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Changes in levels of biomarkers of exposure and biological effect in a controlled study of smokers switched from conventional cigarettes to reduced-toxicant-prototype cigarettes

Christopher J. Shepperd [a], Nik Newland [a], Alison Eldridge [a,*], Linsey Haswell [a], Frazer Lowe [a], Ermioni Papadopoulou [a], Oscar Camacho [a], Christopher J. Proctor [a], Don Graff [b], Ingo Meyer [c]

[a] British American Tobacco, Group Research and Development, Regents Park Road, Southampton SO15 8TL, UK
[b] Celerion, 621 Rose Street, Lincoln, NE 68502, USA
[c] MPS Hamburg GmbH, Kieler Strasse 99-105, Hamburg 22769, Germany

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ABSTRACT

Background: Development of cigarettes that reduce exposure to harmful smoke constituents is a suggested tobacco harm reduction strategy, but robust methods for measurement of change are required. We investigated whether changes in biomarkers of exposure (BoE), effective dose (BoED) and biological effect (BoBE) could be detected after switching from conventional cigarettes to a reduced-toxicant-prototype cigarette (RTP).

Methods: Regular smokers of 6–8 mg ISO tar yield cigarettes were recruited in Hamburg, Germany, and supplied with a conventional 7 mg ISO tar yield cigarette for 2 weeks then switched to the same cigarette with a different tipping paper (control) or the RTP for 6 months. Subjects smoked mostly at home and attended five residential clinic visits where urine and blood samples were collected for analysis. Primary endpoints were changes in specific biomarker levels compared with non-smoker background levels. Changes in daily cigarette consumption were also investigated.

Results: BoE levels in controls generally increased over the study period, whereas most BoE and all BoED significantly declined in RTP smokers. Most BoBE data were similar across groups and/or too variable within individuals to detect changes. Increased daily cigarette consumption was affected by supply of free cigarettes, perceived shorter smoking time per cigarette than usual brands, and perceived reduced harm.

Conclusions: Despite increased cigarette consumption, reductions in BoE and BoED were detectable.

1. Background

The health risks associated with cigarette smoking are correlated with duration of smoking and amount of daily consumption, and cessation reduces an individual's relative risks of tobacco related disease (International Agency for Research on Cancer, 2007; Doll et al., 1994). Thus, tobacco-related health risks are assumed to be due to repeated and sustained exposure to a range

Abbreviations: BoE, biomarker of exposure; BoBE, biomarker of biological effect; BoED, biomarker of effective dose; FDA, Food and Drug Administration; IOM, Institute of Medicine; ISO, International Organisation for Standardisation; ISRCTN, International Standard Randomised Controlled Trial Number; MLE, mouth level exposure; MRTP, modified risk tobacco product; PREP, potential reduced exposure prototype; RTP, reduced toxicant prototype; WHO, World Health Organisation; CPD, cigarettes per day; EOS, end of study; MFCV, modified flue-cured Virginia; CA, cellulose acetate filter material; AFR, amine-functionalised resin; HAC, high activity carbon; CU, CORESTA units; LIP, low ignition propensity; mmWG, mm water gauge; NFDPM, nicotine free dry particulate matter ('tar'); NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, N-nitrosonornicotine; NAB, N-nitrosoanabasine; NAT, N-nitrosoanatabine; PAH, polycyclic aromatic hydrocarbons; BLQ, below quantitation limit; BDL, below detection limit; HMPMA, 3-hydroxy-1-methylpropylmercapturic acid; 3-HPMA, 3-hydroxypropylmercapturic acid; MHBMA, monohydroxybutenyl mercapturic acid; CEMA, 2-cyanoethylmercapturic acid; s-ICAM-1, soluble intercellular adhesion molecule; 8-OHdG, 8-hydroxydeoxyguanosine; SOD, superoxide dismutase; GPx, glutathione peroxidase; hsCRP, high sensitivity C-reactive protein; LDL, low density lipoprotein; HDL, high density lipoprotein; MCP-1, monocyte chemotactic protein 1; LTB4, leukotriene B4; oxLDL, oxidised LDL.

* Corresponding author.
E-mail address: alison_eldridge@bat.com (A. Eldridge).

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of smoke toxicants (Stratton et al., 2001). Reduction of the negative health effects of tobacco use is a clear public-health priority and has led to a series of regulatory and educational initiatives to persuade people not to smoke (World Health Organization, 2011). Despite these efforts, smoking rates in adult populations worldwide remain at 15–25%. Although numbers are declining slowly in many countries (World Health Organization, 2011), the World Health Organization (WHO) has forecast that there will be around 1.5 billion tobacco smokers worldwide in 2050 (World Health Organization, 2002). Current scientific study and public-policy debate, therefore, are concerned with whether public-health gains could arise from reducing exposure to toxicants in people who continue to use tobacco through the development of new tobacco and nicotine products.

In the 2001 Institute of Medicine (IoM) report, *Clearing the Smoke: the scientific basis for tobacco harm reduction* (Stratton et al., 2001), the development of potential reduced-exposure products (PREPs) was suggested as a possible way to achieve tobacco harm reduction. PREPs were defined as products that result in substantial reductions in exposure to one or more tobacco toxicants and that can reasonably be expected to reduce the risk of developing one or more specific diseases or adverse health effects as compared with risks conferred by use of traditional tobacco products. The IoM Report describes the types of scientific studies that might be useful for assessing potential risk reduction offered by the PREPs, including clinical studies, but the optimum study designs are still being considered (Hatsukami et al., 2009). Various researchers have since been trying to develop a validated framework for this research (Hatsukami et al., 2006).

The IoM noted the need to have initiatives related to PREPS overseen by regulators. In the USA, the Food and Drug Administration (FDA) was mandated by law to begin regulating tobacco products in 2009. The legislation includes the possibility of classifying new tobacco products as modified-risk tobacco products (MRTPs) with allowable public claims of reduced risk or exposure to toxicants as compared with traditional tobacco products. The FDA has set out draft guidance (Modified Risk Tobacco Products Applications (Draft Guidance for Industry), 2012) on the science needed to assess MRTPs, which is based partly on further findings from the IoM (Committee on Scientific Standards for Studies on Modified Risk Tobacco Products, 2011), but the optimum methods for determining products' potential to reduce tobacco-related harm remain under development.

Tobacco smoke contains a large number of toxicant species. Whether or not conventional cigarettes can be modified sufficiently to be classified as MRTPs remains unclear. Standardised machine tests, followed by various analytical methods, can collect and measure the levels of these constituents in tobacco smoke under laboratory conditions (Rickert et al., 1986). Nevertheless, cigarettes with relatively low ISO machine-measured tar yields are generally not associated with commensurately reduced health risks, partly because of compensatory smoking behaviour (11–13). Hence, beside *in vitro* and possibly *in vivo* data, assessment of MRTPs requires generating clinical data to determine whether machine-measured reductions in toxic emissions translate to reductions in human toxicant uptake and concomitant reduction of health risks in relation to comparator traditional tobacco products.

It is not entirely clear which toxicants are the most relevant to the development of smoking-associated diseases, whether there is a dose–response relationship between these toxicants and disease or how toxicants may interact in various disease pathways (Fowles and Dybing, 2003). Considerable research is being conducted, particularly with computational toxicology, to refine understanding of priority toxicants (Cunningham et al., 2011). Regulators and interested public health authorities are considering whether reductions in levels of specific cigarette smoke toxicants might yield public-health benefits. The WHO's Study Group on Tobacco Product Regulation has recommended the measurement of 18 toxicants in cigarette smoke and suggested that regulators consider setting progressively lower limits for nine of these (Burns et al., 2008). The FDA's Tobacco Products Scientific Advisory Committee has identified 93 harmful and potentially harmful constituents present in tobacco and tobacco smoke (FDA, 2012). The FDA now requires US tobacco product manufacturers to measure and disclose levels in their products of 20 of these 93 constituents, and has noted that it might in the future propose product standards that include limits on smoke toxicants (FDA, 2012).

The metabolic half-lives of many tobacco-smoke toxicants are short and changes in biomarkers of exposure (BoE) therefore, can be assessed in studies of only a few weeks' duration. We previously reported a 6-week clinical study, in which smokers switched after 2 weeks from conventional cigarettes to another conventional cigarette or to a reduced-toxicant-prototype cigarette (RTP) Shepperd et al., 2013a. The RTP had significantly reduced machine-measured yields of specific smoke toxicants. In the 4 weeks after switching, exposure to toxicants assessed by measurement of urinary BoE was also reduced. Generally, reductions in BoE reflected the machine-measured reductions of toxicants in smoke, and for some toxicants machine-measured and BoE levels reached reductions of more than 70% compared with the conventional cigarette. Sensorially, however, the RTPs were generally less acceptable to smokers than were the conventional cigarettes.

Hatsukami et al. (2006) concluded that no existing biomarkers in smokers were predictive of smoking-related disease. In the absence of such biomarkers, these authors concluded that the best way to evaluate PREPs (and presumably MRTPs) would be to assess reductions in exposure, whilst noting the distinctions between exposure reduction, risk reduction and harm reduction. Thus, a logical approach to assess relations between exposure and risk or harm is to measure biological effects. Biomarkers of biological effect (BoBE) indicate the body's response to exposure to toxicants and indicate early sub-clinical changes that might contribute to subsequent development of disease. We hypothesised that if reductions in exposure to toxicants are maintained over the longer term, BoBE levels will also be reduced by a detectable degree. We therefore developed a new RTP that aimed to reduce levels of specific toxicants whilst maintaining tar and nicotine yields and an acceptable sensory performance and performed this 6-month study to investigate whether the new RTP would alter both exposure and response to toxicants through measurement of potential BoE and BoBE. In addition to measurement of these biomarkers, endpoints included cigarette consumption and sensory impressions of the products. Longitudinal studies of tobacco consumption raise methodological and ethical issues, including accuracy of self-reporting consumption, changes in consumption because of study participation and the maintenance of smoking that may lead to an increase in lifetime risk of smoking-related diseases. For example, in our 6-week study some subjects notably increased the number of cigarettes smoked on the last day compared with average daily consumption throughout the rest of the study, which we believe was associated with their knowledge that provision of free study product was coming to an end (Shepperd et al., 2013a). Thus, in this study we obtained ethics committee approval, allowed subjects the opportunity to quit at any time during the study, and provided cessation advice throughout and after the study.

The primary objective of this study was to assess changes in selected BoE and BoBE within participants and within and between smoking groups after a forced switch from a commercial control cigarette to an RTP cigarette of equivalent International Organization for Standardization (ISO) tar yield. Secondary

Table 1
Construction characteristics, smoke yields (ISO and HCI regimes) and % differences in yield for control and reduced-toxicant prototype cigarettes used in this study.

