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Research paper

Statistical analysis plan for "A randomised, controlled study to evaluate the effects of switching from cigarette smoking to using a tobacco heating product on health effect indicators in healthy subjects"

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ABSTRACT

Tobacco harm reduction strategies aim to substitute smoking with potentially reduced risk products (PRRPs) such as e-cigarettes and tobacco-heating products (THPs). The health benefits of switching from smoking to PRRPs is unknown. A randomised controlled trial is being conducted to increase understanding of the health effects of switching from smoking to a THP in a 12-month long ambulatory study (ISRCTN81075760). Here we describe the study endpoints and the statistical analysis plan. Endpoints are divided into biomarkers of exposure (BoE) to tobacco smoke constituents and health effect indicators related to risk of lung cancer, cardiovascular and obstructive lung disease. These have been selected on the basis of extensive literature evidence. Three primary endpoints, augmentation index (risk factor for cardiovascular disease), total NNAL (linked to lung cancer) and 8-Epi-PGF2α type III (indicator of oxidative stress linked to various diseases), and multiple secondary endpoints will be analysed at 90, 180, and 360 days. Changes from baseline will be compared between study arms by specific contrasts in mixed models. Study wise multiple comparisons adjustments will be performed to account for multiplicity of timepoints and comparisons within timepoints. Generalisability of outcomes will be tested by a sensitivity analysis adjusting for age and gender. Importantly, an ancillary analysis will be performed to assess product compliance during the study based on plasma levels of CEVal, a surrogate marker for acrylonitrile exposure. The rationale underlying the selection of BoEs and health effect indicators, coupled with the statistical analysis plan will be central to understanding the potential health effects of replacing smoking with THP use for one year.

1. Introduction

In the past few decades, smoking prevalence has significantly declined around the world due to regulatory policies and educational campaigns. However, this success has been only partial, and smoking remains one of the most important causes of preventable disease, with the World Health Organization (WHO) estimating that there will be around 1.5 billion smokers worldwide by 2050 [1].

Pharmaceutical products commercialised as nicotine replacement therapies (NRTs) are designed to assist smokers by relieving cravings from nicotine withdrawal and, hence, to increase the likelihood of smoking cessation. However, the nicotine pharmacokinetic profiles of these products, such as patches, gums, sprays and inhalers, are dissimilar to those of conventional cigarettes, with typically lower C_{max} [2,3]. Due to these differences in nicotine pharmacokinetics and rituals associated with smoking, smokers may not find pharmaceutical products as satisfying and have limited effect without behavioural support. Differences in delivery format and pharmacokinetic profiles may be some of the reasons for the limited efficacy of NRT products as aids to smoking cessation [4,5].

Policy-makers have used harm reduction approaches to enhance interventions. These approaches can be particularly beneficial when harm cannot be easily eradicated [6]. It has been suggested that a tobacco harm reduction approach could bring benefits to the overall population by substituting combustible products with products with a lower risk profile [7]. For inhalable products, these potentially reduced risk products (PRRPs) belong to two main categories: vapour products,

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Abbreviations

1-OHP	1-Hydroxypyrene
2-AN	2-Aminonaphthalene
3-HPMA	3-Hydroxypropylmercapturic acid
4-ABP	4-Aminobiphenyl
6MWT	Six Minute Walk Tests
AE	Adverse Event
Ae24h	Amount excreted over 24 Hours
AIx	Augmentation Index
BoE	Biomarker of Exposure
CEMA	2-Cyanoethylmercapturic acid
CEVal	N-(2-cyanoethyl)valine haemoglobin adducts
CI	Confidence interval
CO	Carbon Monoxide
COPD	Chronic obstructive pulmonary disease
dTx	11-Dehydrothromboxane B2
ET-1	Endothelin-1
FDA	Food and Drug Administration
FeNO	Fractional exhaled nitric oxide
FEV1	Forced expiratory volume in 1 s
FMD	Flow-mediated dilation
FVC	Forced vital capacity
HDL	High-density lipoprotein cholesterol
HEMA	2-Hydroxyethylmercapturic acid
HMPMA	3-Hydroxy-1-methylpropylmercapturic acid
HPHC	Harmful and potentially harmful constituents
LDL	Low-density lipoprotein cholesterol
LS	Least squares
MCP-1	Monocyte chemoattractant protein-1
MHBMA	Monohydroxybutenyl-mercapturic acid
MRTP	Modified Risk Tobacco Product
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNN	N-nitrosonornicotine
NO	Nitric oxide
NRT	Nicotine replacement therapy
o-Tol	Ortho-toluidine
PRRP	Potentially Reduced Risk Product
PWA	Pulse wave analysis
PWV	Pulse wave velocity
RHI	Reactive hyperaemia index
sICAM-1	Soluble intercellular adhesion molecule-1
S-PMA	S-Phenylmercapturic acid
THP	Tobacco Heating Product
TNeq	Total nicotine equivalents
WHO	World Health Organization

which are also known as electronic cigarettes or e-cigarettes and tobacco heating products (THPs).

