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Reference change values in concentrations of urinary and salivary biomarkers of exposure and mouth level exposure in individuals participating in an ambulatory smoking study

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ABSTRACT

Background: Modified-risk tobacco products (MRTPs) are being developed that may contribute to tobacco harm reduction. To support reduced exposure or risk claims, a scientific framework needs to be developed to assess the validity of claims and monitor consumers after product launch. We calculated reference change values (RCVs) for biomarker of exposure (BoE): salivary cotinine and hydroxycotinine; and urinary total nicotine equivalents, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and creatinine. Mouth-level exposure (MLE) to nicotine and tar were also recorded in an ambulatory setting to characterise variation among smokers in their everyday environment.

Methods: This non-residential, observational study was conducted over 3.5 years across 10 sites in Germany. Smokers of the same commercial 10 mg ISO tar product were included in the study (N = 1011). Urine samples, questionnaires and cigarette filters were collected every 6 months for a total of seven timepoints.

Results: Greater variability in BoEs was observed compared with confined clinical studies. Gaussian distributed data showed 2-sided values over 100%, which are uninformative for decreases. The proportion of significant changes increased slightly among switchers, probably as a result of additional variability due to the range of products used post-switching. Overall proportions of changes remained small, consistent with literature reporting that when switching to a different tar yield cigarette, smokers partially compensate by changing their smoking behaviour.

Conclusion: Variability estimates and RCVs can be useful for monitoring subjects' BoE and MLE endpoints in longitudinal smoking studies where subjects are followed in their own environment and to aid sample size calculation of studies involving these endpoints.

1. Introduction

Combustible tobacco products have been identified as one of the worldwide leading causes of disease. During the 20th century 100 million deaths were tobacco-related [1]. Despite health awareness campaigns and tobacco control policies, smoking prevalence is $<20\%$ in only seven of the European Union member states (2012) [2].

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In 2001, the US Institute of Medicine issued a report [3] that suggested the development of potential reduced exposure products (PREPs), later termed modified-risk tobacco products (MRTPs) [4], as a complementary approach to tobacco control policies to promote tobacco harm reduction. In their draft MRTP application guidance published in 2012, the U.S. Food and Drug Administration (FDA) defined these products as "*any tobacco product that is sold or distributed for use to reduce harm or the risk of tobacco-related disease associated with commercially marketed tobacco products*"[5].

The potential incentives emerging for reduced exposure or risk claims make it necessary to develop a scientific framework to assess the validity of these claims. In 2012 the FDA Center for Tobacco Products listed 56 research priorities in this area, one of which states: "*What methods and measures best assess biologically relevant changes in harmful and potentially harmful constituents in tobacco products and smoke in both clinical models and humans?*"[6].

Several studies have suggested that differences in levels of biomarkers of exposure (BoEs) to tobacco smoke toxicants can be observed in populations smoking cigarettes containing different levels of toxicants. [7,8] Hatsukami et al. reviewed the usefulness of several established biomarkers used in the assessment of tobacco products and concluded that no existing biomarkers were predictive of tobacco-related disease [9]. However, biomarkers such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) have since been associated with lung cancer, [10] although this has not been validated as a predictor of cancer risk. In the absence of acceptable biomarkers for risk of disease, reductions in BoEs have been suggested as an alternative parameter for the evaluation of MRTP use by consumers, while noting the lack of validation of correlations between exposure reduction and risk reduction [11]. The initial challenge is to determine what constitutes meaningful changes in BoE levels that may lead to reductions in disease risk. Subsequently, risk reduction associated with biomarker reduction could be assessed through prospective epidemiological and surveillance studies [12].

However, BoE levels are variable both within individuals and (more so) between individuals [13]. Such variability is believed to be driven by variations in metabolism [12,14], smoking behaviours, and within-product variation. Furthermore, variations in the yield of smoke constituents can occur when smokers switch between different products with different design specifications [15]. In this complex landscape, Hecht et al. recognised that the ultimate goal should be to aim for the levels of biomarkers observed in non-smokers, who are at lower risk for tobacco-related cancers. However, as this is not likely to be feasible, a realistic but meaningful target level should be set [12].