	Control (v1 and v2)	RTP
Target ISO tar yield (mg)	7	7
Tobacco blend composition	US style	MFVC – 50% washed, extracted & enzyme-treated tobacco/15% tobacco substitute sheet/35% other tobaccos
Blend weight (mg)	598	490
Total cigarette length (mm)	83	83
Cigarette circumference (mm)	24.6	21
Tobacco rod length (mm)	56	46
Filter length (mm)/type	27/Mono CA	37/Triple
Mouth end section length (mm)/type	–	7/Plain CA
Mid-section length (mm)/type	–	10/CA + 20 mg AFR
Tobacco-end section length (mm)/type	–	20/50 mg HAC
Cigarette paper	75 CU LIP	50 CU LIP
Ventilation type	On-machine Laser	'split tipping' (see text)
Ventilation level (%)	33	35
Pressure drop (mmWG)	87	113

	Control (v1 and v2)		RTP		% Yield change (RTP vs control)	
	ISO	HCI	ISO	HCI	ISO	HCI
NFDPM (mg/cig)	7.1	26.3	6.4	17.8	−10	−32
Nicotine (mg/cig)	0.58	1.59	0.64	1.48	10	−7
Carbon Monoxide (mg/cig)	7.4	22.7	5.4	15.7	−27	−31
Puffs per cigarette	7.2	8.2	7.6	9.1	–	–
Tobacco-specific nitrosamines						
NNK (ng/cig)	32.6	79.9	BLQ (<12.4)	28.3	>−62	−65
NNN (ng/cig)	69.5	171.4	9.9	25.4	−86	−85
NAT (ng/cig)	47.4	114.5	21.5	55.9	−55	−51
NAB (ng/cig)	8.9	18.2	2.4	6.5	−73	−64
Aromatic amines and misc nitrogenous						
4-Aminobiphenyl (ng/cig)	1.4	3.1	0.7	1.6	−50	−48
3-Aminobiphenyl (ng/cig)	1.8	4.2	1.0	2.2	−44	−48
2-Aminobiphenyl (ng/cig)	8.1	15.5	4.5	8.7	−44	−44
o-Toluidine (ng/cig)	49.5	101.3	3.1	58.8	−33	−42
Acrylonitrile (μg/cig)	5.6	23.3	BDL (<0.28)	4.7	>−95	−80
Carbonyls						
Acrolein (μg/cig)	43.6	137.0	BLQ	61.6	>−94	−55
Crotonaldehyde (μg/cig)	9.5	46.9	0.99	3.9	−90	−92
Hydrocarbons and PAH						
1,3-Butadiene (μg/cig)	33.0	95.3	4.3	52.5	−87	−45
Pyrene (μg/cig)	41.5	88.3	37.2	70.9	−10	−17
Naphthalene (μg/cig)	257.6	1048.5	54.1	142.0	−79	−86
Fluorene (μg/cig)	141.4	269.2	161.5	238.8	14	−11
Phenanthrene (μg/cig)	99.7	182.4	97.3	163.2	−2	−11

Included are the percent differences in yield of all smoke constituents for which we measured a validated BoE.
Key: ISO = International Organisation for Standardization; HCI = Health Canada Intense; MFCV = modified flue-cured Virginia; CA = cellulose acetate filter material; AFR = amine-functionalised resin; HAC = high activity carbon; CU = CORESTA units; LIP = low ignition propensity; mmWG = mm water gauge; NFDPM = nicotine free dry particulate matter ('tar'); NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN = N-nitrosonornicotine; NAB = N-nitrosoanabasine; NAT = N-nitrosoanatabine; PAH = polycyclic aromatic hydrocarbons; BLQ = below quantitation limit; BDL = below detection limit.

objectives were to report descriptively on changes within participants and within and between smoking groups in further BoE, BoBE and biomarkers of effective dose (BoED), and other measures (quality of life, smoking behaviours, physiological measures, mouth-level exposure (MLE) and sensory perception, not all of which are reported here) and to compare with values for ex-smokers and never-smokers.

2. Materials and methods

This study was a 6-month, single-centre, single-blind, controlled, forced-switch clinical trial conducted by Momentum Pharma Services, Hamburg, Germany, between March and December 2012. The study was designed and conducted in accordance with the ethical principles of the Declaration of Helsinki and the International Committee on Harmonisation for Good Clinical Practice. The protocol was approved by the ethics committee of the Artzekammer, Hamburg. All subjects provided written informed consent. The study is registered (ISRCTN81286286) and the protocol has been published (Shepperd et al., 2013b).

2.1. Products

Three types of cigarettes were manufactured specifically for this study (Table 1), all with target ISO machine-measured tar and nicotine yields of 7.0 and 0.7 mg/cig, respectively. Two control cigarettes were based on British American Tobacco king-size products on sale in Germany at the time of the study, with overall length 83 mm (comprising a 56 mm tobacco rod and 27 mm filter), circumference 24.6 mm, American blend tobacco, and a plain cellulose acetate filter with 33% ventilation achieved by on-machine laser perforation. The two versions of the control product were

identical except that one had cork tipping paper and one had white tipping paper to maintain blinding after switching.

Apart from the ISO yields and overall length, the RTP differed notably from the control products (Fig. 1). The format was demi-slim (circumference 21 mm). This reduction in circumference has been shown to contribute to a reduction in both mainstream and sidestream smoke toxicant yield (Dittrich et al., 2014). The tobacco rod was 46 mm long and contained a blend of 50% washed, extracted and enzyme-treated tobacco (Liu et al., 2011), 15% tobacco substitute sheet (McAdam et al., 2011) and 35% other tobaccos. The filter was 37 mm long and comprised three cellulose acetate segments: the front segment, nearest the tobacco rod, contained 50 mg high-activity carbon (Branton et al., 2011a), the middle segment contained 20 mg amine-functionalised resin (Branton et al., 2011b) and the mouth-end segment was plain cellulose acetate. Filter ventilation was 35%, achieved with a combination of on-machine laser perforation and a 10 mm wide zone of high-porosity paper (Dittrich et al., 2014).

2.2. Subjects

We enrolled 143 regular smokers, 61 ex-smokers and 61 never smokers who responded to advertisements in the local press and on the study clinic website. The advertisement did not include any specific characteristics of the study products. Eligible subjects were healthy adults of any ethnic origin who lived in or around Hamburg. Full details of the inclusion and exclusion criteria are detailed elsewhere (Shepperd et al., 2013b).

Universal inclusion criteria were weight of at least 52 kg (men) or 45 kg (women), body-mass index in the normal range, no abnormal clinical findings, willingness to refrain from consuming alcohol and grilled, fried or barbequed food before in-clinic evaluations, not being pregnant or breastfeeding, and using reliable contraception or being postmenopausal.

To be eligible for the study, smokers had to be aged 23–55 years (i.e. minimum legal age for smoking in Germany (18 years) plus 5 years for the youngest smokers). Maximum age was set at 55 years to avoid age-related effects on BoBE. Smokers had to have smoked 10–30 cigarettes with ISO tar yield 6–8 mg and blend type typical of the German market daily for at least 5 years (current brand for >6 months) and to be willing both to switch to a novel product and to smoke only supplied cigarettes during the study. A urinary cotinine level of >100 ng/mL at screening was required. Ex-smokers had to have not smoked for at least 5 years but previously regularly smoked 10–30 cigarettes per day for at least 5 years and thus were aged 28–55 years. Never smokers had to be aged 28–55 years and not to have smoked more than 100 cigarettes during their lifetime with none smoked in the previous 5 years. A urinary cotinine level of <30 ng/mL at screening was required in ex-smokers and never smokers.

Exceptions to the inclusion criteria were permitted at the discretion of the investigators and sponsor, providing risks to participants were not increased and the realisation of the scientific objectives would not be hindered.

Universal exclusion criteria were: a clinically relevant health condition or abnormal findings on physical examination or acute illness requiring treatment within the previous 4 weeks; participation in a clinical trial within the previous 90 days; donation or loss of $\geqslant$400 mL blood in the past 90 days; donation of plasma within the previous 7 days; regular use of nicotine or tobacco products other than filtered cigarettes; any history of drug or alcohol abuse within the previous 2 years; use of bronchodilators within the previous 12 months; use of any medication that interferes with the cyclo-oxygenase pathway or systemic medication (except hormonal contraception or hormone-replacement therapy) within the previous 14 days; use of any drugs or substances (except tobacco) known to be strong inducers or inhibitors of cytochrome P450 enzymes within the previous 28 days (Shepperd et al., 2013); strenuous physical activity exceeding the normal levels within 7 days before screening or in-clinic evaluations; employment in the tobacco, journalism, public relations, market research or advertising industries; and a positive urine pregnancy test, use of unreliable contraceptive methods or lactation in women of childbearing age. Women who became pregnant during the study were withdrawn.

Smokers were excluded if they self-reported or were observed to be non-inhalers or if they were planning to quit in the next 12 months. Non-smokers were excluded if they smoked at any

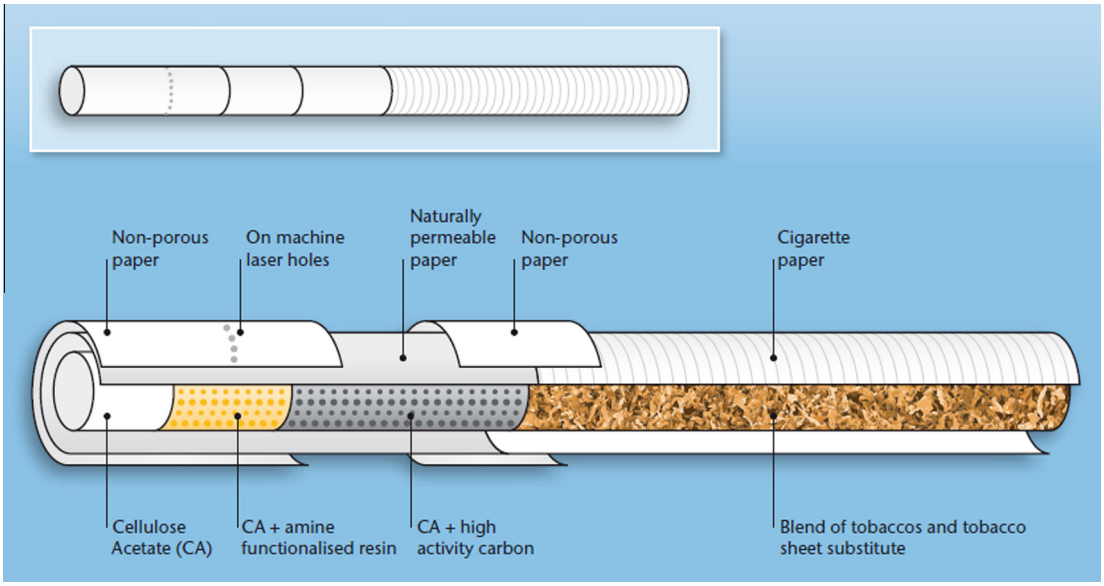

Fig. 1. Schematic of RTP product.

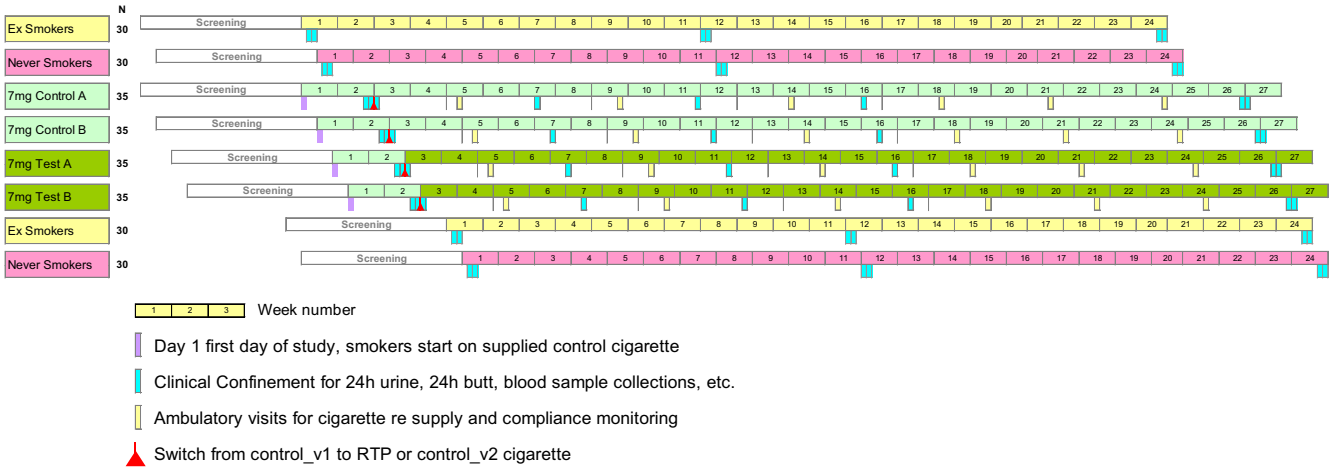

Fig. 2. Study design and scheduled events.

time during the study or if they were regularly exposed to second-hand smoke. Smokers were free to quit smoking at any time, in which case they were withdrawn from the study.

2.3. Study groups and visits

Smokers were assigned to receive the conventional product (control group) or RTP (test group). Ex-smokers and never smokers were enrolled to provide background levels of biomarkers. Subjects were recruited and groups filled according to order of screening, age, gender and availability at the start of the study, and the demographics for all groups were matched as far as possible. To ensure that enough participants were recruited to all groups, subject availability was assured and groups were well matched for age and gender, full randomisation was not possible.