A number of studies have been carried out on e-cigarettes including analysis of emissions and toxicological analysis in comparison to conventional cigarettes. These studies indicate reductions in chemical toxicants [127] as well as reduced levels of DNA damage [128], mutagenicity [129,130], cytotoxicity [131], and carcinogenicity [132]. In addition, clinical studies have demonstrated that when smokers switch to e-cigarettes there are substantial reductions in exposure to selected cigarette smoke toxicants [133,134].

THPs consist of a two-part system comprising a tobacco-containing consumable and an electronic heating device that heats tobacco, typically to temperatures lower than 345 °C, to avoid combustion [8]. Due to the absence of tobacco combustion, significantly fewer chemical toxicants are formed, however, nicotine is still released with the inhaled aerosol [9,10]. Given the presence of a cigarette-like hand-to-mouth action, and the presence of nicotine in the aerosol, THPs are expected to provide a more familiar experience to smokers helping them to transition from combustible cigarettes to a PRRP.

Compared with e-cigarettes, there has been less published research investigating the properties of THPs; however, available data generated from laboratory smoking machines and short-term biomarker of exposure studies have revealed significant reductions in emissions and exposure, respectively, to many chemical toxicants found in cigarette smoke [120,122]. Smoking health risks are linked to the amount of cigarettes smoked and the duration that consumers have smoked for [11], this relationship is not necessarily linear, however, a significant reduction of repeated and sustained exposure to cigarette smoke is expected to have a beneficial effect on health outcomes. If widely adopted and if these products prove to substantially reduce risk, this strategy might offer substantial public health gains by providing smokers, who would otherwise continue to smoke, with alternative sources of nicotine, but with similar rewarding effects, i.e., the substitution of cigarettes by PRRPs.

Measuring biomarkers of exposure (BoEs) associated with cigarette smoke exposure is informative as it could, for example, demonstrate how far using a PRRP instead of smoking reduces a user's exposure to certain toxicants found in cigarette smoke. However, how these changes in BoEs translate into changes in risk for smoking related diseases is still unknown. Therefore, additional biomarkers that go a stage further and could potentially indicate changes in disease development and health outcomes are investigated. This report describes the statistical approaches and rationale used to investigate both BoEs and health effect indicators in a 12-month long ambulatory study of smokers who switch from cigarettes to a THP, contextualised against smokers who cease cigarette smoking, and individuals who have never smoked [12].

2. Methods

2.1. Study design

The full protocol describing this study has been recently published [12]. In brief, this is a multi-centre randomised switching study where participants are recruited from three populations:

Continue to smoke/THP population (n = up to 280);

Intend-to-quit population (n = 190);

Never smoker population (n = 40).

Subjects in the continue to smoke/THP population will be randomised to continue smoking commercially manufactured filter cigarettes and/or roll your own (n = up to 80, Arm A) or randomised to the THP glo™ coded as THP1.1(RT) (n = 200, Arm B). Subjects in the intend-to-quit population will be allocated to the assisted smoking cessation arm (Arm D). Subjects in the never smoker population will be assigned to the never smoker arm (Arm E). Main endpoint assessments will take place at days 90, 180 and 360 from baseline.

2.2. Sample size determination

The target of 50 subjects for Arms A, B and D was based on the primary biomarker, Augmentation Index (AIx), requiring the largest sample size to observe differences between the test and control product. Specifically, the power calculation was based on the number of subjects required to perform a contrast based on the F-statistic with 90% power between the arm using the THP product and the continue to smoke arm at day 360. For this calculation, we assume an expected change in this

biomarker of 80% with respect to changes observed in subjects completely quitting smoking [13]. Hence, the AIx expected means were 25.7% and 17.5% for the smoker and THP arms, respectively, with a common standard deviation of 12.4%. The significance level was adjusted for timepoint multiplicity using the O'Brien-Fleming sequential approach with $\alpha = 0.0471$ at the end of the study.

The objective is to complete the study with at least 50 subjects in each arm with the exception of never smokers, for which 30 was considered sufficient to characterise a never-smoker benchmark [124]. The number of subjects allocated to each group is based on the expected attrition rate in this 12-month ambulatory study. Attrition rates in Arm A (continue to smoke) and Arm E (never smoker) are expected to be low based on our previous experience with ambulatory smoking studies [14]. However, it has been observed that significant attrition rates occur in smoking cessation studies [15], and it is expected that switching completely to a new product may also lead to study withdrawal. Therefore, additional subjects were assigned to these study arms to account for the anticipated attrition rates.

The main assessment timepoints are Day 90 (±3 days) for BoE endpoints only and, Day 180 (±2 weeks) and Day 360 (±2 weeks) for all endpoints.

2.3. Randomisation

Some endpoints assessed in this study have been shown to be affected by demographic characteristics such as gender and age. For example, NNAL has been found to be correlated to age and gender [126]. Aiming to mitigate potential confounding effects due to unbalanced demographics, the study arms are randomized by using four separate randomisation lists for gender combinations and age 40 years as a threshold. This generated four lists of subjects at each centre (males $\leq$ 40, males $>$ 40, females $\leq$ 40 and females $>$ 40 years). Within each list, blocks of 8 are used to allocate 2 subjects to Arm A (continue to smoke) and 6 to the Arm B (switch from smoking to THP).

