end of the 24-hour period and will be thoroughly mixed before providing aliquots for analyses. Blood samples will be taken either by direct venepuncture or from a cannula placed in a forearm vein. The total volume of blood to be drawn from each subject will be less than 100 mL, inclusive of that required for clinical laboratory and biomarker assessments. CEVal assessment will be performed on washed erythrocytes derived from 5 mL whole blood.

2.7.2. BoEs

The BoEs assessed will be: total NNAL, TNeq, MHBMA, HMPMA, 3-HPMA, total NNN, 3-OH-B[a]P, and S-PMA in urine. Total NNAL, total NNN, TNeq, 3-OH-B[a]P and all

mercapturic acids (MHBMA, HMPMA, 3-HPMA and S-PMA) analysis will be carried out at Analytisch-biologisches Forschungslabor (ABF) GmbH, Planegg, Germany as previously described [67, 68].

2.7.3. BoPHs and Physiological Assessments

The BoPHs assessed will be: COHb, HDL, s-ICAM1 and WBC in blood, 8-Epi-$PGF_{2\alpha}$ Type III and 11-dTX B2 in urine, FeNO in exhaled breath and the physiological assessments CIMT and FEV1%pred. HDL and WBC will be analysed at an RPL-nominated pathology/clinical laboratory using cobas® 8000 c 702 and Sysmex XN20/XN10 automated analyser systems, respectively. 8-Epi-$PGF_{2\alpha}$ Type III and 11-dTX B2 analysis will be carried out at ABF as previously described [69]. In addition, COHb analysis will be carried out at ABF according to a published method [70] with modifications. Briefly, 100 µL of whole blood is spiked with 50 µL of in-situ generated internal standard (IS) solution (saturated whole blood containing ^{13}COHb) and 1.4 mL of water. CO is released with the addition of 200 µL of potassium hexacyanoferrate solution ($K_3[Fe(CN)_6]$ 200 g/L) at 55°C for 30 minutes in a head space vial. 1 mL of the head space is injected into a model 6890 GC interfaced to a model 5973 mass selective detector (Agilent Technologies, Waldbronn, Germany) equipped with a multi-purpose autosampler (Gerstel, Mülheim, Germany). Chromatographic separation is achieved on a RT-Msieve 5A PLOT capillary column (30 m x 0.32 mm ID, 30 µm thickness; Restek, Bad Homburg, Germany). The injector temperature is set to 150°C with a split of 9:1 and a constant helium flow of 1.9 mL/minute. An isotherm temperature program (45°C) is applied for chromatographic separation. MS detection is performed in the selected ion monitoring mode with electron impact ionization. The transfer line temperature is set to 280°C with a source temperature of 230°C and a quadrupole temperature of 150°C. The mass fragment used for quantification is m/z of 28 (IS: 29) and m/z of 12 (IS: 13) as qualifier. The retention of CO is 5.8 minutes. Celerion AG, Zürich, Switzerland will conduct the plasma s-ICAM1 analysis using ELISA (DuoSet ELISA kit, R&D systems, Minneapolis, USA). CIMT will be assessed by ultrasound; the measurement will be performed on a 10 mm section of the distal portion of the common carotid artery, on both sides of the neck, at least 5 mm from the carotid bulb. The mean, standard deviation and maximum thickness of the intima-media will be recorded. FEV 1% pred will be measured by spirometry assessment (without a bronchodilator) in accordance with procedures of the American Thoracic Society/European Respiratory Society [71], values will be standardised to the Global Lungs Initiative predictive values. Participants will not be allowed to eat for 2 hours or smoke or vape within the 1 hour prior to spirometry assessments. FeNO will be measured using an appropriate device such as the Vivatmo Pro (Bosch Healthcare Solutions, Waiblingen, Germany).

2.7.4. Quality of Life Questionnaire

Quality of life will be self-reported via the RAND 36-Item Short Form Health Survey questionnaire [72].

2.7.5. Subject Compliance

Compliance with smoking abstinence in the previous 6 months in EC users and former smokers will be assessed by measurement of CEVal in erythrocytes as previously described [73] and analysis will be conducted by ABF. Urinary creatinine analysis will be carried out at Celerion as previously described [67].