On days 1, 31, 62, 95, 124, 144 and 165 smokers visited the clinic to collect supplies of cigarettes sufficient for the next ambulatory period. To ensure the results and conclusions from this study were realistic, smokers were required to smoke as naturally as possible and therefore were permitted to smoke *ad libitum* throughout the study. Initial numbers of cigarettes supplied were based on self-reported daily consumption at screening or during the previous ambulatory period, plus two packs (Fig. 2); subjects were not told at screening that the supply would be based on these values. Smokers also attended the study clinic for periods of clinical confinement, during which cigarettes were issued singly on request after the filter from the previous cigarette had been returned. This ensured complete collection of filters and accurate data on cigarette consumption. If daily consumption increased during the study, the supply was reviewed with the individual and adjusted accordingly.

On day 1, all smokers received cork-tipped control cigarettes to smoke in their usual manner. On the evening of day 12 smokers entered the clinic for the first period of clinical confinement, when 24 h urine, blood and exhaled breath samples were collected for analysis. Subjects also underwent physical examinations, pulmonary function tests, electrocardiography and urine cotinine testing with NicAlert™ (Palico, Rotkreuz, Switzerland) and completed questionnaires on quality of life (to be reported elsewhere) and smoking sensory experience. On the evening of Day 14 smokers were switched to either the white-tipped control product or the RTP, which they continued to smoke for the remainder of the study. They attended the clinic for further 24–48 h periods of clinical confinement on days 46, 77, 109 and 182.

Ex-smokers and never smokers attended the clinic for three 48 h periods of clinical confinement, starting on days 2, 78 and 163. During these visits, 24 h urine and blood samples were collected and they underwent physical examinations, pulmonary function tests, electrocardiography and urine cotinine testing and completed questionnaires on quality of life, diet and lifestyle.

2.4. Determination of endpoints

2.4.1. Selection of biomarkers

The selection of BoE and BoED was based on confirmed smoke compositional changes in the RTP as compared with the control product and the availability of validated biomarker methodologies. All but one BoE were included in the primary objectives of this study due to a reasonable expectation of change following a switch to the RTP that was based on prior results. Urine mutagenicity was deemed exploratory and, therefore, was included as a secondary endpoint. BoBE selection was based on literature searches for possible associations between biomarkers and biological processes related to exposure to smoke toxicants. Although this was not a cessation study, the switching period of 6 months was based on information from such studies, which indicated that measurable changes in the levels of some BoBE can be seen within this 6 months. Thirty-four BoBE were identified as potentially useful to measure. Some BoBE listed in the protocol (Shepperd et al., 2013) were not included for methodological reasons: all levels of interleukins 6 and 8 and tumour necrosis factor alpha were below the limit of quantification, and baseline levels of matrix metalloproteinases 1 and 9, tissue inhibitor of metalloproteinase 1 and vascular endothelial growth factor were not obtained due to delays in analysis that compromised the stability criteria. The most promising four BoBE (F2-isoprostanes [8-iso-PGF2 type lll and 8-iso-PGF2 Type Vl], white blood cells and s-ICAM-1) were assigned to the primary objective because of their reported association with smoking-related disease and abilities to differentiate between smoking status and demonstrate reversibility upon smoking cessation (Lowe et al., 2009; Lowe et al., 2013). All other BoBE were considered exploratory and were assigned to the secondary objectives.

2.4.2. Bioanalysis

Whilst resident in the clinic, 24 h urine samples were collected from subjects for calculation of daily output of urinary biomarkers (concentrations * total 24 h urine volume). At baseline and the end of the study, two 24 h samples were collected to ensure a complete

sample was available for analysis for each timepoint (Fig. 2). We measured 23 BoE and five BoBE in urine. The methods utilised for measurement of BoE for aromatic amines and polycyclic aromatic hydrocarbons have been reported elsewhere (Riedel et al., 2006; Ramsauer et al., 2011). Methods for BoE for nicotine and metabolites, tobacco-specific nitrosamines, 1,3-butadiene, crotonaldehyde, acrolein and acrylonitrile were adapted from published methods (Roethig et al., 2007; Kavvadias et al., 2009; Urban et al., 2003; Scherer et al., 2007; Ding et al., 2009). References to all urinary BoBE methodologies can be found elsewhere (Haswell et al., 2014). Urine mutagenicity was determined by use of reverse mutation in YG1024, a histidine-requiring strain of *Salmonella typhimurium* (Agurell and Stensman, 1992).

Of note, the nicotine BoE comprised nicotine plus five metabolites, summed to provide 'total nicotine equivalents' (TNeq). Similarly, for naphthalene, two metabolites (1- and 2-hydroxy naphthalene) and for phenanthrene, five metabolites (1-, 2-, 3-, 4- and 9-hydroxy phenanthrene) were determined and summed to provide 'total OH naphthalene' and 'total OH phenanthrene' respectively.

Blood samples were collected in blood-collection tubes for clinical laboratory tests (safety data) (Shepperd et al., 2013b) and to estimate concentrations of 29 additional BoBE (Haswell et al., 2014) and two biomarkers of effective dose (BoED), which were haemoglobin adducts of 2-cyanoethylvaline and 4-aminobiphenyl (van Sittert, 1996; Lewalter and Gries, 2000).

Exhaled carbon monoxide levels were determined with an EC50 Micro III Smokerlyzer monitor (Bedfont Scientific, Maidstone, UK).

2.4.3. Cigarette consumption and mouth-level exposure (MLE)

Self-reported cigarette consumption was recorded at screening. During ambulatory periods subjects recorded consumption as cigarettes per day (CPD) in hand-held electronic diaries on smartphone devices (supplied and administered by CRF Health, Helsinki, Finland). Subjects also answered questions about other products smoked during ambulatory periods and an 'honesty policy' was employed to ensure accurate reporting of non-compliance without fear of exclusion from the study. On specified ambulatory days, subjects collected all butts from cigarettes smoked and returned these along with unsmoked cigarettes which was compared with diary data to further assess compliance. During clinical confinement periods CPD was recorded by clinic staff on the basis of the number of spent filters returned. Collected spent filters were sent to the sponsor for part-filter analysis to assess MLE (Charles et al., 2009). These values were compared with ISO machine-smoking values.

2.4.4. Sensory questionnaire

A previously used questionnaire about sensory experience of cigarette smoking (Shepperd et al., 2013b) was administered with slight modifications. Smokers completed the questionnaire on a tablet device (supplied and administered by CRF Health, Helsinki, Finland) at baseline, immediately after the product switch and during all confinement visits. Magnitude and liking of each parameter were scored for the following sensory features: acceptability, draw effort, amount of smoke, satisfaction, irritation, nicotine impact, taste amount and quality, mouth dryness and aftertaste.

2.4.5. Cigarette consumption questionnaire

Soon after switching, clinic staff reported widespread increases in daily consumption which was reported to the ethics committee and an independent Data Safety Monitoring Board (DSMB). The DSMB recommended that the study continue, consumption monitoring during the study be increased, that subjects be administered a questionnaire to ascertain the reasons for the consumption increases, and that consumption be monitored for 8 weeks following study completion. In addition the amount and intensity of smoking cessation advice was increased. The ethics committee agreed with these measures and all were implemented in the study.

The questionnaire comprised two parts: a free-text response section for all subjects who self-reported any changed consumption, whether that was an increase or a decrease and a section in which each subject was asked to rate the level of applicability on a four-point scale for each of a series of specified factors that possibly led to changed consumption.

2.5. Statistical analysis

Sample size calculations were based on estimates of expected differences and standard deviations for F2-isoprostanes, white blood cells and s-ICAM-1 reported in the literature for BoBE (16 studies) and on previously observed data for BoE (Shepperd et al., 2013a). Calculations were made with MINITAB software (version 15) and based on one-way ANOVA. A sample size of 50 yielded at least a 80% power to identify differences between study groups for 75% of reported estimates. Literature searches for the BoBE in the secondary endpoint were uninformative and, therefore, sample size was assumed to be the same as for the primary objective. To allow for an anticipated attrition rate of 16% among smokers and to make sure at least 50 participants in each smoking group completed the study, we aimed to enrol 70 participants per smoking group. Owing to lower expected attrition in the ex-smokers and never-smoker groups, we aimed to recruit 60 to each.

Statistical analyses and data management were performed by Celerion, Lincoln, NE, USA. The primary endpoint was change in biomarker levels, and secondary endpoints included cigarette consumption, MLE to nicotine and tar, and sensory experience. These were examined by computation of group biomarker levels at baseline, midpoints and at the end of the study. The data were checked to ensure that model assumptions were satisfied. No transformations were required. For primary endpoints ANOVA was used to identify differences both within and between groups and across time points. To account for the possibility of changes in cigarette consumption over time or because of the switch, ANCOVA was also performed, with CPD as a covariate. For the secondary outcomes, exploratory analysis and repeated-measures ANOVA was used to identify differences over time within and between smoker groups and products. The significance level was set at 5%, and Tukey's HSD post hoc test was used to account for multiplicity.

Table 2
Characteristics of participants by study group.

	Control smokers	RTP smokers	Ex-smokers	Never-smokers	Total
Enrolled (*n*)	67 (100%)	76 (100%)	61 (100%)	61 (100%)	265 (100%)
Completed (*n*)	56 (83.6%)	58 (76.3%)	58 (95.1%)	57 (93.4%)	229 (86.4%)
Gender					
Male	28 (50%)	27 (46.6%)	29 (50%)	29 (50.9%)	113 (49.3%)
Female	28 (50%)	31 (53.4%)	29 (50%)	28 (49.1%)	116 (50.7%)
Age					
Mean (SD)	39.9 (8.75)	39.7 (9.31)	42.7 (7.75)	42.4 (7.25)	
Median (min, max)	43.0 (24, 54)	40.0 (23, 55)	44.0 (28, 55)	44.0 (28, 55)	
Race					
Caucasian	56 (100%)	58 (100%)	58 (100%)	55 (96.5%)	
Non-caucasian	0	0	0	2 (3.5%)	

Table 3
In-clinic cigarette consumption and nicotine and NFDPM mouth-level exposure (MLE).

	Control smokers				Test smokers			
	Baseline $n = 56$	Midpoint $n = 55$	EOS $n = 56$	% Change (EOS vs baseline)	Baseline $n = 58$	Midpoint $n = 54$	EOS $n = 58$	% Change (EOS vs baseline)
CPD	18.26	22.50	21.65	18.6	19.79	23.72	22.27	12.5
	$p < 0.001$				$p < 0.001$			
		$p = 0.309$				$p = 0.083$		
		$p = <0.001$				$p = 0.002$		
MLE nicotine (mg/24 h)	23.44	27.10	27.70	18.2	25.63	39.82	39.66	54.7
	$p = 0.009$				$p < 0.001$			
		$p = 0.671$				$p = 0.908$		
		$p = 0.002$				$p < 0.001$		
MLE NFDPM (mg/24 h)	272.44	323.26	306.24	12.4	297.67	370.16	345.69	16.1
	$p < 0.001$				$p < 0.001$			
		$p = 0.240$				$p = 0.090$		
		$p = 0.019$				$p < 0.001$		

Key: CPD = cigarettes per day; MLE = mouth-level exposure; NFDPM = nicotine-free dry particulate matter ('tar'); EOS = End of study (day 183).

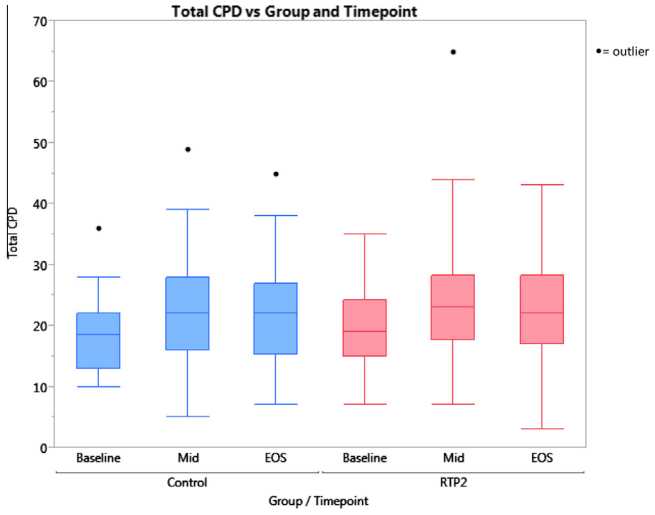

Fig. 3. Cigarette consumption (in-clinic data).