2.4. Analysis populations

The *randomised population* is defined as all subjects who were assigned to a study arm and had at least one valid assessment of a biomarker variable.

The *per-protocol population* is defined as all subjects who had a valid assessment of a biomarker variable and completed the study without major protocol deviations.

All statistical analyses will be performed on the randomised and per-protocol populations.

2.5. Product compliance

Subject compliance is a crucial aspect of every clinical study as it has a large bearing on the outcome. Subject compliance to their assigned arm will be extremely important for the assessment of biomarker changes during this study. Compliance will be particularly important for subjects switching to the THP (Arm B) and ceasing to smoke (Arm D), where a full switch to the THP or complete abstinence from smoking is intended respectively. If subjects fail to comply with this and continue smoking, potentially alongside the investigational product, they are not likely to experience the full change in biomarker levels or may even experience no changes at all.

In such a long ambulatory study, self-reported cigarette consumption is not likely to be a reliable means of determining subjects' cigarette use. Furthermore, the clinical assessments typically used for this purpose have a short half-life and may not be able to detect smoking, even if it has occurred a few days earlier, thus, longer term biomarkers indicative of cigarette consumption are required. To enable identification of potential non-compliance, we will use *N*-(2-cyanoethyl)valine haemoglobin adducts (CEVal), a biomarker of exposure to acrylonitrile. Acrylonitrile is not expected to be present in the THP emissions, or in much lower concentrations than cigarettes [9], and CEVal has a long half-life, based on the red blood cell life cycle which is between 90 and 120 days in the circulation in healthy individuals. Therefore, it is expected to take several months before concentrations of CEVal fall to levels comparable to those of never smokers. This property could potentially make CEVal a suitable biomarker of compliance for this study.

Based on a previous study where participants were switched to a prototype combustible product [14], we computed thresholds for CEVal for the main assessment timepoints of the study (data not published). Based on the concentration of acrylonitrile in the emissions of the products, the THP product in the current study is expected to outperform the prototype combustible product used in the previous study, and thus CEVal concentrations are expected to be lower than those observed in the previous study. Thresholds are based on percentiles with the exception of potential THP solus use at Day 360, which has been defined as the maximum concentration among never smokers observed in the previous study [14] because it was higher than the suggested threshold from switching. Therefore, this is a conservative approach in which some non-compliant subjects may still be classified as potential THP solus users or complete cessation (Arm D). The thresholds proposed to guide assessment of lack of compliance are summarised in Table 1.

These thresholds are speculative and not validated, therefore, it is unknown how many subjects will fall within each category. A minimum of 30 subjects in a category is considered necessary to provide statistical power for most of the BoEs. In order to reach 30 subjects for statistical analysis, a stepwise merging method will be taken, starting in the lower categories (the more likely compliant subjects), and statistical analysis will be performed only if there is a minimum of 30 subjects in a group. Starting from the lowest category, this category will be merged with the next category until the minimum number of 30 subjects is reached. This approach may yield up to two groups (S + D and D + H) if individual categories do not reach n = 30. Note that merging S + D + H would be the same as a per-protocol analysis. If the number of subjects in a group is still below 30 after merging, no further analyses will be performed for that group.

3. Rationale for study outcomes

Smoking is known to be a risk factor for several chronic diseases [111]. However, its mechanisms are complex and depend, to a great extent, on exposure and duration of tobacco smoke exposure; nevertheless, other still unknown factors contribute to disease development because not all smokers develop disease [11]. Within this context, the predictability of disease is limited and currently there is no qualified panel of endpoints that can predict smoking-related disease development.

Epidemiological studies have been able to demonstrate the harm caused by smoking by assessing disease prevalence among people with different smoking habits [111]. In the longer term, whether PRRPs realise their potential and become reduced risk products is also likely to be revealed through epidemiology. Given that these products have been commercially available only relatively recently, epidemiological data will not be available for many years and it is critical to provide the most complete information to policy-makers and consumers to help them

Table 1
Proposed thresholds to guide assessment of lack of compliance.

Category	Day 90	Day 180	Day 360
Highly Likely Smoking (H)	>164 pmol/g Hb	>112 pmol/g Hb	>78 pmol/g Hb
Potential Dual Use (D)	[78, 164 pmol/g Hb]	[54, 112 pmol/g Hb]	[35, 78 pmol/g Hb]
Potential Solus Use (S)	<78 pmol/g Hb	<54 pmol/g Hb	<35 pmol/g Hb

make informed decisions now. Therefore, this study investigates several health effect indicators that are thought to be related to various smoking-related disease pathways.

Due to the long-term complex nature of disease development associated with smoking and the complex nature of tobacco smoke, which contains more than 6500 compounds [16], a large number of endpoints have been included in this study. The endpoints include BoEs to investigate exposure to cigarette smoke toxicants, whereas the health effect indicators and physiological measures characterise biological functions that are known to be perturbed by tobacco smoke. Where there are no qualified health effect indicators to predict the onset of smoking-related disease, multiple indicators have been included to characterise trends in each area of interest and progressive stages of disease development.