Reference change values (RCVs) are used in clinical settings to compare two sequential analytical results [16]. RCVs are defined as the minimum critical difference that must be exceeded between two sequential results for their difference to be considered significant, rather than due to random variation [17]. An advantage over classic population reference intervals is that RCVs allow assessment of significant changes within the accepted population intervals, i.e. a subject's analyte levels can lie within the expected ranges, but be classified as a significant change. RCV methodology assumes that analytes fluctuate randomly within individuals around a homeostatic set point following a normal distribution [17]. However, the log-normal distribution has since been found to be more appropriate for many analytes [18]. Hammond et al. have reported high correlation of cigarette consumption over time within smokers [38] which would lead to homeostatic levels of biomarkers of exposure. Therefore, we consider that the RCVs methodology could provide valuable information to monitor these biomarkers between consecutive timepoints.

RCVs have been applied in a wide range of clinical settings and endpoints [19,20]. RCVs for biomarkers of smoke exposure were calculated in a clinical confinement setting to assess metabolic and behavioural variation in subjects who switched between their conventional cigarette and an MRTP [21]. In this study, RCVs for biomarkers were calculated based on the variability observed in an ambulatory setting in which subjects were free to switch between cigarette products. It was expected that the biomarkers of tobacco smoke exposure and mouth-level exposure assessed in this non-invasive unrestricted setting would more closely reflect real variation among smokers [22]. The urinary biomarkers measured in this study were chosen for their specificity to tobacco smoke exposure and smoking behaviour. For example, NNAL has been shown to be sensitive to changes in exposure to tobacco smoke [23] and has been linked to lung cancer [10], whilst cotinine is frequently used in smoking studies to assess compliance. [24] Mouth-level exposure (MLE) endpoints have proved useful in assessing smoking intensity, showing correlations with urinary and salivary nicotine metabolites [25,26].

Here, we report RCVs calculated in conditions representative of those expected during monitoring potential MRTPs. The sensitivity of these references was evaluated by quantifying significant changes between successive observations when subjects switched to products of a lower ISO tar and nicotine yield.

2. Methods

2.1. Study design

A non-residential, observational, ambulatory study was conducted over 3.5 years across 10 sites in Germany (Berlin, Cologne, Dresden, Gelsenkirchen, Hamburg, Leipzig, Munich, Nuremberg, Rostock and Stuttgart). The study was conducted in accordance with the principles documented in the Declaration of Helsinki and the International Conference on Harmonisation Guidelines for Good Clinical Practice (trial registration ID: ISRCTN95019245). All subjects provided written informed consent and the study protocol and informed consent forms were approved by the Ethics Committee of Bayerischen Landesärztekammer.

Full details of the study protocol have been described previously [27]. Briefly, all subjects were daily smokers of

Table 1
Baseline demographics.

	Number of subjects (N = 1011)					
	Gender		**Age, years**			
	Male	**Female**	**21–29**	**30–39**	**40–49**	**50–64**
n (%)	652 (64)	359 (36)	418 (41)	353 (35)	173 (17)	67 (7)
	Age (years)			**Daily consumption*** **(cig)**		
Mean (SD)	33 (9)			13.3 (6.2)		

Cig, cigarette; N, total number of subjects; n, number of subjects in the group.
* Daily consumption is the average of daily collected cigarette tips (cig).

8 cigarettes or more. All participants smoked the same brand for at least 6 months before enrolment. To promote homogeneity within and between subjects, consumption inclusion criterion was used. Only applicants smoking 8 cigarettes or more were considered to participate in the study and those subjects attempting to quit were removed from the study. Subjects were free to withdraw from the study at any time without specifying the reasons for withdrawal. Once withdrawn they were not allowed to return. Withdrawn subjects were not replaced. Participants were assessed at seven timepoints, including baseline, at 6-month intervals. At each timepoint, participants attended three clinical visits over a 12-day period, during which 24-h urine samples, saliva samples, and spent cigarette filter tips were collected and cigarette consumption questionnaires were completed. Participants were allowed to miss timepoints and were permitted to switch cigarette brands or alter their cigarette consumption at their will.

Subjects commencing the study were smokers of the same commercial 10 mg ISO tar product ('conventional cigarette'). The age and gender frequency distributions of the subjects at baseline are displayed in Table 1. Details of the full analyses of biomarkers can be found in a previous publication [28]. Aiming to palliate the introduction of variability due to product differences, RCVs were calculated based on those subjects smoking the initial 10 mg brand for the whole period of the study or up to the timepoint previous to the first switching event.