3. Results

3.1. Subjects

Characteristics of subjects are shown in Table 2. A total of 265 subjects entered the study: 143 smokers, 61 ex-smokers and 61 never smokers. Sixty-seven smokers were allocated to the control group and 76 to the RTP group. Overall, 229 completed the study (56 control smokers, 58 RTP smokers, 58 ex-smokers and 57 never smokers). Of the 36 subjects who discontinued early, 10 withdrew because of serious adverse effects resulting in hospitalisation and/or surgery. The medically qualified principal investigator considered that none of these SAEs were related to the study or products. Two additional subjects were withdrawn because of non-compliance, two due to protocol violations, one due to a progressive disease, one because of pregnancy and 20 for other reasons that were not classified as adverse events. Age and gender were well matched across all groups, taking into account the different minimum age for smoker and non-smoker groups (Table 2).

3.2. Cigarette consumption

In clinic CPD increased by three (18.6%) or four (12.5%) cigarettes per day overall in the control and test groups, respectively after the switch (Table 3 and Fig. 3). Subsequently, clinical confinement CPD periods remained consistently in the range of 21–23 cigarettes per day in both groups. The greatest change was seen from baseline to the midpoint of the study after which CPD was stable for the remainder of the study (Fig. 3). This increase in CPD must be borne in mind when considering the biomarker data.

As described above, the increase in cigarette consumption was unexpected and prompted an alert to the ethics committee and set up of a DSMB, both of whom recommended study continuation but an increase in consumption monitoring (including post study) and implementation of a questionnaire to ascertain the reasons for the change. Frequent monitoring using an electronic diary generated multiple time points during ambulatory periods (in addition to the consumption data acquired in the clinic). Data for all time points are tabulated, and presented graphically, in Supplementary data: Appendix 1. However, in keeping with the biomarker data (see below) we focus on the clinical CPD data here (Table 3 and Fig. 3).

Immediately after the study was completed, mean daily consumption returned to pre-study levels, and by around 8 weeks of post-study follow-up had dropped to 17 cigarettes per day in the control group and 16 cigarettes per day in the test group (Supplementary data: Appendix 1).

3.3. Biomarkers

Due to the large amount of data generated in this study, we present only the baseline and end-of-study biomarker values. Full biomarker data are provided as Supplementary data: Appendix 2. Here can be found the biomarker levels at all time points, and the results of the statistical comparisons within and between groups at all time points.

3.3.1. Biomarkers of exposure and effective dose

Levels of BoE varied little throughout the study in ex-smokers and never smokers (Supplementary data: Appendix 2) and therefore only the means are shown here (Table 4). In smokers, levels of the nicotine BoE TNeq were largely as expected from the combination of the increased consumption over the course of the study (Table 4) and the 10% difference in nicotine yield between the control and RTP product (Table 1), with increases of 10.8% in the control group, and 25.5% in the RTP (Test) group by the end of the study.

Significant increases from baseline were seen in the control group for the BoEs NNK, NAB, NAT, 4-aminobiphenyl, 3-aminobiphenyl, 2-aminonapthalene, crotonaldehyde, acrolein, acrylonitrile, fluorene, naphthalene and carbon monoxide (Table 4, Fig. 4), which were largely in keeping with the increase in daily consumption. For one nitrosamine (NNN) and one aromatic amine (*o*-toluidine), and one vapour phase constituent

Table 4
LS Means (95% Confidence Intervals) and EOS vs baseline comparisons (*p*-values) for urinary biomarkers of exposure and effective dose.

Smoke constituent	% Yield change (RTP vs control)	BoE	Control smokers			Test smokers			Ex-smokers	Never-smokers
			Baseline (smoking control v1)	EOS (smoking control v2)	% Change* (EOS vs baseline)	Baseline (smoking control v1)	EOS (smoking RTP)	% Change* (EOS vs baseline)	Mean	Mean
Nicotine	10	TNeq (mg/24 h)	14.8 (13.4–16.3)	16.4 (14.9–17.9)	10.8	14.1 (12.7–15.5)	17.7 (16.2–19.1)	25.5	−0.13	−0.10
			$p = 0.004$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
NNK	>−62	NNAL (ng/24 h)	189.3 (170.7–208.0)	208.2 (189.6–226.8)	10.0	174.4 (155.9–192.8)	105.6 (87.1–124.1)	−39.4	3.0	2.5
			$p = 0.003$ ($n = 56$)			$p < 0.001$ ($n = 57$)				
NNN	−86	NNN (ng/24 h)	10.8 (8.8–12.8)	10.7 (8.7–12.7)	−0.9	7.9 (5.9–9.9)	2.8 (0.8–4.9)	−64.6	0.5	0.6
			$p = 0.956$ ($n = 56$)			$p < 0.001$ ($n = 55$)				
NAB	−73	NAB (ng/24 h)	27.1 (24.0–30.2)	34.0 (30.8–37.1)	25.5	26.2 (23.2–29.3)	14.9 (11.8–18.0)	−43.1	1.6	1.7
			$p = <0.001$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
NAT	−55	NAT (ng/24 h)	167.9 (148.5–187.3)	209.3 (189.9–228.7)	24.7	159.0 (139.9–178.1)	114.6 (95.5–133.6)	−27.9	2.6	3.3
			$p = <0.001$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
4-Amino biphenyl	−50	4-Amino biphenyl (ng/24 h)	17.6 (16.1–19.1)	21.0 (19.5–22.5)	19.3	16.8 (15.3–18.2)	14.0 (12.5–15.5)	−16.7	2.6	2.4
			$p = <0.001$ ($n = 55$)			$p < 0.001$ ($n = 58$)				
3-Amino biphenyl	−44	3-Amino biphenyl (ng/24 h)	7.2 (6.5–8.0)	7.9 (7.2–8.7)	9.7	7.2 (6.5–8.0)	5.0 (4.2–5.7)	−30.6	0.4	0.6
			$p = 0.038$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
o-Toluidine	−33	o-Toluidine (ng/24 h)	162.6 (150.3–174.9)	162.1 (149.8–174.4)	−0.3	153.9 (141.8–166.0)	148.3 (136.2–160.3)	−3.6	71.3	63.3
			$p = 0.938$ ($n = 56$)			$p = 0.377$ ($n = 58$)				
2-Amino Napthalene	−44	2-Aminonapthalene (ng/24 h)	26.6 (24.4–28.9)	29.7 (27.4–31.9)	11.6	25.6 (23.4–27.8)	23.0 (20.8–25.2)	−10.2	1.0	0.4
			$p < 0.001$ ($n = 56$)			$p = 0.002$ ($n = 58$)				
Croton-aldehyde	−90	HMPMA (μg/24 h)	472.3 (425.9–518.8)	538.0 (491.6–584.5)	13.9	530.4 (484.7–576.1)	139.4 (93.8–185.1)	−73.7	99.5	180.8
			$p = 0.008$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
Acrolein	>−94	3-HPMA (μg/24 h)	1355.1 (1215.5–1494.8)	1605.3 (1465.6–1744.9)	18.5	1314.7 (1177.3–1452.1)	869.4 (732.0–1006.8)	−33.9	208.4	281.2
			$p < 0.001$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
1,3-Butadiene	−87	MHBMA (ng/24 h)	4035.2 (3441.4–4628.9)	4191.4 (3597.7–4785.2)	3.9	4115.0 (3530.6–4699.3)	2860.2 (2275.9–3444.5)	−30.5	121.4	144.7
			$p = 0.315$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
Acrylonitrile	>−95	CEMA (ng/24 h)	183.0 (164.5–201.6)	229.6 (211.1–248.2)	25.5	185.0 (166.7–203.2)	78.9 (60.6–97.1)	−57.4	−1.28	−1.25
			$p < 0.001$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
Pyrene	−10	1-OH Pyrene (ng/24 h)	393.2 (347.1–439.4)	296.2 (250.1–342.4)	−24.7	389.9 (344.5–435.3)	274.8 (229.4–320.2)	−29.5	145.0	116.3
			$p < 0.001$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
Fluorene	14	2-OH Fluorene (ng/24 h)	3464.0 (2956.8–3971.2)	4711.5 (4204.3–5218.6)	36.0	2747.5 (2248.6–3246.4)	4977.2 (4478.3–5476.2)	81.1	899.5	874.4
			$p < 0.001$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
Naphthalene	−79	Total OH Naphthalene (ng/24 h)	23.3 (20.1–26.5)	33.3 (30.2–36.5)	42.9	23.2 (20.1–26.3)	35.9 (32.8–39.0)	54.7	3.7	3.5
			$p < 0.001$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
Phenanthrene	−2	Total OH Phenanthrene (ng/24 h)	831.2 (729.5–933.0)	844.3 (742.6–946.0)	1.6	748.4 (648.3–848.5)	892.1 (792.0–992.2)	19.2	462.8	496.1
			$p = 0.787$ ($n = 56$)			$p = 0.003$ ($n = 58$)				

Table 4 (*continued*)

Smoke constituent	% Yield change (RTP vs control)	BoE	Control smokers			Test smokers			Ex-smokers	Never-smokers
			Baseline (smoking control v1)	EOS (smoking control v2)	% Change* (EOS vs baseline)	Baseline (smoking control v1)	EOS (smoking RTP)	% Change* (EOS vs baseline)	Mean	Mean
Carbon Monoxide	−27	Exhaled CO (ppm)	15.6 (14.0–17.3)	21.4 (19.7–23.0)	37.2	21.87 (20.3–23.5)	17.67 (16.1–19.3)	−19.2	0.98	1.16
			$p < 0.001$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
4-Amino biphenyl	−50	4-Aminobiphenyl Hb Adduct (pg/g Hb)	59.5 (53.9–65.2)	75.2 (69.5–80.8)	26.4	64.2 (58.6–69.8)	62.2 (56.7–67.8)	−3.1	9.4	10.5
			$p < 0.001$ ($n = 56$)			$p = 0.343$ ($n = 58$)				
Acrylonitrile	>−95	2-cyanoethylvaline Hb Adduct (pmol/g Hb)	104.2 (92.2–116.3)	162.4 (150.4–174.4)	55.9	121.2 (109.5–133.0)	73.6 (61.9–85.3)	−39.3	−0.7	−1.22
			$p < 0.001$ ($n = 55$)			$p < 0.001$ ($n = 58$)				

Key: LS = least square; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN = N-nitrosonornicotine; NAB = N-nitrosoanabasine; NAT = N-nitrosoanatabine; HMPMA = 3-hydroxy-1-methylpropylmercapturic acid; 3-HPMA = 3-hydroxypropylmercapturic acid; MHBMA = monohydroxybutenyl mercapturic acid; CEMA = 2-cyanoethylmercapturic acid.

* Under ISO machine-smoking conditions.

(1,3-butadiene) no significant changes were seen from baseline to the end of the study and the BoE for one smoke constituent (pyrene) significantly decreased in the control group. The levels of BoEs in non-smokers were in keeping with published data (Shepperd et al., 2013a; Gregg et al., 2013).

In the test group smoke yields for all of these toxicants were reduced compared with the control product, and significant reductions were also seen in the levels of all BoE, except *o*-toluidine, which again showed no significant change. The reductions in BoE were generally less than those in smoke yields because daily consumption increased, but reductions for some BoE were substantial in both the particulate and vapour phase (NNN, 64.6%, crotonaldehyde, 73.7% and acrylonitrile, 57.4%). Of note is the reduction in the level of NNAL, the biomarker for NNK, for the test group. This urinary biomarker has a long half-life (typically 10–18 days (Goniewicz et al., 2009) and so the reduction in level provides some indication of subject compliance during the ambulatory periods, despite the known consumption increase.