Overall, the study outcomes can be broadly split into BoEs to tobacco smoke constituents (Table 2) and health effect indicators relating to i) lung cancer risk, ii) cardiovascular risk and iii) obstructive lung disease risk (Table 3). Comprehensive reviews of many of the biomarkers included in this study and their applicability to the assessment of novel tobacco and nicotine products which serve as good point of reference for a broad overview of the area, have been conducted by Scherer and Peck [17,123].

Three endpoints were chosen as primary endpoints: total NNAL, AIx and 8-Epi-prostaglandin F2α type III (8-Epi-PGF2α type III). Their relevance to tobacco-related disease outcomes are explained in the following sections.

3.1. Biomarkers of exposure

To investigate whether reduced exposure is sustained, biomarkers (urinary and exhaled breath) were selected in accordance with the Food and Drug Administration (FDA) list of harmful and potentially harmful constituents (HPHCs) of tobacco and tobacco smoke, with the exception of pyrene, which is used as a surrogate BoE for benzo[a]pyrene [18,19].

Total NNAL has more commonly been used as a BoE for exposure to 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) a tobacco specific-nitrosamine. However, this was included as a primary end-point in this study because of its potential to cause DNA damage and association with cancer [21].

3.2. Health effect indicators

Health effect indicators can be broadly split into the areas of i) lung cancer risk, ii) cardiovascular risk, and iii) obstructive lung disease risk. The development of each of these diseases is an on-going process over time and involves many different mechanistic networks; therefore, health effect indicators have been selected that cover various stages of the disease development process. These range from biomarkers of immediate biological response to exposure to chemical toxicants (e.g. oxidative stress) to biomarkers which are related to pathological processes which take longer to manifest (e.g. arterial stiffness). The aim of this approach is to yield data on whether or not shorter-term biological changes are associated with longer term changes of direct relevance for smoking-related disease development. The health effect indicators included in this study are described in further detail below.

3.2.1. Biomarkers of oxidative stress

Oxidative stress has been described as *"an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage"* [22], and is reported to be a significant factor behind the development of all three of the above disease risk areas [23–25]. Oxidants are known to directly and indirectly damage DNA, which increases the risk of permanent DNA mutations and subsequently neoplasia under suitable local conditions [26,27]. Furthermore, oxidative stress is known to contribute to impaired vasodilation of vascular tissue, of relevance to arterial stiffening and hypertension [28] and chronic inflammatory states in vascular tissue and the lung, of relevance to the development of atherosclerosis and chronic obstructive pulmonary disease (COPD) [29,30].

To assess the primary objective of the study, 8-epi-prostaglandin F2α type III (8-epi-PGF2α); an isoprostane and product of lipid peroxidation [27] will be measured in urine. Given the numerous smoking data available of 8-isoprostanes and smoking, its fairly consistent change upon smoking cessation (and in smaller sample sizes), its decline upon THP use and link to smoking-related diseases including a potential link to hypertension [28] we included 8-epi-prostaglandin F2α type III as a primary outcome in this study.

3.2.2. Biomarkers of inflammation

Acute and chronic inflammation are hallmarks of tissue damage and the developmental stages of vascular and obstructive lung disease, respectively [23,25]. Inflammation also has numerous roles in carcinogenesis and tumour progression [31,32]. Persistent exposure to chemical toxicants, radical species, and physical and microbial insults can lead to persistent damage, unresolved inflammation, and subsequently tissue re-modelling over time, as the body adapts to protect itself from chronic noxious stimuli [33]. Examples of tissue re-modelling are the development of atherosclerotic lesions and arterial stiffening of relevance for cardiovascular disease [33], metalloproteinase release, fibrosis and emphysema of relevance for obstructive lung disease [34], squamous cell metaplasia and epithelial to mesenchymal transition of relevance for

Table 2
Biomarkers of exposure.

Biomarker	Abbreviation	Associated toxicant/compound	Matrix
Carbon monoxide[b]	CO	Carbon monoxide	Exhaled breath
Total nicotine equivalents (nicotine, cotinine, 3-hydroxycotinine and their glucuronide conjugates)[b]	TN_{eq}	Nicotine	Urine (24-h)
Total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol[a]	Total NNAL	Metabolite of the smoke toxicant 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	Urine (24-h)
Total *N*-nitrosonornicotine[b]	Total NNN	NNN	Urine (24-h)
3-Hydroxypropylmercapturic acid[b]	3-HPMA	Acrolein	Urine (24-h)
3-Hydroxy-1-methylpropylmercapturic acid[b]	HMPMA	Crotonaldehyde	Urine (24-h)
S-Phenylmercapturic acid[b]	S-PMA	Benzene	Urine (24-h)
Monohydroxybutenyl-mercapturic acid[b]	MHBMA	1,3-butadiene	Urine (24-h)
2-Cyanoethylmercapturic acid[b]	CEMA	Acrylonitrile	Urine (24-h)
1-Hydroxypyrene[b]	1-OHP	Pyrene	Urine (24-h)
2-Hydroxyethylmercapturic acid[b]	HEMA	Ethylene oxide	Urine (24-h)
4-Aminobiphenyl[b]	4-ABP	4-aminobiphenyl	Urine (24-h)
2-Aminonaphthalene[b]	2-AN	2-aminonaphthalene	Urine (24-h)
Ortho-toluidine[b]	o-Tol	Ortho-toluidine	Urine (24-h)

[a] NNAL is included as a primary endpoint due to its association with cancer development [20,21].
[b] Secondary endpoints.