2.8. Safety

Participant safety will be monitored by assessment of vital signs, physical examination, 12-lead electrocardiogram (ECG), and clinical laboratory assessments (biochemistry, haematology, virology, and urinalysis). Adverse events (AEs) and serious adverse events (SAEs) will be monitored during the clinic stay and by telephone 7 days after discharge. If the study is stopped due to an AE, it will not be recommenced without reference to the study ethics committee. All AEs will be recorded on the participant's CRF and coded in accordance with the latest version of the Medical Dictionary for Regulatory Activities and tabulated by system organ class and preferred term. Severity will be classified as mild (does not cause significant discomfort or change in activities of daily living, symptoms are easily tolerated), moderate (causes inconvenience or concern to the participant, interferes with activities of daily living but such activities may be continued), or severe (significantly interferes with activities of daily living to the point where they cannot be continued, or the participant is incapacitated). The numbers and percentages of participants reporting at least one AE, SAE, or an AE leading to withdrawal from the study, and by the numbers and percentages of participants with AE by severity will be reported.

Participants who develop an AE at any time during the study will be followed up until any required evaluations have returned to baseline or until the PI has determined that these events are no longer clinically significant. AEs reported after 7 days will be followed up until no further attention is required. At this point, the study will be completed. The ethics committee will be informed of study completion within 90 days of the last participant's final study procedures.

2.9. Statistical Analysis

All primary endpoints were assessed by literature review. Of these, s-ICAM1 showed the most variability in terms of mean ratios and coefficients of variation (CVs). The ratio of means for s-ICAM1 between current and former smokers was 0.697–0.847 in identified studies, and CVs were 24.5–34.1%. Based on these data, a sample size calculation was performed using PROC POWER (SAS version 9.4) to enable assessment of differences between EC users and current smokers in this study. It was assumed that EC users have a ratio below 1 when compared to smokers and set a mean ratio of 0.847 with CV of 27.1–32.8% based on data from Haswell et al. [74]. These values would yield β=0.2 and α=0.05. Thus, it was determined the study would require 84–

120 participants to complete across the two groups to demonstrate a significant difference. This number would provide power of 0.806. Since the split between EC users and current smokers is not planned to be equal, the total for these combined groups will be set at a minimum of 120. The aim is to recruit 140 participants across the EC users and current smoker groups, allowing for up to 20 non-compliant EC users (as determined by CEVal assessment) while still ensuring the 120 minimum is met. For the former and never smoker groups approximately 40 of each will be recruited, as this was considered sufficient to characterise biomarker levels in these populations. It is anticipated the attrition will be low as participants are enrolled on the same day as screening and discharged the following day. If participants withdrawn prematurely from the study, irrespective of the reason, lead to a drop below these pre-planned numbers, they may be replaced to ensure the minimum values are met.

Datasets will be generated from the final study database. A detailed statistical analysis plan describing the full methodology will be written and finalised before database lock. In general, continuous variables will be presented by means of descriptive statistics (n, mean, standard deviation, median, and minimum and maximum) and categorical variables will be presented by means of frequency tables. Appropriate statistical tests will be used to compare group means for each primary and secondary objective between smokers and exclusive EC users in the per-protocol population and the CEVal-compliant population. The safety analysis will be performed in all participants. Analyses will be performed with SAS software.

2.10. Data Management

Full details of data management methods will be documented in a data management plan, which will be written and finalised prior to collection of the first data. Completeness of the participants' records, accuracy of recording on the CRFs, adherence to the study protocol and to good practice guidelines, and progress of enrolment will be checked throughout the study by an independent clinical research associate. CRFs will serve as the source documents for reviewing data collection procedures.

Data will be captured on source paper data documents and then entered in the electronic data capture system by staff at the clinical site. Data entry will undergo quality control checks. Any discrepancies will be resolved in the database. Following all data validation steps, the PI or designee will electronically sign the completed electronic data prior to database lock. All primary sources and copies of data generated by the study site (e.g., laboratory records, CRFs, data sheets, correspondence, photographs, and electronic records) that are necessary for the reconstruction and evaluation of the study report will be retained in the study site's archive for 25 years after completion of the study.