Smoke yields of PAHs varied greatly. For instance, reductions were seen for pyrene (−10%) and napthalene (−79%) in RTP smoke compared with control smoke, whereas little change or slight increases were seen in smoke yields for others (phenanthrene −2% and fluorene 14%; Tables 1 and 4). Levels and changes in PAH BoE were also variable. Pyrene BoE decreased significantly in the both smoking groups (Control -24.7%, Test −29.5%) whereas substantial increases were seen in BoE levels for fluorene (Control 36.0% and Test 81.1%) and naphthalene (Control 42.9%, Test 54.7%) (Table 4). The BoE for phenanthrene increased largely in line with CPD and tar MLE results for the test group but did not change for the control group smokers despite the similar CPD increase (Table 3, Table 4).

For the BoED, levels of urinary 4-aminobiphenyl and the haemoglobin adduct increased in controls by 19.3% and 26.5%, respectively, whereas in RTP smokers a 16.7% decrease was seen in urinary 4-aminobiphenyl with no significant change in the haemoglobin adduct (Table 4). Levels of urinary acrylonitrile biomarker and 2-cyanoethlyvaline haemoglobin adduct increased in controls (25.5% and 55.9%, respectively) but decreased in RTP smokers (−57.4% and −39.3%, respectively; Table 4). The haemoglobin adducts have long body residence times (approximately 120 days Scherer et al., 2014) and so this reduction provides some further evidence of compliance for those subjects switched to the RTP.

Fig. 5 shows substantial separation between smokers and never smokers in urine mutagenicity at the end of the study compared with baseline. A significant reduction in urine mutagenicity was seen in the RTP group at the end of the study.

3.3.2. Biomarkers of biological effect

Among the BoBE in the primary objectives, levels of 8-iso-PGF2α type III were not significantly different between baseline and end of study (EOS) in both the control and RTP group, whereas levels of 8-iso-PGF2α type VI showed a significant increase in the control group smokers ($p = 0.003$), but not in the RTP group. Mean absolute levels of both 8-iso-PGF2α types III and VI were higher than those in ex-smokers and never smokers (Table 5, Fig. 6). With respect to white blood cell counts, there was no significant change between baseline and EOS for both the control and RTP group, although higher absolute values were observed in the RTP group at both time points compared to those in the control group smokers (Table 5, Fig. 6). Finally, levels of s-ICAM-1 significantly increased in both the control and RTP groups over the course of the study ($p < 0.001$), with higher absolute levels observed in the RTP group at both baseline and EOS compared to the control group smokers (Table 5, Fig. 6), which suggest a potential product-related effect.

Most BoBE from the secondary objectives yielded little useful data, with many showing no differences between smokers and

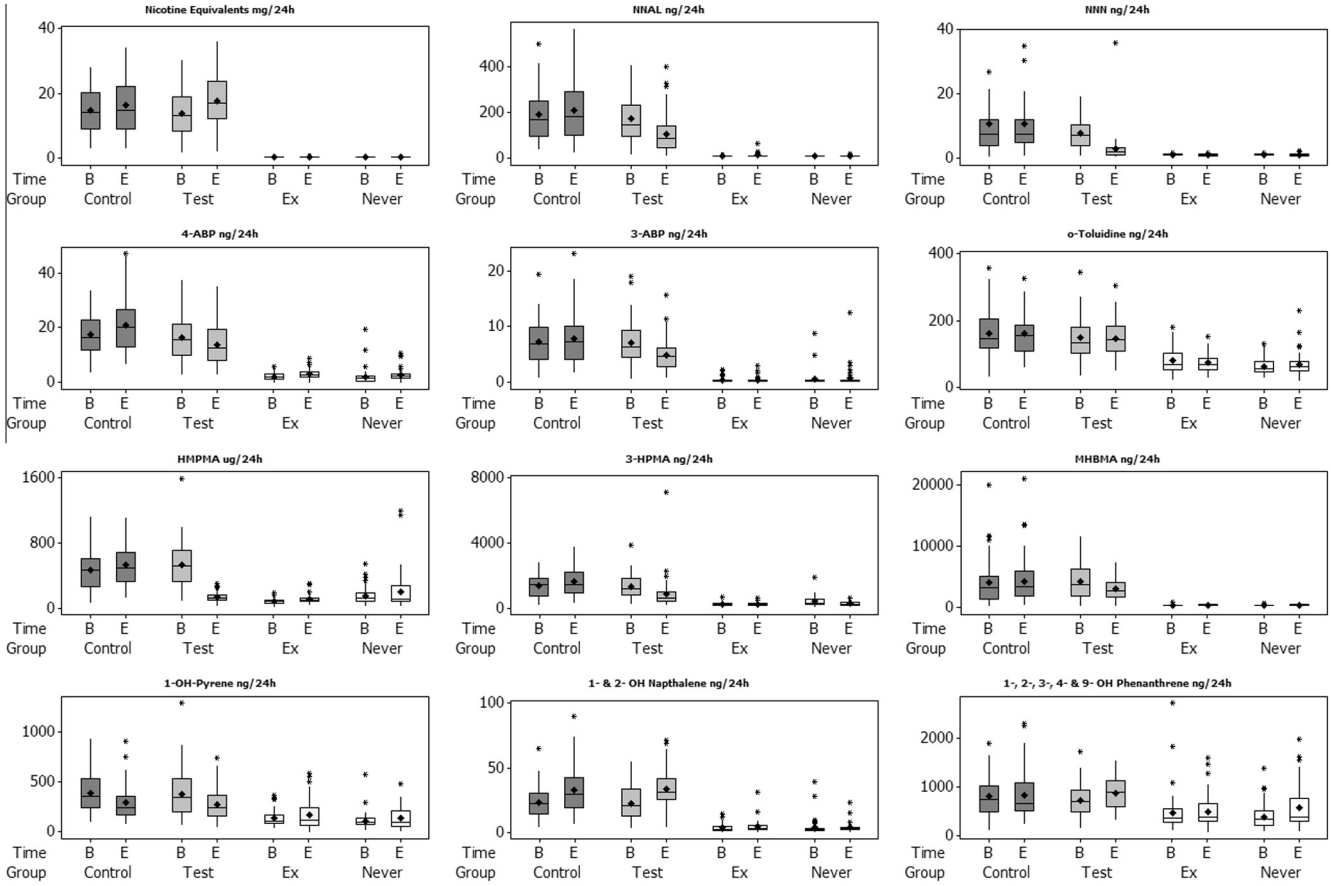

Fig. 4. Boxplots of selected biomarkers of exposure.

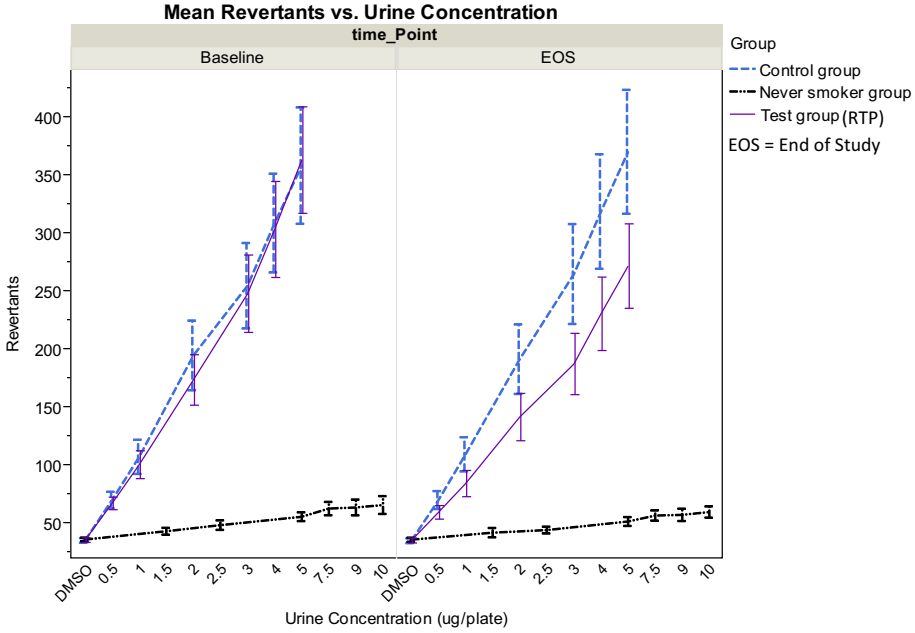

Fig. 5. Urine mutagenicity results.

non-smokers and/or too much variability to draw clear conclusions. To help identify potentially meaningful changes in the secondary BoBE we focused on those that satisfied the following criteria (See Supplementary data: Appendix 2 for cross-sectional comparisons):

- The BoBE showed a significant difference between smokers of the control product and never-smokers between no less than two out of three time points (baseline, mid-point, end of study).

Table 5
LS Means (95% confidence intervals) and EOS vs baseline comparisons (p-values) for biomarkers of biological effect (BoBE).

BoBE	Matrix	Method	Control smokers			Test smokers			Ex-smokers	Never-smokers
			Baseline (smoking control v1)	EOS (smoking control v2)	% Change (EOS vs baseline)	Baseline (smoking control v1)	EOS (smoking RTP)	% Change (EOS vs baseline)	Mean	Mean
F2-isoprostane (8-iso-PGF2 Type III) (ng/24 h)	Urine	LC–MS/MS	244.6 (206.6–282.5)	287.4 (249.5–325.4)	17.5	252.9 (215.5–290.2)	261.0 (223.6–298.2)	3.2	152.0	143.8
			$p = 0.057$ ($n = 56$)			$p = 0.716$ ($n = 58$)				
F2-isoprostane (8-iso-PGF2 Type VI) (ng/24 h)	Urine	LC–MS/MS	1483.0 (1339.6–1626.4)	1672.1 (1528.7–1815.5)	12.8	1551.2 (1410.1–1692.2)	1453.2 (1312.1–1594.3)	−6.3	1396.1	1256.6
			$p = 0.003$ ($n = 56$)			$p = 0.111$ ($n = 58$)				
White blood cells ($\times 10^9$ cells/L)	Blood	Flow cytometry	7.0 (6.6–7.4)	7.3 (6.9–7.6)	4.3	7.7 (7.3–8.1)	7.7 (7.3–8.1)	0.0	5.7	5.5
			$p = 0.090$ ($n = 56$)			$p = 0.946$ ($n = 58$)				
s-ICAM-1 (ug/L)	Plasma	ELISA	303.6 (265.4–341.8)	427.0 (388.9–465.2)	40.6	371.4 (333.9–409.0)	593.8 (556.3–631.3)	59.9	266.5	305.3
			$p < 0.001$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
8-OHdG (ng/24 h)	Urine	LC–MS/MS	4708.8 (4184.7–5232.9)	4905.5 (4381.4–5429.6)	4.2	5117.3 (4601.6–5633.0)	4266.9 (3751.2–4782.7)	−16.6	4882.5	4609.4
			$p = 0.300$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
cis-thymidine glycol (ng/24 h)	Urine	LC–MS/MS	2638.8 (2378.1–2899.5)	2245.5 (1984.8–2506.2)	−14.9	2653.9 (2397.4–2910.4)	2316.1 (2059.6–2572.6)	−12.7	2308.7	2217.8
			$p < 0.001$ ($n = 56$)			$p = 0.001$ ($n = 58$)				
11-dehydroxy thromboxane B2 (ng/24h)	Urine	LC–MS/MS	807.5 (711.2–903.9)	895.7 (798.7–992.6)	10.9	840.4 (748.2–932.6)	679.4 (587.1–771.6)	−19.2	709.3	609.6
			$p = 0.056$ ($n = 51$)			$p < 0.001$ ($n = 57$)				
Neutrophil count (10^9 cells/L)	Blood	Flow cytometry	3.83 (3.54–4.13)	3.92 (3.62–4.21)	2.3	4.33 (4.04–4.62)	4.23 (3.93–4.52)	−2.3	3.02	2.91
			$p = 0.516$ ($n = 56$)			$p = 0.406$ ($n = 58$)				
Monocyte count (10^9 cells/L)	Blood	Flow cytometry	0.56 (0.52–0.60)	0.62 (0.58–0.66)	10.7	0.64 (0.60–0.69)	0.62 (0.58–0.66)	−3.1	0.52	0.47
			$p < 0.001$ ($n = 56$)			$p = 0.185$ ($n = 58$)				
SOD activity to Hb ratio (U/umol)	Erythrocytes	Enzyme Activity	3.60 (3.31–3.89)	3.55 3.25–3.84)	−1.4	4.83 (4.55–5.12)	4.20 (3.92–4.49)	−13.0	3.98	3.70
			$p = 0.791$ ($n = 56$)			$p = 0.002$ ($n = 58$)				
GPx activity to Hb ratio (nm/min/um)	Erythrocytes	Enzyme Activity	326.5 (306.0–347.5)	297.3 (276.8–318.4)	−8.9	327.8 (307.8–348.6)	287.6 (267.5–308.4)	−12.3	352.3	343.1
			$p < 0.001$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
Glutathione reductase activity to Hb ratio (nm/min/um)	Erythrocytes	Enzyme Activity	24.8 (20.9–28.8)	16.2 (12.2–20.1)	−34.7	36.2 (32.3–40.1)	7.3 (3.4–11.2)	−79.8	18.1	21.8
			$p = 0.002$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
Catalase activity to Hb ratio (nm/min/um)	Erythrocytes	ELISA	2263.8 (2108.5–2419.1)	898.4 (743.1–1053.7)	−60.3	1142.6 (989.9–1295.2)	1243.0 (1090.3–1395.7)	8.8	1240.6	1051.1
			$p < 0.001$ ($n = 56$)			$p = 0.337$ ($n = 58$)				
Malondialdehyde to Hb ratio (nmol/mmol)	Erythrocytes	LC–MS/MS	137.5 (104.0–170.9)	216.9 (183.5–250.3)	57.7	102.4 (69.5–135.2)	277.5 (244.6–310.3)	171.0	155.0	161.0
			$p < 0.001$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
Ascorbic acid (umol/L)	Plasma	LC–MS/MS	54.0 (49.5–58.5)	54.0 (49.5–58.5)	0	55.3 (50.9–59.8)	48.6 (44.1–53.0)	−12.1	60.4	60.7
			$p = 0.982$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
Dehydroascorbic acid (umol/L)	Plasma	LC–MS/MS	24.2 (22.7–25.8)	23.0 (21.4–24.5)	−5.0	24.6 (23.1–26.1)	22.5 (21.0–24.0)	−8.5	25.04	26.29
			$p = 0.225$ ($n = 56$)			$p = 0.047$ ($n = 58$)				
Total antioxidant capacity (mmol/L)	Serum	Colorimetric	1.51 (1.49–1.54)	1.59 (1.56–1.62)	5.3	1.54 (1.51–1.56)	1.64 (1.61–1.66)	6.5	1.54	1.57
			$p < 0.001$ ($n = 56$)			$p < 0.001$ ($n = 58$)				