2.2. Urinary and salivary biomarkers

The total number of smoked cigarettes was recorded during the 24-h urine collection period. Urine was collected in 2 L polyethylene containers and stored in a cool bag with two frozen cooling elements during the collection period. The volume of urine was estimated from the weight and a nominal specific gravity factor of 1.018, prior to the removal of aliquots for biomarker analysis. The levels of nicotine equivalents [29] (defined as the molar sum of nicotine, cotinine, 3′-hydroxycotinine (OH-cotinine), nicotine-N-glucuronide, cotinine-N-glucuronide and trans-3′-hydroxycotinine-O-glucuronide), NNAL [30] and creatinine [31] were measured in the urine samples.

Saliva samples were obtained under the supervision of a study nurse using Salivettes® (Sarstedt, Numbrecht, Germany) and salivary levels of cotinine and OH-cotinine were measured [29]. All methods, with the exception of creatinine, were validated according to the U.S. FDA Guidance for Industry [32].

2.3. Estimation of MLE to tar and nicotine

Cigarette filters were collected during the 24-h urine collection period, for subsequent determination of MLE to tar and MLE to nicotine [25,26]. Machine-derived nicotine and tar yields were determined using the ISO regime (ISO 3308:2000). Human exposure to mainstream smoke varies due to differences in smokers' behaviour, including puff volume, number of puffs and overall consumption. Heterogeneous smoking behaviours can lead to variability in exposure [33]. In this study, NNAL and MLE to tar and nicotine were also assessed on a per cigarette basis, to evaluate whether adjustments per cigarette impact variability.

2.4. Statistical analyses

RCVs [16,34] were calculated based on data from smokers who smoked the 10 mg ISO tar yield cigarette for at least two consecutive timepoints. Of the initial 1011 subjects, 852 subjects had two or more consecutive observations, which translated into approximately 4000 consecutive data entries used to calculate RCVs. Skewness of biomarker distributions was assessed by comparing median and arithmetic means in both raw and log-transformed data, as suggested by Fokkema et al. [18] Prior to RCV calculation, samples were examined for outlier detection using Tukey's interquartile outlier detection method, as suggested by the Clinical and Laboratory Standards Institute [35]. Homoscedasticity was assessed with Levene and O'Brien's tests. All RCVs were calculated with 5% probability error.

Potential differences in biomarker levels by age and gender (Table 1) were assessed using repeated measures analysis of

variance (ANOVA) to investigate whether partitioning of RCVs by age and gender categories would be appropriate [34]. The repeated measures ANOVA was performed in SAS 9.3 software (SAS Institute, Cary, NC, USA) with the PROC MIXED statement and subject as random effect. RCVs were calculated by category based on the results of the differences found in the repeated measures ANOVA using the approach of Fraser and Harris for Gaussian distributed measurements [36,37] and Fokkema's approach [18] for log-normal distributed data.

Switching events were classified as a side-switch (SS) if subjects changed to a cigarette brand with an ISO tar yield of 8–10 mg, or a down-switch (DS) if subjects switched to products with an ISO tar yield of ≤ 7 mg.

3. Results

A total of 55 samples were identified as having outliers for four or more analytes and were removed from the analyses. Comparisons of means and medians of the normal and log-normal representations of the data showed that only salivary cotinine and urinary nicotine equivalents approximated the normal distribution, while the rest of the analytes were better represented by the log-normal transformation.

Correlation in cigarette consumption across timepoints was up to 0.75–0.86 for the closest timepoints and it gradually decreased to reach a minimum of 0.62 for the farthest i.e. between baseline and timepoint 6. This is aligned with correlations in consumption reported in the literature (0.91) [38].

Analytical coefficients of variation were calculated for the MLE to nicotine and tar measurements based on 3 replicates for each subject at each timepoint (only the 333 subjects smoking the initial 10 mg product for the whole duration of the study were used for this analysis). The analytical report for urine and saliva markers records measures of performance (coefficient of variation, CV) for the analytical imprecision (CV_A) based on 3 concentrations and $n=5$. CV_As were 33% and 32% for MLE to tar and nicotine, respectively. CV_As calculated from analytical imprecision in salivary markers were: 4.2% for Cotinine, 3.6% for OH-Cotinine. CV_As for urine markers were 10.1% for nicotine equivalents, 6% for NNAL and 3% for Creatinine.