3. Discussion

The aerosol of ECs contains substantially fewer and greatly reduced levels of HPHCs [21], indicating that these nicotine products may have a role to play in a tobacco harm reduction approach [14, 24]. To determine whether the decrease in HPHCs in the aerosol of ECs translates to a reduction in toxicant exposure and potential health risks for EC users relative to cigarette smokers, this cross-sectional study will compare BoEs and BoPHs between individuals who have been exclusively using ECs for at least 6 months, and current, former and never-smokers. The results of the study are anticipated to add to the current knowledge of the potential for ECs to reduce toxicant exposure and potential health risks for individuals who do not want to quit nicotine use.

In the past few years, studies have begun to document BoEs associated with EC use by a number of approaches. Early research on first-generation ECs noted reductions in CO, cotinine and 3-HPMA among smokers switching to EC use for 4 weeks [25], and significantly lower urinary metabolites of acrolein, crotonaldehyde and 1,3-butadiene in EC users than in smokers [75]. Short-term studies have also assessed biomarkers among smokers switching to exclusive EC use in a controlled environment, noting decreases in 23 BoEs after exclusive EC use for 5 days [76], and substantial reductions in the BoEs NNAL, 3-HPMA, MHBMA, S-PMA and COHb after exclusive use of a pod-type EC for 6 days [77].

Recently, in a novel approach, EC users and smokers were recruited online and urine/blood samples were collected at local clinics to assess BoE and BoPH in these user groups [78]. Total NNAL, 3-HPMA, COHb and TNeq were up to 86% lower in EC users than in smokers, while the BoPHs 11-dTX B2, 8-Epi-$PGF_{2\alpha}$ Type III and s-ICAM1 were also lower in EC users, suggesting that EC users may have lower health risks than cigarette smokers. Similarly, s-ICAM1 and 8-Epi-$PGF_{2\alpha}$ Type III were found to be lower among EC users than among cigarette smokers in Wave 1 (2013-2014) of the PATH study [79].

This study will continue to build on these data, providing data on 17 BoEs and BoPHs in EC users and current smokers. In contrast to many of the above studies, which relied solely on participants self-identifying as EC users or smokers, the present study has the strength that participants who self-report as exclusive EC users or former smokers will have their compliance assessed using CEVal [80]. In addition, it will measure basal BoEs and BoPHs in regular users of an EC that they have chosen, rather than in smokers who have been asked to switch to a specific investigational product for a short period of time [76, 77]. Lastly, the total number of participants recruited to the study (n=220) is substantially higher than that in many of the previous studies due to the cross-sectional study design [25, 75].

We also note some limitations to the study design. The present study is cross-sectional, providing an assessment of the study population at a specific time. This approach can be both time and cost effective compared to other types of study designs. Vuse/Vype closed systems ECs have been available in the UK since 2017 with sufficient numbers of exclusive ePod and/or ePen3 users to make a cross-sectional study

design feasible. Although this study design will allow us to compare the levels of several BoEs and BoPHs between EC users and current smokers, no definite information about cause-and-effect relations will be obtained. In contrast, a longitudinal study involves repeatedly assessing the same subjects to determine potential changes that occur over a period of time. Longitudinal studies involving novel tobacco and nicotine products can involve switching a population of smokers from combustible cigarettes to novel products and assessing potential reductions in BoEs and favourable changes in BoPHs over time. These studies typically follow subjects for 3-6 months [41, 80, 81], as although changes can be observed in BoEs in shorter timescales the BoPHs often require a longer time of observation. A longitudinal study design allows the evaluation of changes at both the group and individual level, but both the length of the study and the repeated assessments does make longitudinal study more expensive and time-consuming relative to a cross-sectional study.

As for many BoPHs utilised in smoking and switching studies, those such as WBC and s-ICAM1 are not specific for smoking-related diseases; they may be modulated by other risk factors and associated lifestyle choices such as diet and exercise, in addition to smoking [36-40, 48-57], and these factors cannot be fully accounted for.

4. Conclusion

Given the interest in encouraging smokers to switch to ECs as part of a tobacco harm reduction approach, this is a timely study that will provide information on biomarkers of tobacco toxicants and potential health risks among regular and relatively long-term users (> 6 months) of ECs.

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