(*continued on next page*)

Table 5 (*continued*)

BoBE	Matrix	Method	Control smokers			Test smokers			Ex-smokers	Never-smokers
			Baseline (smoking control v1)	EOS (smoking control v2)	% Change (EOS vs baseline)	Baseline (smoking control v1)	EOS (smoking RTP)	% Change (EOS vs baseline)	Mean	Mean
hsCRP (males) (mg/L)	Serum	Turbidity	1.07 (0.26–1.87)	1.84 (1.03–2.65)	72.0	1.48 (0.63–2.32)	1.16 (0.32–2.00)	−21.6	0.92	1.59
			$p = 0.168$ ($n = 28$)			$p = 0.587$ ($n = 26$)				
Total cholesterol (mg/dL)	Serum	Enzyme Activity	188.0 (177.5–198.5)	203.8 (193.3–214.3)	8.4	198.1 (187.8–208.5)	203.5 (193.2–213.8)	2.7	198.0	194.7
			$p < 0.001$ ($n = 56$)			$p = 0.170$ ($n = 58$)				
LDL (mg/dL)	Serum	Derived by Friedewald's equation	115.3 (106.4–124.2)	125.2 (116.3–134.1)	8.6	124.8 (116.0–133.6)	127.4 (118.6–136.2)	2.1	119.6	115.2
			$p = 0.002$ ($n = 56$)			$p = 0.393$ ($n = 58$)				
HDL (mg/dL)	Serum	Enzyme Activity	48.3 (44.7–51.9)	51.3 (47.7–54.8)	6.2	48.7 (45.2–52.2)	52.6 (49.1–56.1)	8.0	56.3	57.9
			$p = 0.017$ ($n = 55$)			$p = 0.002$ ($n = 58$)				
Triglycerides (mg/dL)	Serum	Enzyme Activity	117.1 (101.6–132.7)	124.6 (109.0–140.1)	6.4	122.6 (107.2–137.9)	117.4 (102.1–132.7)	−4.2	114.4	107.5
			$p = 0.151$ ($n = 56$)			$p = 0.309$ ($n = 58$)				
Fibrinogen (mg/dL)	Plasma	Coagulation	289.4 (277.0–301.8)	300.0 (287.6–312.3)	3.7	295.6 (283.5–307.8)	291.7 (279.5–303.8)	−1.3	273.1	288.1
			$p = 0.090$ ($n = 56$)			$p = 0.516$ ($n = 58$)				
MCP-1 (ng/L)	Plasma	Multiplexed ELISA	120.5 (111.9–129.2)	105.6 (97.0–114.3)	−12.4	142.3 (133.7–150.8)	149.2 (140.7–157.7)	4.8	106.7	103.3
			$p < 0.001$ ($n = 56$)			$p = 0.106$ ($n = 58$)				
Neutrophil elastase (ng/mL)	Plasma	ELISA	32.2 (29.9–34.5)	11.8 (9.5–14.2)	−63.4	25.4 (23.1–27.7)	10.9 (8.6–13.2)	−57.1	13.0	12.4
			$p < 0.001$ ($n = 56$)			$p < 0.001$ ($n = 58$)				
LTB4 (ng/L)	Plasma	ELISA	14.2 (7.4–21.1)	10.0 (3.2–16.9)	−29.6	14.0 (7.3–20.7)	8.7 (2.0–15.4)	−37.9	23.6	16.9
			$p = 0.346$ ($n = 56$)			$p = 0.223$ ($n = 58$)				
oxLDL (U/L)	Plasma	ELISA	76.8 (71.4–82.1)	77.1 (71.8–82.5)	0.4	72.1 (66.8–77.4)	69.4 (64.1–74.6)	−3.7	63.2	59.5
			$p = 0.885$ ($n = 56$)			$p = 0.284$ ($n = 58$)				

Key: LS = least square; s-ICAM-1 = soluble intercellular adhesion molecule; 8-OHdG = 8-hydroxydeoxyguanosine; SOD = superoxide dismutase; GPx = glutathione peroxidase; hsCRP = high sensitivity C-reactive protein; LDL = low density lipoprotein; HDL = high density lipoprotein; MCP-1 = monocyte chemotactic protein 1; LTB4 = leukotriene B4; oxLDL = oxidised LDL.

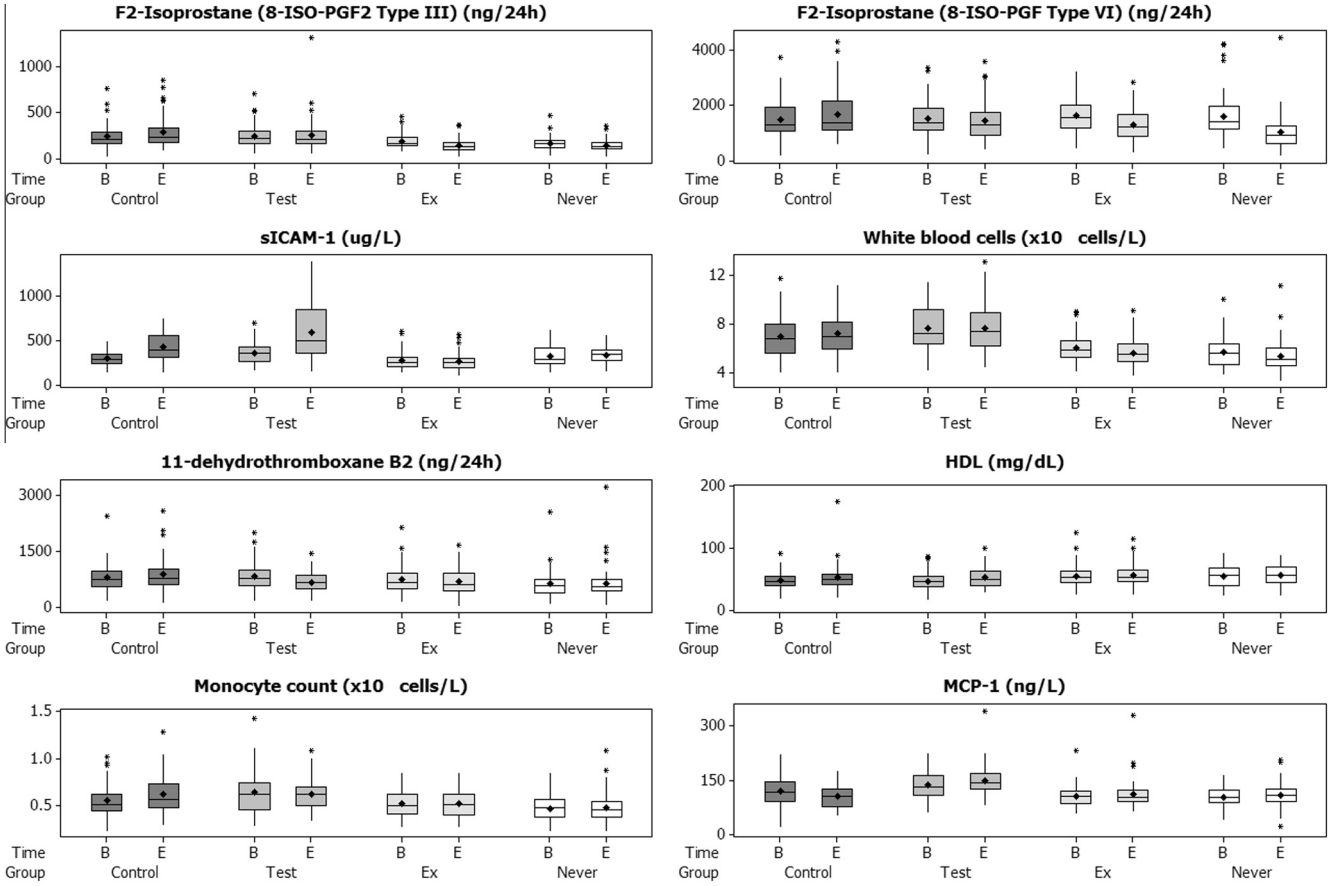

Fig. 6. Boxplots of selected biomarkers of biological effect.

- The BoBE showed no significant difference over time in never-smokers between no less than two out of three time points (baseline, mid-point, end of study).

Thus, these biomarkers are differentially useful or show longitudinal stability. The biomarkers that satisfied these criteria were 11-dehydrothromboxane B2, monocyte count, monocyte chemotactic protein-1 (MCP-1) and HDL cholesterol (Table 5).

Levels of urinary 11-dehydrothromboxane B2 were significantly lower in the RTP group at the EOS compared to baseline ($p < 0.001$), whereas the smoking control group showed no significant difference between baseline and EOS, which suggest a product-related effect following the switch to the RTP. Mean absolute levels of 11-dehydrothromboxane B2 in the RTP group at EOS were comparable to the pooled levels in the ex-smoker group, but were higher than those in the never smoker group. Monocyte counts increased significantly between baseline and EOS in the control group ($p < 0.001$), but not in the RTP group. Mean absolute monocyte counts were higher in both smoking groups compared to former and never-smokers. Plasma MCP-1 levels were significantly lower in the smoking control group ($p < 0.001$) and showed no significant difference in the RTP group, between baseline and EOS. Mean absolute levels of MCP-1 were higher in both smoking groups compared with those in non-smokers. Levels of HDL cholesterol increased significantly in both the control group ($p = 0.017$) and the RTP group ($p = 0.002$) from baseline to EOS, and approached the mean absolute levels observed in non-smokers by EOS.