In Table 2, RCV_S are displayed stratified by age and gender, showing significantly different means. The minimum differences between consecutive timepoints for the log-normal approach were non-symmetric. The RCV_{UP} indicates the minimum significant increase between two consecutive timepoints and RCV_{DOWN} indicates the minimum significant decrease (indicated by the negative sign). For normally distributed analytes (salivary cotinine and urinary nicotine equivalents), the between-subject (CV_G) and within-subject marker variation to smoke exposure (CV_I) are reported as CVs. Arithmetic means and geometric means are provided for normal and log-normal distributed analytes, respectively. Homoscedasticity was confirmed for most of the age-gender groups with exception of MLE to tar and nicotine when measured on a per cigarette basis.

The percentages of consecutive observations falling outside the RCVs are shown in Table 3, presented by smoker type and timepoint change relative to the previous timepoint (i.e. timepoint 1 indicates measurements between baseline and timepoint 1; timepoint 2 indicates measurements between timepoint 1 and 2, etc.). These percentages were calculated with respect to the number of pairs of consecutive observations recorded with smoker type determined as the smoker type at the end of the interval. These calculations include those observations previously categorised as outliers. The numbers of down- and side-switchers experiencing a significant change in analyte concentration are recorded in Table 4.

In order to facilitate comparisons with biomarker levels reported in the literature, the overall median and geometric mean NNAL corrected for creatinine were calculated (218.3 pg/mg, 191.6 pg/mg respectively). The geometric mean of NNAL on a per cigarette basis was 16.5 ng while mean and median nicotine adjusted for creatinine were 10,167.7 ng/mg and 9311.3 ng/mg. Other ways of reporting these measurements are available on request.

4. Discussion

Over 1000 subjects were monitored for 3.5 years in a non-residential clinical, ambulatory smoking study. In this non-confined and unrestricted setting, RCVs were calculated to aid evaluation of biomarker levels between consecutive observations that may be undertaken during potential MRTP monitoring. Increased variability was observed compared with a confined clinical study that reported a symmetric RCV of 68% for NNAL (ng/cig) [21]. This previous estimate was similar to the RCVs for decreases (RCV_{DOWN}) that we observed here, with values between 65% and 70%, but clearly underestimated the significant increases observed in ambulatory studies (up to 229%). Similar wide references were yielded for most of the upper RCVs calculated by Fokkema's approach [18], with many of the analytes needing to reach a 2-fold change to be considered significant.

In 1988, Gowans and Fraser [49] published a report on the biological variation of serum and urine creatinine. They found that serum measurements had a high index of individuality and therefore they were not very useful to calculate population level references. While creatinine in urine was not different while studied as concentration it was clearly different in output terms. These conclusions [49] agreed with our findings, and highlighted the need for stratification by gender. They also indicated that creatinine expressed in terms of the output could produce useful references at population level, with indices of individuality of 1.42 and 1.83 for men and women respectively.

Table 2
RCVs of analytes by demographic category showing significant differences between their means. Between-subject (CV_G) and within subject variation (CV_I) are represented as coefficients of variation (CV).

Analyte	**Salivary cotinine (ng/mL)**					**Urinary nicotine equivalents (mg/24 h)**			
n	Group	Mean*	CV_G	CV_I	RCV	Mean*	CV_G	CV_I	RCV
87	Male < 30	263	46%	33%	± 92%	12.1	50%	46%	± 130%
85	Male 30–39	301	52%	33%	± 93%	14.2	52%	43%	± 120%
43	Male ≥ 40	338	49%	28%	± 78%	16.3	41%	39%	± 110%
32	Female < 30	218	54%	44%	± 123%	10.1	57%	48%	± 134%
35	Female 30–39	257	55%	42%	± 117%	11.5	57%	44%	± 123%
51	Female ≥ 40	287	48%	29%	± 81%	13.2	44%	40%	± 112%