Table 3
Health effect indictors.

Study endpoint	Disease pathway	Summary description	Related references
8-Epi-prostaglandin F2α type III[a]	Oxidative stress	Surrogate outcome measure of oxidative burden in the body, which may be indicative of future disease risk. A product of lipid peroxidation [25] will be measured in urine	[17,54–58]
4-Hydroxy-2-nonenal	Oxidative stress	A product of lipid peroxidation [57]	[17,60]
Homocysteine	Oxidative stress	Known to deplete endothelial antioxidant levels [59]	[17,62]
3-Nitrotyrosine	Oxidative stress	A product of nitrosative stress following the interaction of tyrosine residues with peroxynitrite [61]	[17,64]
White blood cell count	Inflammation	Marker of general inflammation; elevated blood levels are associated with the development of atherosclerosis	[17,54]
High sensitivity C-reactive protein	Inflammation	Marker of general inflammation; elevated blood levels are associated with the development of atherosclerosis	[17]
Monocyte chemoattractant protein-1 (MCP-1), E-selectin, and soluble intercellular adhesion molecule-1 (sICAM-1)	Inflammation	Associated with endothelial dysfunction and adhesion of immune cells to the vascular endothelium, a critical step in the formation of atherosclerotic lesions [63]	[17,54]
Tissue plasminogen activator	Coagulation	A serine protease found on endothelial cells that catalyses the conversion of plasminogen to plasmin, a major enzyme responsible for the breakdown of blot clots [64]	[65–71]
Plasminogen activator inhibitor-1	Coagulation	A member of the serine protease inhibitor (serpin) family, and a major physiologic inhibitor of serine proteases such as tPA [70]	[68,72–74]
Fibrinogen	Coagulation	The soluble precursor to insoluble fibrin (the major constituent of blood clots); it also supports platelet aggregation [73]	[17,54,75–77]
11-Dehydrothromboxane B2 (dTx)	Coagulation	A metabolite of TxA2, which is a potent activator of platelets with thrombogenic and vasoconstrictive properties; dTx has been implicated in endothelial dysfunction, atherosclerosis, type II diabetes and hypertension [76]	[78–81]; reviewed in Refs. [17,82]
Augmentation index (AIx) and pulse wave velocity (PWV)[a]	Physiological measures: arterial stiffness	Pulse wave analysis (PWA) assesses changes in blood pressure in major arteries during the cardiac cycle. AIx and PWV are two key outputs from PWA with relevance to arterial stiffness	[17,83–89]
Reactive hyperaemia index	Physiological measures: endothelial dysfunction	Finger plethysmography will be used to monitor peripheral reactive hyperaemia as a surrogate measure of flow-mediated dilation (a marker of endothelial dysfunction) [39]	[90–93]
Brachial systolic and diastolic blood pressure	Physiological measures: blood pressure	Chronically elevated blood pressure defines hypertension, a known risk factor for cardiovascular disease	[94–98]
Endothelin-1 (ET-1)	Vascular tone	A potent vasoconstrictor released by endothelial cells that acts upon vascular smooth muscle via the Endothelin A receptor to induce prolonged vasoconstriction. It also acts upon endothelial cells via the endothelin B receptor to induce nitric oxide (NO) production (promoting vasodilation), hence acting as a counterbalance to its own primary effects [97]	[99–105]
6-min walk test (6MWT)	Physiological measures	The 6MWT is a submaximal exercise test used to quantify functional exercise capacity in clinical populations; it is commonly used as an outcome measure for treatment of COPD and cardiovascular disorders	[106–108]
Serum lipids (HDL/LDL/total cholesterol and triglycerides)	Atherosclerosis	Atherosclerosis is a well-known risk factor for cardiovascular events. Accumulation of LDL cholesterol in blood vessel walls is a hallmark of the condition, while athero-protective HDL cholesterol levels are reduced	[17,48–51,53,109,110]
Lung spirometry, FEV1, FEV1/FVC ratio	Lung function: spirometry	Airflow limitation is a major characteristic of COPD. Smoking is well-known to accelerate a decline in forced expiratory volume in 1 s (FEV1) over time [109]; coupled with forced vital capacity FVC (generating the FEV1/FVC ratio), it diagnoses and defines the severity of COPD [110]	[17,113–115]
Fractional exhaled nitric oxide (FeNO)	Nitric oxide bioavailability	Endogenous NO plays an important role in the vasculature and the airways, and is generated by NO synthases. Smoking reduces the generation of NO directly by oxidising critical amino acid residues of NO synthases, and indirectly by reducing bioavailability of enzymatic cofactors (e.g. tetrahydrobiopterin), causing uncoupling of the enzymes [26]	[17,116–119]

[a] Primary endpoints; All other endpoints are secondary endpoints.

lung carcinogenesis [35]. These phenotypes are generally accepted to be pathological in nature and are pre-cursor steps to overt disease.

The inflammatory biomarkers included in this study support the secondary and exploratory objectives of the study.