Unlike BoE, the levels of some BoBE in non-smokers did change over time and, therefore, pooled mean values are presented for reference (Table 5).

3.4. Mouth level exposure (MLE)

MLE changed in the control group only in line with the increase in CPD. Under ISO machine smoking conditions the RTP delivered 10% more nicotine and 10% less tar than the control product (Table 1). In the study, however, an increase of 55% was seen for MLE to nicotine in the RTP group (Table 3), obviously due to increased intensity of smoking as well as increases in CPD. However, this increase was not fully reflected in the biomarker data.

Biomarker and MLE endpoints were also assessed using ANCOVA. CPD was consistently found to be a significant covariate in the statistical models evaluating the BoE and MLE endpoints, but generally was not a significant covariate in the statistical models evaluating the BoBE. In most cases, similar high-level interpretations (group * period interaction, group and period effects) were found when CPD was included in or excluded from the statistical model.

3.5. Sensory questionnaire

The sensory questionnaire data are summarised in Fig. 7. For the control group, most scores did not change throughout the study period except for overall acceptability, appearance and rod length acceptability which were significantly lower than at baseline, and irritation which was higher immediately after the switch. Post-switch control group scores did not change for the remainder of the study. In the RTP group, scores for all attributes (except for nicotine impact ('kick') sensation, mouth/throat irritation, mouth

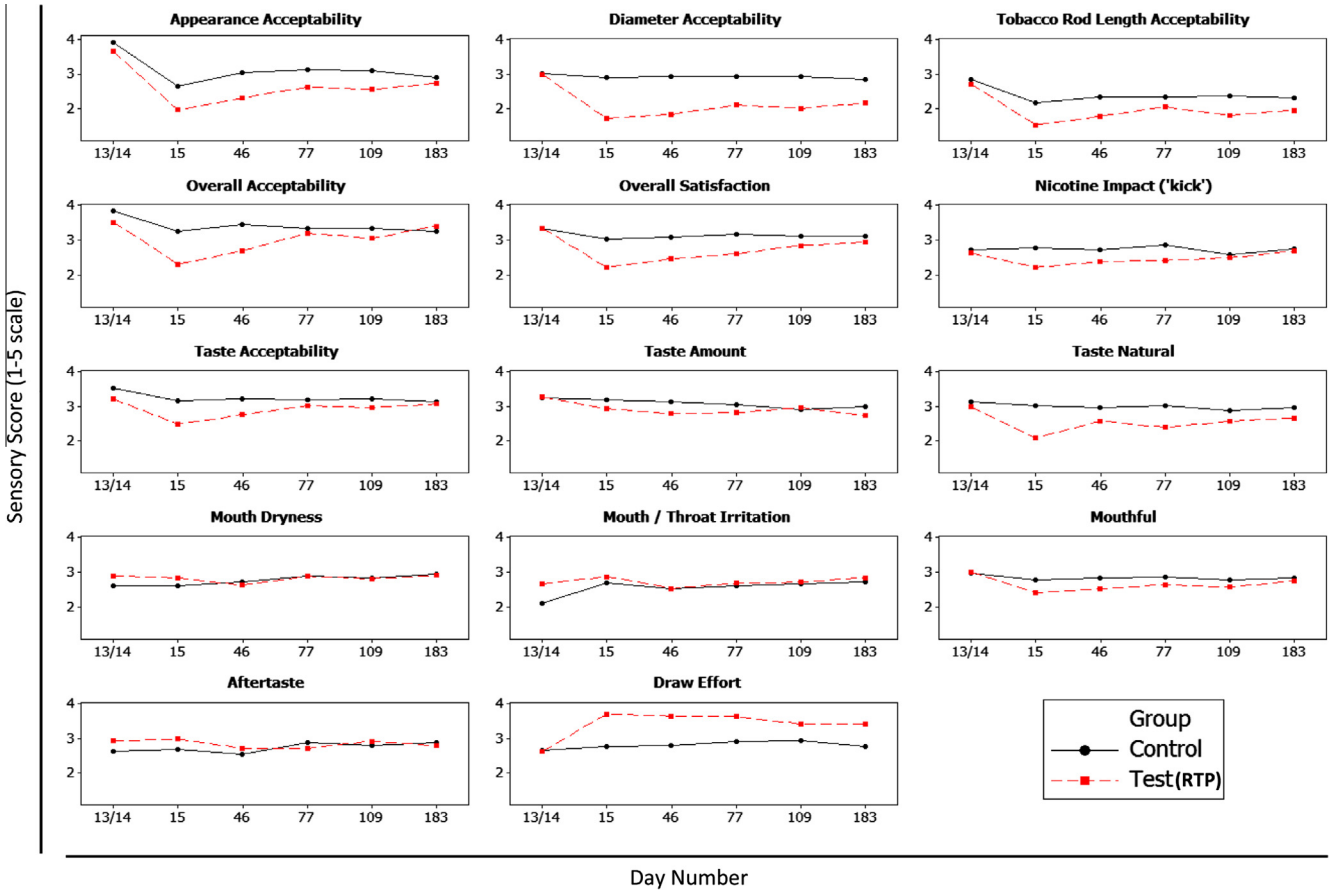

Fig. 7. Mean sensory questionnaire scores for both products by study group.

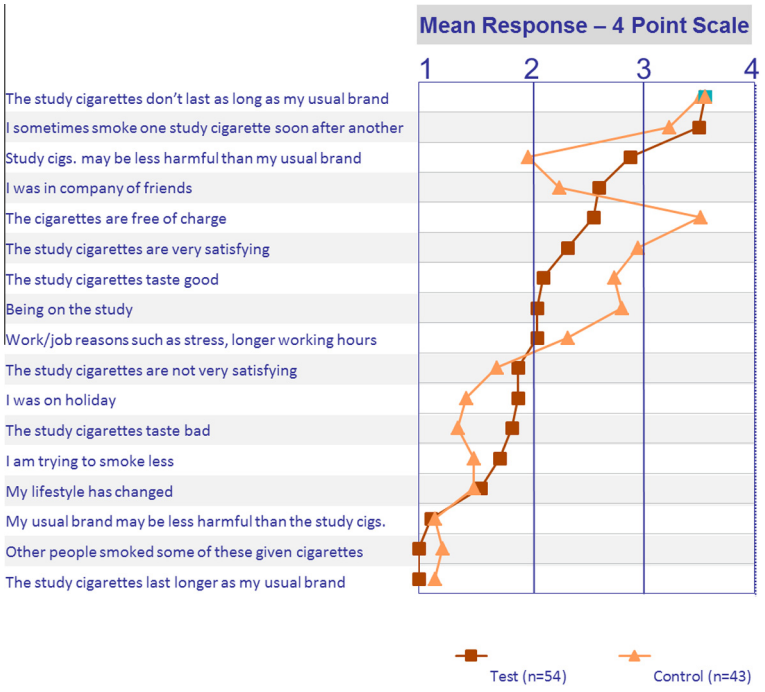

Fig. 8. Scores for pre-specified possible reasons for increased daily cigarette consumption. Questions are translated from the original German.

dryness, aftertaste and amount of taste) changed significantly in a negative direction immediately after the switch. However, sensory perception of the RTP product improved during the study in the direction of baseline.

3.6. Cigarette consumption questionnaire

Changes in mean CPD were reported by 54 subjects in the control group and 43 in the test group. In the free-text part of the questionnaire, 26 (49%) subjects in the control group cited the availability of free cigarettes as being an important factor in the increase. Twenty-two (40%) described the post-switch control cigarettes as smoking faster, seeming "lighter", shorter or containing less tobacco. In the test group, 33 (76%) subjects reported that an important factor for increased consumption was the shorter and slimmer format of the product, and 17 (39%) said that reduced sensory experience was a factor.

Among the scored statements, response to the statement "The study cigarettes don't last as long as my usual brand" received the highest score in both groups (Fig. 8). In the control group, the other highest-scoring reasons for smoking more cigarettes were "I sometimes smoke one study cigarette soon after another", "The cigarettes are free of charge", "The study cigarettes are very satisfying", "The study cigarettes taste good", and "Being on the study". In the test group the next highest-scoring reasons were "I sometimes smoke one study cigarette soon after another", "Study cigarettes may be less harmful than my usual brand", "I was in company of friends" and "The cigarettes were free of charge" (Fig. 8).

4. Discussion

The primary objective of this study was to estimate changes in levels of BoE and BoBE in smokers switched from a conventional cigarette to an RTP. Previous work demonstrated that the switching of smokers from a control to an RTP cigarette for 4 weeks resulted in significant reductions in exposure to toxicants (as determined by BoE levels) that were largely in line with changes in smoke composition (Shepperd et al., 2013a). Increased CPD meant that we anticipated that reductions in BoE might not be as great as seen in the short-term study (Shepperd et al., 2013a) and, therefore, that potential changes in BoBE might be partially negated. The switch to the RTP nonetheless resulted in significant reductions in many BoE levels. The end-of-study levels for the four tobacco-specific nitrosamines, three of the four aromatic amines and all the vapour-phase toxicants (crotonaldehyde, acrolein 1,3-butadiene and acrylonitrile) were significantly reduced in the RTP group compared with levels at baseline. Reductions in these BoE were on average around 70% of those in the 6-week study, but were sustained throughout the 6-month study period. In the control group BoE levels for these toxicants unexpectedly increased by up to 25.5% over the duration of the study, probably because of increased CPD. These increases, however, allowed us to investigate whether decreases and increases in certain BoE are associated with any changes in BoBE.

With the exception of 1-OH pyrene, the BoE for the PAHs generally increased in the control and RTP groups. The RTP machine-smoking ISO naphthalene yields were 79% lower than those for the control product. However, after smokers switched to the RTP, levels of the naphthalene BoE increased by approximately 54.7%. A somewhat similar effect was also seen in the 6-week study, where large reductions in naphthalene smoke yield in the RTP resulted in no significant change to the related BoE (Shepperd et al., 2013a). The discrepancy between the large reduction in naphthalene smoke yield but no change or increase of its BoE in both studies cannot be explained at present, although the influence of dietary sources of PAH's cannot be ruled out. Similarly, the RTP fluorene smoke yields showed a modest increase compared with control (14%), whereas the BoE level increased substantially (81%). In this case, no similar effect was seen previously and, apart from dietary influences, we have no explanation for this anomaly (Shepperd et al., 2013a). It could be possible to extend the MLE method to include PAHs and this additional evidence may provide some explanation for these findings.

Urine mutagenicity in smokers has previously been shown to differ significantly from that in non-smokers (Kado et al., 1983), and our results confirm this. Urine mutagenicity in the RTP group was significantly reduced compared with that in the control group and is likely to be in keeping with the reduction in exposure to mutagenic species in smoke. Indeed, the bacterial mutagenicity of particulate matter from the RTP cigarettes have been shown to be significantly lower than the control cigarette (Crooks et al., 2015).

For the BoED, moderate correlations were found between the haemoglobin adducts and their parent compound in the smoke. In 2008 Gyorffy and colleagues (Gyorffy et al., 2008) proposed a model to describe the biomarker continuum for genotoxicants that included biomarkers of internal dose (typically urinary biomarkers), effective dose (adducts with macromolecules), and early biological effect. In this study, we found a significant reduction in levels of 2-cyanoethylvaline haemoglobin adducts between baseline and EOS in the test group which corresponds to the reduction in the level of the urinary biomarker for acrylonitrile, CEMA. However, no significant change in 4-ABP haemoglobin adducts was seen in the test group at the end of study. This may have been because the toxicant reductions in the smoke were relatively modest, and were likely off-set by the increase in consumption. A comprehensive analysis of the correlation between haemoglobin adducts for acrylonitrile and 4-aminobiphenyl and their corresponding urinary metabolite has been reported elsewhere (Scherer et al., 2014).