Analyte	**Salivary OH-Cotinine (ng/mL)**					**Urinary NNAL (ng/24 h)**			
	Group	Mean	CV	RCV_{UP}	RCV_{DOWN}	Mean	CV	RCV_{UP}	RCV_{DOWN}
	Male < 30	54.7	39%	183%	−65%	188	43%	211%	−68%
	Male 30–39	61.9	37%	170%	−63%	249	41%	197%	−66%
	Male ≥ 40	85.8	35%	160%	−61%	333	37%	170%	−63%
	Female < 30	44.7	39%	183%	−65%	149	40%	190%	−66%
	Female 30–39	56.2	44%	219%	−69%	195	38%	175%	−64%
	Female ≥ 40	85.8	34%	152%	−60%	266	39%	180%	−64%

Analyte	**MLE Nicotine (mg/24 h)**					**MLE Tar (mg/24 h)**			
	Group	Mean	CV	RCV_{UP}	RCV_{DOWN}	Mean	CV	RCV_{UP}	RCV_{DOWN}
	Male < 30	18.9	29%	120%	−54%	229	29%	120%	−54%
	Male 30–39	22.4	27%	108%	−52%	273	27%	109%	−52%
	Male ≥ 40	24.5	25%	96%	−49%	300	24%	94%	−48%
	Female < 30	15.9	27%	108%	−52%	191	26%	105%	−51%
	Female 30–39	18.2	28%	113%	−53%	221	27%	111%	−53%
	Female ≥ 40	20.6	25%	97%	−49%	252	24%	94%	−48%

Analyte	**Urinary NNAL (ng/cig)**					**Urinary creatinine (mg/24 h)**			
	Group	Mean	CV	RCV_{UP}	RCV_{DOWN}	n Mean	CV	RCV_{UP}	RCV_{DOWN}
	Male < 30	14.1	45%	228%	−69%				
	Male 30–39	17.1	45%	229%	−70%	215+ 1517+	31%+	130%+	−57%+
	Male ≥ 40	20.7	40%	194%	−66%				
	Female < 30	11.6	43%	216%	−68%				
	Female 30–39	13.8	39%	182%	−65%	118++ 1038++	28%++	114%++	−53%++
	Female ≥ 40	18.4	42%	208%	−68%				

Analyte	**MLE Nicotine (mg/cig)**					**MLE Tar (mg/cig)**			
n	Group	Mean	CV	RCV_{UP}	RCV_{DOWN}	Mean	CV	RCV_{UP}	RCV_{DOWN}
87	Male < 30	1.42	15%	51%	−34%	17.2	16%	57%	−36%
128	Male > 30	1.55	14%	47%	−32%	18.9	15%	52%	−34%
32	Female < 30	1.25	14%	47%	−32%	15.0	14%	48%	−33%
86	Female > 30	1.37	15%	50%	−33%	16.7	15%	53%	−34%

Cig, cigarette; CV, coefficient of variation; CV_G, between-subject coefficient of variation; CV_I, within-subject coefficient of variation; h, hours; MLE, mouth-level exposure; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; RCV, reference change value; RCV_{UP} indicates the minimum significant increase between two consecutive timepoints; RCV_{DOWN} indicates the minimum significant decrease between two consecutive timepoints, n indicates the number of smokers that remained using the initial 10 mg for the whole duration of the study (7 measurements per subjects).

* Mean values for salivary cotinine and urinary nicotine are arithmetic means; mean values for all other analytes are geometric means.

+ Male subjects (all ages).

++ Female subjects (all ages).

BoE and MLE displayed consistent associations with age and gender across analytes, with higher levels in males and in older subjects. Differences in MLE observed between genders could be explained by puffing topography [38], with females yielding lower mean MLE to tar and nicotine across all age categories, for both per cigarette and 24-h values. These gender differences confirmed patterns previously observed [39]. Smoking intensity also appeared to increase with age. MLE measures experienced less variability than BoEs, especially on a per cigarette basis. However, heteroscedasticity between

Table 3
Percentage of observations showing a significant change in analyte concentration by smoker type and timepoint.