3.2.3. *Biomarkers of coagulation*

Coagulation is a critical component of tissue repair in the body, however, in some circumstances such as atherosclerotic plaque rupture, coagulation can be very dangerous. In haemostasis, blood vessel walls are lined with antithrombotic mediators, which inhibit platelet activation and coagulation. However, the subendothelial layer is highly thrombogenic. When damage occurs to the endothelium, these thrombogenic factors can activate platelets and initiate the formation of a thrombus. If thrombi are not broken down, they may become lodged in key blood vessels supplying nutrients to the heart and brain, for example. Such occlusions can often lead to myocardial infarction and stroke [36]. Hypercoagulability or thrombophilia is the increased tendency of blood to thrombose, placing affected individuals at a greater risk of thrombotic disease [37]. Tobacco smoking has been reported to induce a hypercoagulation state, where smokers may be more at risk to blood clot formation [38].

3.2.4. *Physiological measures*

Pulse wave analysis (PWA) assesses changes in blood pressure in major arteries during the cardiac cycle, and its potential application to clinical research and treatment has been reviewed by Hametner and Wassertheurer [39]. PWA has been employed mainly in the study of vascular ageing (arterial stiffness) and hypertension [40]. AIx and pulse

wave velocity (PWV) are two key outputs from PWA that have relevance for arterial stiffness and are included as outcomes in this study. Essentially, AIx is a function of the difference between the primary aortic pressure wave just after a heartbeat and the reflected pressure wave from that heartbeat, received back at the central aorta from the distal aortic bifurcation in the pubic area. It is usually normalised to 75 beats per minute to account for variation in heart rate among individuals. Smoking studies involving AIx and PWV are increasing in number of late. Kim et al. [87] reported that current smokers had significantly higher AIx than never smokers and that ex-smokers had significantly lower AIx compared to current smokers. Xue et al. [90] reported that both AIx and brachial/ankle PWV decreased in healthy smokers, following a period of 3 months of smoking cessation, and further improved at a 12-month follow-up. Given the links to cardiovascular risk prediction and potential development of hypertension [28] as well as consistent reversibility upon smoking cessation, AIx was included as a primary outcome in the study. PWV is the speed in m/s at which the pressure waveform traverses major arteries. Taken together, these metrics provide a useful insight into arterial stiffness. PWV was included as a secondary outcome, as although a favourable performance was observed in literature, questions remain over the length of time required to observe meaningful changes in smoking cessation studies.

Finger plethysmography will be used to monitor peripheral reactive hyperaemia in the study groups as a surrogate measure of flow-mediated dilation (FMD; a marker of endothelial dysfunction). Occlusion of the brachial artery for a period of 5 min restricts blood flow to the forearm, resulting in ischaemia. Upon release of the occlusion, the resulting surge in blood flow increases endothelial sheer stress and correspondingly increases production of nitric oxide from endothelial cells, resulting in vasodilation of the distal arteries. This change is captured in the reactive hyperaemia index (RHI), which is calculated on the EndoPAT™ device. In general, RHI values below 2 are categorized as endothelial dysfunction [41].

The 6-min walk test (6MWT) is a simple and low-cost submaximal exercise test used to quantify the functional exercise capacity in clinical populations and, is commonly used as an outcome measure for treatment of COPD and cardiovascular disorders. Longer distances in the 6MWT are associated with higher exercise capacity [42]. Both patients with COPD and cardiovascular-related disorders are known to record lower distances in the 6MWT [43–47], which is symptomatic to poor oxygen bioavailability in the circulation during exercise.

3.2.4.1. Biomarkers of atherosclerosis. Hypercholesterolaemia is a well-known risk factor for cardiovascular disease, and statin therapy has been shown to normalise low-density lipoprotein cholesterol (LDL) levels and reduce cardiovascular risk. However, many people remain at high risk even when their level of LDL has been reduced by aggressive treatment with statins, and this is thought to be due to low levels of high-density lipoprotein cholesterol (HDL) [48]. Lipids such as triglycerides and LDL are well known to accumulate in blood vessel walls, forming plaques, during the process of atherosclerosis. This accumulation is associated with macrophage infiltration, smooth muscle cell phenotypic switching (to a macrophage-like phenotype), leading to the formation of foam cells, macrophagic cells packed with lipid deposits, which persist in the plaque [49]. HDL cholesterol has been reported to be athero-protective in nature. Not only does HDL promote cholesterol efflux from vessel walls [50], it also has been reported to reduce oxidation and inflammation and improve endothelial function and repair [51].

3.2.5. Biomarkers of lung function

COPD is a chronic inflammatory condition of the large and small airways [112], and alveoli that is defined as chronic airflow obstruction that is progressive and only partly reversible [52]. It is associated with severe airflow limitation, mucous hypersecretion, impaired mucociliary clearance, coughing, wheezing and poor gaseous exchange. Smoking is a major risk factor for the disease, but other environmental/occupational hazards are also known to contribute to its development [53].

4. Statistical analysis approaches

Toxicant emissions of the investigational THP product have been shown to be significantly reduced compared to those from cigarette smoke [9], therefore, it is expected that the endpoints chosen in this study will change for the participants who switch to the investigational product compared with those who continue to smoke. Additionally, the cessation arm will provide a benchmark to evaluate the direction and magnitude of those changes.