The BoBE measured are implicated in key processes that contribute to the development and progression of smoking-related diseases, including oxidative stress, inflammation and DNA damage. For instance, elevated levels of F2-isoprostanes have been associated with smoking-related diseases via oxidative-stress-mediated mechanisms (Morrow, 2005; Rahman, 2005). Levels of urinary and plasma isoprostane are higher in smokers than in non-smokers (Lowe et al., 2013), but decrease significantly after smoking cessation (Flores et al., 2004; Morrow et al., 1995; Chehne et al., 2002; Oguogho et al., 2000). Despite the lack of change in the levels of 8-iso-PGF2α type III following a switch to the RTP, levels of this BoBE between smokers and non-smokers continued to be significant throughout the study ($p < 0.001$, See Supplementary data: Appendix 2). With respect to 8-iso-PGF2α type VI, the data show elevated levels in the control smoking group and no change in the RTP group at the end of study. However, there is significant heterogeneity in the data to the point where 8-iso-PGF2α type VI was unable to discriminate between current and never-smokers at baseline (Supplementary data: Appendix 2). Hence, data for 8-iso-PGF2α type VI are not robust enough to draw any clear conclusions.

Elevations in white blood cell counts have been associated with smoking and smoking-related disease (Frohlich et al., 2003; Lao et al., 2009), and decreases have been reported following smoking cessation (Frohlich et al., 2003; Blann et al., 1997; Hammet et al., 2007). In this study, there was no significant difference over time in either the smoking control or RTP group. Given that a significant difference between smokers and non-smokers' WBC count was maintained for each time point over the course of the study (see Supplementary data: Appendix 2), this gives a degree of confidence

in the stability of the BoBE over time, and suggests that there was no product-related effect. The monocyte counts followed a very similar pattern to WBC, which indicates that the test product had no specific effects on this BoBE.

11-dehydrothromboxane B2 is a urinary metabolite of the potent platelet agonist and vasoconstrictor, thromboxane A2. Raised concentrations of the urinary metabolite are associated with platelet activation and smoking status (Wennmalm et al., 1991; Lowe et al., 2009; Nowark et al., 1987; Frost-Pineda et al., 2011) and decrease significantly following smoking cessation (Rangemark et al., 1993; Saareks et al., 2001) or a switch to an electrically heated tobacco product (Roethig et al., 2008). Throughout the study period, levels of 11-dehydrothromboxane B2 in the control group remained significantly higher than in the never-smoker group and became a significantly different from values in the ex-smoker group at the study midpoint and EOS time points (see Supplementary data: Appendix 2). This finding could potentially be due to the increase in cigarette consumption during the study in the smoking control group. Interestingly, despite the increase in cigarette consumption also reported in the RTP group, levels of 11-dehydrothromboxane B2 significantly decreased over the course of the study. These data suggest that the chemical yield reductions in the RTP product may have been partly responsible for the reductions in 11-dehydrothromboxane B2 levels, and could contribute to elevations in 11-dehydrothromboxane B2 generally observed in smokers of conventional cigarettes. Levels of the tobacco-specific nitrosamine NNK were shown to be significantly reduced in the RTP from machine-smoked chemistry data (Table 1), and data from this study showed significantly lower levels of urinary NNAL (a urinary metabolite of NNK) in the RTP group than in the smoking control group. Huang et al. (2011) recently reported that NNK increased the synthesis of thromboxane A2 in human lung cancer cells, which was associated with prolonged cellular survival. Hence, the relative reductions in the RTP NNK smoke yields and corresponding reductions in urinary NNAL could explain why levels of 11-dehydrothromboxane B2 were lower in the Test group than the control group at the end of the study. If this association were proven, then thromboxane A2-mediated cell survival in lung cells could be a possible mechanism by which NNK induces its reported carcinogenic effects. Although this is an interesting hypothesis, caution should be exercised when relating cause to effect by association alone, and further studies are needed to fully explore this hypothesis to understand any potential causal relationship and its implications for respiratory disease.

Recruitment of inflammatory cells from the vascular system is an essential part of the damage-repair process in endothelial dysfunction and tissue injury, and elevations in levels of s-ICAM-1 and MCP-1 are implicated in the development of atherosclerosis (Piemonti et al., 2009; Deo et al., 2004; Gross et al., 2012; Kusano et al., 2004). With respect to plasma MCP-1 levels, a significant reduction was observed in the control group, whereas in the RTP group values did not differ significantly between baseline and EOS. Clear conclusions are difficult to draw from these data due to the apparent variability in the smoking groups. A significant difference between smokers and non-smokers was maintained until the mid-point of the study, however at the EOS time-point, MCP-1 levels sharply increased in the RTP group, whereas levels sharply decreased to that of non-smoker levels in the control group (see Supplementary data: Appendix 2). This finding suggests the switch to the RTP had no clear favourable effects in terms of MCP-1 levels. With respect to plasma s-ICAM-1, initial baseline comparisons of smokers and non-smokers showed no significant difference in levels of s-ICAM-1 (Haswell et al., 2014). However, following the product switch, levels of s-ICAM-1 in the control and RTP groups were consistently higher than non-smokers ($p < 0.001$, see Supplementary data; Appendix 2). Absolute levels of s-ICAM-1 in the RTP group at EOS were substantially higher than those in the control group. These data suggest that the RTP had a potentially adverse effect on the test population. The biological rationale for such an increase is not clear. Given the lack of discrimination between smokers and non-smokers for s-ICAM-1 at baseline (in contrast to published literature) and the apparently higher s-ICAM-1 levels in ex-smokers than in never smokers also at the end of the study (see Supplementary data: Appendix 2), it is possible that methodological variance might be responsible for the differences, however this hypothesis is difficult to confirm. Assuming that the method is sound, it is clear that the RTP had no beneficial effects in terms of s-ICAM-1 levels.

Finally, modulation of the serum lipid profile in smokers is associated with development of cardiovascular disease. HDL cholesterol levels are significantly lower in smokers compared with non-smokers (Chelland Campbell et al., 2008) and increase in people who have quit smoking (Gross et al., 2012; Ohsawa et al., 2005; Hata and Nakajima, 2000) or switched to an electronically heated tobacco product (Roethig et al., 2008). Accordingly, we found consistently lower concentrations of HDL cholesterol in smokers than in non-smokers. The significant increase in HDL levels seen in both groups of smokers during the study may have been due to reasons other than smoking, such as dietary influences.

For BoBE, switching to the RTP only demonstrated one potentially beneficial effect with decreased levels of 11-dehydrothromboxane B2, and one potentially adverse effect with increased s-ICAM-1 levels This general lack of response in BoBE levels to a reduction in toxicant exposure was highlighted in a similar study by Sarkar et al. who reported no significant changes in selected cardiovascular disease-related BoBE following a forced switch from a conventional cigarette to a cigarette containing activated carbon in the filter (Sarkar et al., 2008). There are various possible explanations for the BoBE findings. The study period of 6 months might not have been long enough to detect any meaningful changes, despite literature reports indicating changes in BoBE levels following smoking cessation occurred within the time-frame of this study. Additionally, few or none of the toxicants reduced in the RTP might affect the mechanistic pathways linked to the measured BoBE, and reductions in other toxicants could prove to be of greater importance for these specific end-points. A further possibility is that samples for measurement of BoBE were taken during clinical confinement. Despite increased CPD during these periods, subjects reported even greater daily cigarette consumption during ambulatory periods (Supplementary data: Appendix 1) and, therefore, the potential reductions in exposure in the RTP group might be overestimated and increases in exposure in the control group underestimated. Some important drivers of disease are respiratory irritants and greater reductions in these toxicants than achieved might be needed to affect BoBE levels. Perhaps of greater concern is that the RTP group might have experienced an adverse effect related to increased s-ICAM-1 levels, despite experiencing reductions in various BoE, including several currently under consideration for regulatory monitoring and possibly mandated lowering. Finally, although the study was sufficiently powered to detect changes in the primary objective BoBE over time, based upon smoking cessation data, it may not have been sufficiently powered to detect changes in subjects who continue to smoke, albeit switching to a product with reduced machine smoked toxicant yields. Indeed, the inclusion of a cessation arm in this study would have clarified the true utility of the BoBE included. If no changes in BoBE level had been seen following complete cessation, then none would be seen following the switch to the RTP. This limitation should be addressed in future work.

Our data do not challenge the basic premise that smoking-related diseases are caused by sustained repeated

exposure to toxicants or that risks are dose related and reduce on cessation of smoking. They do however, suggest that selective toxicant lowering/removal from combustible tobacco products may have relatively little effect on health risks, or may have small positive or negative effects. Our data suggest that more research is needed on the potential for modifying health risks through changes in toxicant levels in cigarette smoke.

In the previous 6-week study, apart from a slight uplift in CPD in some smoking groups on the last day of that study, consumption changed little. In this 6-month study significant increases in cigarette consumption were seen in both smoker groups. The greatest increases were seen during the ambulatory periods of the study, whereas during days of clinical confinement when clinic staff allocated cigarettes and collected butts to record consumption, increases were modest (never more than on average three or four more cigarettes than at screening). We cannot be absolutely certain how much consumption increased overall owing to self-reporting and the possibility of stockpiling or sharing cigarettes with others during ambulatory periods, but the numbers of cigarette butts collected at these times generally corroborated consumption levels recorded in the diaries. No smoker increased beyond a limit of 60 cigarettes per day and the study continued despite the observed increase in CPD.

In the test group, the possibility that the RTPs were less harmful than conventional cigarettes was given as a key reason given for smoking more, but this reason did not score highly in the control group. The subjects were informed of the purpose of the study before volunteering to participate, received information about toxicants in smoke and learned that the research was being conducted to study the potential health effects of reducing toxicants in smoke. Subjects in the test group might have connected the unusually long filter with potential risk reduction, but masking of this fundamental change in the product was not possible. Further study is required of how such product risk information is best communicated to potential consumers and of how product appearance influences consumption patterns. Such work would support the FDA requirement that information on the levels of harmful and potentially harmful constituents in tobacco smoke for cigarette brands sold in the USA be clearly communicated to the public. Research has shown, however, that smokers have little understanding of such toxicants despite knowing that smoking is extremely harmful (Hammond et al., 2006).

The increased CPD was similar in the control and RTP groups. Subjects in both groups scored the reason "The cigarettes are free of charge" highly in the questionnaire. We believed when we designed the study that we would need to provide all the cigarettes at no cost and to supply, within reason, as many products as the subjects required to ensure that the ambulatory aspect of the study would be as naturalistic as possible as well as to optimise compliance with smoking only study cigarettes. Restriction of study cigarettes to the amount typically smoked at screening might have lessened the risk of increased consumption and should be explored in future studies. Alternatively, we could increase the stipend for volunteering but require that some of money be used to purchase study cigarettes. As the RTPs were sensorially different from the smokers' regular brands, however, use of the money to supplement with and/or return to conventional cigarettes would be difficult to control.

5. Conclusions

Our aim was to test the hypothesis that the reduced exposure to cigarette smoke toxicants obtained through switching to an RTP from a conventional cigarette would reduce levels of potential biomarkers of smoking-related disease, as assessed through measurement of BoBE. An unexpected increase in daily cigarette consumption, apparently driven substantially by the provision of free cigarettes, made assessment difficult because the reductions in exposure from the RTP were generally less than in our previous study (Shepperd et al., 2013b), although they were still quite large for some toxicants. Additionally, the increased consumption led to the control group having greater exposure to toxicants during the study than normal. Nevertheless, these unexpected exposure patterns had no major effect on the BoBEs that distinguish smokers from ex-smokers and never smokers. Greater reductions in RTP toxicant yields and/or a longer study period might show more substantial effects, but more research would be needed to demonstrate this. Additionally, we believe our findings provide useful insights into study design for potential MRTPs and indicate areas of research to investigate effective modes of communication to the public of information regarding tobacco smoke toxicants and MRTPs.

Competing interests

CJS, NN, AE, LH, FL, EP, OC and CJP are current employees of British American Tobacco and the work was funded by British American Tobacco (Investments) Ltd, United Kingdom. DG is currently employed by Celerion, United States of America, who were involved in project and data management plus statistical analysis and reporting of the study. IM was the Principle Investigator for the study and at the time the study was employed by Momentum Pharma Services in Hamburg, Germany.

Authors' contributions

All authors contributed to the development of the study protocol, aspects of study conduct and the preparation of the manuscript. All authors state that the work was funded by British American Tobacco. All authors read and approved the final version of the manuscript.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.yrtph.2015.04.016.

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