Smoker type	Timepoint	Direction of change	n	Creatinine	Cotinine	OH-cotinine	Nicotine (cig)	Tar (cig)	Nicotine (day)	Tar (day)	NNAL (day)	Nicotine equivalent	NNAL (cig)
Conventional cigarette (10 mg ISO tar)	1	↑	741	4.3%	2.2%	3.8%	5.0%	5.0%	5.4%	5.3%	2.4%	1.3%	4.7%
		↓		9.6%	1.9%	6.6%	2.4%	2.3%	5.4%	5.5%	6.1%	5.9%	5.1%
	2	↑	614	6.5%	2.8%	5.5%	2.6%	2.9%	4.2%	4.6%	2.6%	5.0%	2.1%
		↓		7.2%	2.0%	2.9%	4.6%	3.3%	3.6%	3.4%	7.2%	2.1%	7.3%
	3	↑	527	8.5%	1.9%	3.6%	3.8%	3.2%	3.2%	3.6%	7.6%	3.4%	7.0%
		↓		5.1%	2.5%	4.6%	4.2%	3.4%	5.1%	4.9%	3.2%	1.7%	2.8%
	4	↑	469	7.5%	6.2%	5.1%	2.6%	2.1%	2.1%	2.3%	7.2%	1.9%	10.0%
		↓		2.8%	1.7%	2.1%	4.7%	4.5%	3.6%	3.8%	0.9%	2.8%	0.4%
	5	↑	436	3.2%	2.1%	1.6%	3.0%	3.2%	1.8%	2.5%	3.2%	1.4%	2.8%
		↓		7.8%	7.8%	8.0%	5.0%	5.0%	3.0%	3.4%	4.8%	4.8%	4.4%
	6	↑	395	5.1%	1.3%	6.1%	4.6%	2.8%	2.8%	2.5%	2.3%	4.6%	2.3%
		↓		2.0%	2.3%	2.5%	2.3%	2.8%	2.0%	3.5%	3.3%	1.3%	2.8%
Side-switchers	1	↑	39	5.1%	0%	7.7%	7.7%	12.8%	7.7%	7.7%	7.7%	0%	17.9%
		↓		5.1%	2.6%	7.7%	7.7%	5.1%	15.4%	10.3%	5.1%	5.1%	5.1%
	2	↑	58	1.7%	5.2%	6.9%	3.4%	0%	3.4%	3.4%	3.4%	1.7%	3.4%
		↓		10.3%	3.4%	0.0%	3.4%	3.4%	1.7%	1.7%	8.6%	1.7%	6.9%
	3	↑	62	3.2%	0%	0%	3.2%	1.6%	3.2%	1.6%	0%	0%	3.2%
		↓		8.1%	4.8%	3.2%	9.7%	3.2%	1.6%	0%	4.8%	0%	4.8%
	4	↑	71	4.2%	9.9%	2.8%	2.8%	0%	2.8%	2.8%	9.9%	1.4%	9.9%
		↓		1.4%	0%	0%	4.2%	4.2%	5.6%	4.2%	0%	2.8%	0%
	5	↑	72	0%	2.8%	1.4%	1.4%	0%	1.4%	1.4%	1.4%	0%	4.2%
		↓		4.2%	9.7%	9.7%	4.2%	6.9%	4.2%	2.8%	1.4%	5.6%	0%
	6	↑	77	3.9%	1.3%	7.8%	3.9%	2.6%	2.6%	1.3%	3.9%	3.9%	5.2%
		↓		3.9%	5.2%	5.2%	1.3%	3.9%	2.6%	3.9%	1.3%	0%	0%
Down-switchers	1	↑	40	5.0%	0%	0%	0%	0%	7.5%	5.0%	0%	0%	5.0%
		↓		12.5%	0%	15.0%	0%	2.5%	2.5%	2.5%	5.0%	12.5%	5.0%
	2	↑	72	6.9%	1.4%	8.3%	4.2%	4.2%	5.6%	4.2%	0%	2.8%	0%
		↓		2.8%	0%	6.9%	5.6%	2.8%	4.2%	4.2%	5.6%	1.4%	11.1%
	3	↑	73	9.6%	1.4%	1.4%	2.7%	0%	2.7%	0%	6.8%	1.4%	5.5%
		↓		6.8%	0%	2.7%	6.8%	5.5%	6.8%	6.8%	4.1%	0%	2.7%
	4	↑	71	5.6%	2.8%	11.3%	8.5%	9.9%	5.6%	7.0%	8.5%	0%	9.9%
		↓		2.8%	0%	0%	7.0%	5.6%	5.6%	5.6%	1.4%	0%	1.4%
	5	↑	74	0%	0%	1.4%	0%	1.4%	5.4%	2.7%	1.4%	0%	1.4%
		↓		2.7%	4.1%	10.8%	8.1%	8.1%	2.7%	4.1%	1.4%	1.4%	1.4%
	6	↑	74	5.4%	0%	8.1%	4.1%	1.4%	6.8%	4.1%	1.4%	0%	0%
		↓		2.7%	0%	2.7%	2.7%	4.1%	0%	0%	1.4%	1.4%	2.7%