The concentration of biomarkers, especially BoEs, is linked to smoking behaviour and consumption [14,120]. To favour comparability between arms, primary and secondary endpoints will be assessed as the absolute change from baseline, where baseline is defined as the last value measured prior to commencement of the subject's randomised Arm (Day 0), including unscheduled readings. For each subject, the change from baseline will be calculated by subtracting their individual baseline value from the value at a subsequent timepoint.

Primary and secondary endpoints will be statistically assessed at three time points during the study: Day 90, Day 180 and Day 360. Any primary or secondary endpoints producing statistically significant results at an early timepoint will not be statistically analysed at subsequent timepoints. However, descriptive statistics will be presented for all endpoints at all timepoints.

Urine biomarkers will be expressed as amount excreted over 24h (Ae_{24h}) according to the formula:

$$Ae_{24h}\ [g] = \text{Urine concentration (g/mL)} * \text{urine volume (mL)}$$

4.1. Analysis of primary endpoints

Total NNAL is a urinary biomarker and therefore will be reported as Ae_{24h}. 8-Epi-PGF2α type III will be analysed in 24h urine collection but it will also be reported as corrected by creatinine, simply by dividing each subject's value by their own creatinine concentration measured at the same timepoint. Before statistical analysis, AIx requires normalisation to a heart rate of 75 bpm, which provides AIx75 (%) using the formula:

$$AIx75 = AIx - \left(\left[\frac{(75 - HR)}{10}\right] * 4.8\right).$$

Changes from baseline for all three aforementioned biomarkers will be compared between the THP arm and continue to smoke arm by using specific contrast in a regression model including baseline values and arm as the main effects. Data will be examined and may be transformed to adhere to distributional assumptions associated with statistical tests.

Multiple comparisons adjustments are essential in studies with numerous endpoints to control for Type I error inflation. As explained in section 2.2, to retain an overall significance level of 0.05 we considered the "Multiple Endpoints in Clinical Trials" guidance provided by the US Food and Drug Administration (FDA) [121]. Following this guidance, the overall significance level has been adjusted for each time point using the O'Brien-Fleming method. This provides significance levels of $\alpha = 0.0006$, 0.0151 and 0.0471 for Days 90, 180 and 360 respectively. At Day 90, only BoEs are expected to show a significant change; therefore, only total NNAL will be statistically assessed with $\alpha = 0.0006$. For statistical analyses performed at Day 180 the α level will be equally distributed between primary endpoints. However, if any of the primary endpoints are found to be statistically significant at Day 90 or 180, they will not be assessed in subsequent timepoints, as appropriate, and the assigned α level will be equally distributed between the remaining endpoints. At day 360, $\alpha = 0.0001$ has been assigned to total NNAL and

8-Epi-PGF$_{2\alpha}$ type III and the remaining alpha level (0.0469) will be used to assess AIx.

Generalisability of outcomes will be explored by performing a sensitivity analysis in which the previous model is adjusted for age and gender. This secondary analysis model includes baseline, age (as continuous), gender, arm, and the interaction of gender and arm as fixed effects. In addition, clinical site will also be included as a random effect. If the interaction effect between gender and study arm is found to be significant, specific differences within arm genders will be assessed by contrasts using available α level for each endpoint and timepoint.

An ancillary analysis will also be performed for categories of product compliance based on CEVal concentrations. Contrasts from a regression model with change from baseline as a dependent variable and independent variables including CEVal categories/groups and the control arm continue to smoke (Arm A) will be used to compare each of the product compliance groups to Arm A.

For all analysis, least squares (LS) means and 95% confidence intervals (CIs) for each group (groups based on CEVal compliance analysis) will be presented, along with differences, CIs and p-value for each comparison. If the data are log-transformed before analysis, the results will be back-transformed to provide geometric LS means and 95% CIs, as well as geometric LS mean ratios and CIs for each comparison.

4.2. Analysis of secondary endpoints

Secondary endpoints can be found in Tables 2 and 3 marked with *. Total nicotine equivalents TNeq is a compound endpoint formed as a summation of different nicotine metabolites:

$$\begin{aligned} TN_{eq}\left[mg/24h\right] = \big(&\text{free nicotine }\left[\mu mol/L\right] + \text{nicotine} - \text{glucuronide }\left[\mu mol/L\right] \\ &+\text{free cotinine }\left[\mu mol/L\right] + \text{cotinine} - \text{glucuronide }\left[\mu mol/L\right] \\ &+\text{free trans} - 3' - \text{hydroxycotinine }\left[\mu mol/L\right] \\ &+\text{trans} - 3' - \text{hydroxycotinine} - \text{glucuronide }\left[\mu mol/L\right]\big) \\ &*162.2\ \left[\mu g/\mu mol\right]*(\text{urine volume (L)}/1000) \end{aligned}$$

Due to the nature of the assessment and practicalities in clinic, the procedure for exhaled CO, a short-term indicator of cigarette consumption, will be performed at different time points in contrast with the rest of the study endpoints: Days 120, 150, 210, 240, 300 and 330. Means for the paired values 120 + 150, 210 + 240 and 300 + 330 will be calculated and analysed with the other secondary endpoints as the nominal Days 90, 180 and 360, respectively.