Cig, cigarette; n, total number of consecutive measurements belonging to that smoker type at that timepoint; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol.

Table 4
Number of observations showing a significant change in analyte concentration by smoker type and timepoint.

Smoker type	Timepoint	Direction of change	n_s	Creatinine	Cotinine	OH-cotinine	Nicotine (cig)	Tar (cig)	Nicotine (day)	Tar (day)	NNAL (day)	Nicotine equivalent	NNAL (cig)
Side-switcher	1	↑	39	2	0	3	3	5	3	3	3	0	7
		↓		2	1	3	3	2	6	4	2	2	2
	2	↑	30	0	1	1	1	0	1	1	1	0	1
		↓		1	2	0	2	2	1	1	1	1	2
	3	↑	9	0	0	0	0	0	0	0	0	0	0
		↓		0	1	0	1	1	0	0	0	0	1
	4	↑	15	0	1	1	0	0	2	1	0	0	1
		↓		0	0	0	2	2	3	2	0	0	0
	5	↑	10	0	1	1	0	0	0	0	0	0	0
		↓		0	1	1	0	2	1	0	0	0	0
	6	↑	9	1	1	1	0	0	0	0	1	1	1
		↓		0	0	0	1	2	1	1	0	0	0
Down-switcher	1	↑	40	2	0	0	0	0	3	2	0	0	2
		↓		5	0	6	0	1	1	1	2	5	2
	2	↑	39	2	1	4	1	1	2	2	0	0	0
		↓		0	0	4	4	2	2	2	3	1	6
	3	↑	11	2	0	0	1	0	0	0	0	1	0
		↓		1	0	0	1	1	0	0	1	0	0
	4	↑	11	0	1	1	1	1	1	1	1	0	3
		↓		0	0	0	2	2	2	2	0	0	0
	5	↑	8	0	0	0	0	0	1	0	0	0	0
		↓		0	0	1	1	2	0	0	0	0	0
	6	↑	6	0	0	0	0	0	1	0	0	0	0
		↓		0	0	0	0	0	0	0	0	0	0

Cig, cigarette; n_s, number of smokers switching to that smoking type between that timepoint and the previous timepoint; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol

subjects was observed for the MLE measures, as per cigarette measurements are likely to enhance differences in smoking behaviour, and therefore these estimates should be considered with caution.

We observed a high level of concordance in BoEs with values published previously. For example, Lowe et al. [40] reported NNAL levels with a median of 198.67 pg/mg (220.11 pg/mg corrected for creatinine) and a geometric mean of 162.52 pg/mg (200.75 pg/mg corrected for creatinine), depending on average cigarette consumption. These values are comparable with the median of 218.3 pg/mg and geometric mean of 191.6 pg/mg observed in this study. NNAL levels were also comparable with previously published data on a per cigarette basis, with an overall geometric mean of 16.5 ng/cig. [21] Nicotine equivalents adjusted for creatinine, produced a mean value of 10167.7 ng/mg and a median of 9311.3 ng/mg, which are also comparable with data published previously. [39] In saliva, mean cotinine ranged from 218 to 338 ng/mL, which is in line with the range reported in the literature for smokers of 15–20 cigarettes per day. [41] Smoking 'compliance' was high, with only 118/4124 observations below the 7 ng/mL cotinine threshold, which can differentiate between smokers and non-smokers with a sensitivity of 92.3% and a specificity of 89.7% [40].

The general trends observed for MLE measurements by age and gender were also observed for the BoEs. Similarly to our study, a trend for increased NNAL by age was also observed by Agaku et al. [42] Although we observed higher levels of NNAL in men than in women, this trend was reversed when NNAL was adjusted for creatinine (data not shown), which is in agreement with results observed by Agaku et al. [42] However, NNAL variability, expressed as CVs in Table 1, did not reduce when it was adjusted for cigarette consumption per day, possibly due to the long half-life of NNAL (10–18 days) [43]. In fact, NNAL has been suggested as an indicator of exposure to the smoke constituent 4-(methylnitro-samino)-1-(3-pyridyl)-1-butanone (NNK) over weeks or months [44]. Consequently, a per cigarette adjustment based on the average cigarettes consumed when the samples were taken may not be appropriate.

The percentage of observations experiencing a significant change in analyte concentration by smoker type appeared to be consistent for the conventional cigarette group (Table 3). More changes were observed than those expected by chance, which could have been due to changes in cigarette consumption and/or perhaps variable compliance in urine collection. In the conventional cigarette group, creatinine appeared to be the analyte with the highest percentage of significant changes, which may indicate issues with urine collection, although this is not of concern. Across analytes, the percentage of significant changes increased for down-switchers and side-switchers compared with the conventional cigarette group. This could be due to the additional variability introduced by product switching (Table 4). Differences in blend chemistry and design features between products could have contributed to the increase in significant changes in analytes observed in the down-switchers and side-switchers [8,41]. This was especially noticeable at Timepoint 1, when most switching events occurred. In general, the percentage of changes were small and remained relatively stable for the remainder of the study, as expected from literature on switching studies and compensation [38]. Hence, these data suggests that RCVs could be useful for monitoring BoE and MLE endpoints in smoking studies in which subjects are followed in their everyday environment. It has been demonstrated that smoking compensation among smokers prevents biologically significant changes occurring when switching products [38] and the RCV approach appeared to confirm this, by detecting some changes when switching products and consumption, but without highlighting a large proportion of false positives.

Based on recently published guidance [48] in designing and reporting studies in biological variation there are several shortcomings to our study. Reference values were calculated for markers of exposure to a single brand of cigarettes and within the German population. How transferable these results are to other populations with different underlying distributions, for example due to ethnicity, is unknown. Røraas et al. [47] carried out a simulation study to facilitate the assessment of estimates of biological variation based on the ratio between analytical and inter-individual variability. Unfortunately, we could only calculate analytical coefficients of variation for the MLE to nicotine and tar measurements based on technical replicates of the study samples while, for the rest of the analytes, only estimates based on imprecision were available. This presents some limitations when calculating confidence intervals and the power of the study. However, due to the large number of subjects completing our study and the number of measures available for each subject, even considering CV_As to be 3-fold higher than those estimated from precision, the power calculated from the tables of Røraas et al. [47] would be still above 0.90 in all cases.

With respect to the two approaches used for calculating the RCVs, the classical approach [16] yields decreasing references over 100%, which is counterintuitive and therefore the alternative approach estimates [18] seem more reasonable. However, upper reference values reached over 200% using Fokkema's approach [18], which seems high in practical terms compared with previous clinical data [21], but it may just reflect the intrinsic variability of an ambulatory study.

Only two consecutive observations were compared in this study because when RCVs are used for more than two consecutive observations, the number of false positives increases [34]. Recently, methods based on computer simulation models have been suggested for assessing more than two serial results in a unidirectional [45] and bidirectional [46] context. These could be used during monitoring to assess changes over more than two consecutive timepoints.

5. Conclusion

We reported variability estimates and reference change values from a 3.5 year ambulatory study in healthy smokers. These data have proved to be useful for monitoring BoE and MLE endpoints in smoking studies where subjects are followed in their everyday environment i.e. outside a clinical setting. The estimates in this report can be used to estimate appropriate

sample size in studies involving these endpoints.

Conflict of interests

OMC, JS, KP and AC are employees of British American Tobacco.

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Ethical approval

The ethics committee of Bayerischen Landesärztekammer approved this study (trial registration ID: ISRCTN95019245).

Guarantor

OMC.

Contributorship

KP and JS conceived the study. JS and AC were involved in protocol development and sponsor's project management. OMC analysed the data and wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript.

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