Secondary endpoints will be analysed by following the same approaches used for the primary endpoints, however, the significance level used to perform statistical tests will be determined by the α level remaining after statistical analysis of primary endpoints. This approach implies that if none of the primary endpoints yield statistically significant results, then statistical analysis of the secondary endpoints will not be performed. Additionally, further multiple comparisons adjustments will be carried out among secondary endpoints by using Holms' method [125].

4.3. Handling missing data

No data imputation will be performed for missing data. Taking a conservative approach, for biomarker concentrations below the limit of detection or quantification (e.g., <20 ng/mL), the urine concentration will be replaced by half the limit of detection or quantification respectively (e.g., 10 ng/mL) prior to calculation of the amount excreted. Similarly, if the urine concentration is above the upper limit of quantification (e.g., >500 ng/mL), then the urine concentration will be replaced by the upper limit of quantification (e.g., 500 ng/mL) prior to calculation of the amount excreted.

4.4. Safety data

Adverse events (AEs) will be classed as occurring in one of two periods:

Pre-randomisation – any AE that starts after the subject has provided written informed consent and that resolves prior to 06:00 on Day 0, or an AE that starts prior to 06:00 on Day 1 and does not increase in severity after 06:00 on Day 0.

Exposure Period – any AE that occurs after 06:00 on Day 1 or that is present prior to 06:00 on Day 0 and becomes more severe after 06:00 on Day 0.

All AEs will be listed. Onset times post-product use will be calculated from the last product administered for Arms A to B, and from 06:00 on Day 1 for Arms D and E.

AEs occurring in the exposure period will be summarised by the arm that the subject is randomised to, by severity, and by relationship to the product. The frequency of AEs (i.e., number of AEs, number of subjects experiencing an AE, and percentage of subjects experiencing an AE) will be summarised by the arm the subject is randomised to, and by MedDRA system organ class and preferred term. Summary and frequency AE tables will be presented for all causalities and for those AEs considered to be related to the product (those that have a relationship of possibly related or related). Any severe or serious AEs will be tabulated. If an AE changes severity ratings, it will be included only once under the maximum severity rating in the summaries.

5. Discussion

Most smoking-related diseases are a consequence of continued exposure to tobacco smoke toxicants, but it can take many years for smoking-related diseases to develop in susceptible individuals. Epidemiological substantiation of PRRP efficacy as a less risky alternative to smoking may take decades due to the time lag between exposure and disease outcome. Therefore, studies are required that focus on the potential of PRRPs to deliver, in the shorter term, reduced health effects relative to continued smoking to smokers that ultimately are likely to manifest in a reduction in the incidence of smoking-related disease. The data generated from such studies will provide valuable information to guide public health decision-making in the present day and may help to enable consumers to make a more informed choice on the use of nicotine products available to them.

This report describes the design and statistical analysis approach of a randomised, open-label, parallel group switching study to assess the effects of replacing smoking with a THP for 1 year. The primary objective of the study is to evaluate changes in BoEs and health effect indicators among healthy volunteers using the investigational THP product relative to continuing to smoke over a 1-year period.

The study will investigate three primary endpoints: total NNAL, which has been linked to lung cancer [20,21]; 8-Epi-PGF2α type III, which is an indicator of oxidative stress that can lead to various diseases [17,54–58]; and AIx, which has been used as an indicator of arterial stiffness, a risk factor for cardiovascular disease [17,83–89]. Secondary endpoints have been chosen to provide a broad picture of disease mechanisms that are linked to the primary outcomes and have been shown to be negatively affected by smoking (e.g., inflammation, coagulation, blood pressure and lung function). The study also presents a well-established BoE panel to investigate reductions in exposure to known tobacco smoke toxicants. Current literature suggests that reductions in toxicant exposure, combined with favourable changes in health effect indicators, are likely to lead to health benefits for smokers who completely replace smoking with a PRRP [7]. Ultimately, such changes are likely to indicate a reduction in smoking-related disease risk over time. Given the lack of qualification of these health effect indicators to predict the onset of smoking-related disease, the study will characterise this by measuring them in smokers who quit smoking by conventional recommended methods. Because smoking cessation is a

globally recognised approach to reduce smoking-related disease risk, and the epidemiology of the health consequences of quitting are well documented, these data will contextualise the health effect changes in smokers switching to the investigational product.

Great efforts have been made to provide a framework that will facilitate transparency and critical appraisal of the results. For example, the study has been designed to facilitate comparative assessment, including a cessation group as the gold standard. Critically, product compliance is monitored by using both diaries of self-reported product use and biomarkers of compliance. In addition to this statistical analysis plan, the protocol has been published [12], and we are fully committed to publish the results from the study. Best practices have been followed by providing appropriate sample size calculation to satisfy the main hypothesis and adjusting for multiple timepoint and endpoint assessments.

In conclusion, we present in this manuscript the design and statistical analysis approach of a randomised, open-label, parallel group switching study assessing the effects of replacing smoking with a THP for one year. This study is an essential element to understand the potential health effects as a result of switching from conventional and roll-your-own cigarettes to the investigational THP product glo™ and will be an important addition to the growing evidence evaluating THP products.

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