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REVIEW ARTICLE

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Review of biomarkers to assess the effects of switching from cigarettes to modified risk tobacco products

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ABSTRACT

Context: One approach to reducing the harm caused by cigarette smoking, at both individual and population level, is to develop, assess and commercialize modified risk alternatives that adult smokers can switch to. Studies to demonstrate the exposure and risk reduction potential of such products generally involve the measuring of biomarkers, of both exposure and effect, sampled in various biological matrices.

Objective: In this review, we detail the pros and cons for using several biomarkers as indicators of effects of changing from conventional cigarettes to modified risk products.

Materials and methods: English language publications between 2008 and 2017 were retrieved from PubMed using the same search criteria for each of the 25 assessed biomarkers. Nine exclusion criteria were applied to exclude non-relevant publications.

Results: A total of 8876 articles were retrieved (of which 7476 were excluded according to the exclusion criteria). The literature indicates that not all assessed biomarkers return to baseline levels following smoking cessation during the study periods but that nine had potential for use in medium to long-term studies.

Discussion and conclusion: In clinical studies, it is important to choose biomarkers that show the biological effect of cessation within the duration of the study.

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Introduction

The Biomarkers Definition Working Group in 2001 defined a biomarker as "a characteristic that can be objectively measured and evaluated as an indicator of normal conditions and disease processes or of pharmacological responses" (Biomarkers Definitions Working Group 2001). Clinical biomarkers can be grouped by function.

Diagnostic – for the identification of disease or abnormal pathology, differential diagnosis and monitoring the response to therapy (Hijazi *et al.* 2013, Holdenrieder 2016).

Prognostic – as an indicator of disease prognosis. In oncology it is generally accepted that prognostic markers are patient/tumour factors that affect patient outcome independently of treatment administered (Tsao *et al.* 2015).

Predictive – a biomarker is predictive if the treatment effect is different for biomarker-positive patients compared with biomarker-negative patients (Ballman 2015, Tsao *et al.* 2015).

Most of the biomarkers in this review can be considered biomarkers of effect. They consist of measurable biochemical, physiologic, behavioural or other alteration in an organism that, depending on the magnitude, can be associated with an established or possible health impairment or disease. Some may also be considered biomarkers of exposure: a chemical, its metabolite or the product of an interaction between a chemical and some target molecule or cell that is measured in the human body (Committee on Human Biomonitoring for Environmental Toxicants 2006).

In summary, disease-related biomarkers give an indication of the probable effect of treatment on a patient (predictive biomarker), if a disease already exists (diagnostic biomarker) or how such a disease may develop in an individual case regardless of the type of treatment (prognostic biomarker) (Ballman 2015).

Biomarkers have demonstrated their value in early efficacy and safety evaluation and met scientific acceptance in basic and clinical research as primary endpoints for evaluation. The relevance of specific biomarker use is strengthened through evidence showing repeatedly to predict clinical outcomes (Strimbu and Tavel 2010). There is consensus that such surrogates for clinical outcomes require rigorous qualification and assessment. But even when this has been done, in line with best available knowledge, failures in clinical studies can occur. For example, suppression of arrhythmias as a surrogate endpoint for decreased morbidity due to cardiovascular disease (CVD) resulted in the approval of two drugs that in later studies were shown to increase morbidity (Fleming and DeMets 1996). In many instances, not all treatment effects are fully accounted for by a single biomarker (Biomarkers

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Definitions Working Group 2001). Therefore, for complex diseases, such as Chronic Obstructive Lung Disease (COPD), it has been suggested that a panel of biomarkers might prove more accurate (Leung and Sin 2013, Sun *et al*. 2016) and that a selection of biomarkers might increase the probability of success in clinical trials (Carroll 2016).

Expert opinion is unanimously in support of the fact that smoking cessation is the best way to reduce the risks of cigarette smoke-related diseases including COPD, CardioVascular Disease (CVD) and lung cancer. Smokers who stop smoking between the ages of 25–34 years do not differ in their life expectancy to those who have never smoked (Jha *et al*. 2013). For many smokers looking for better alternatives to cigarettes, the use of non-burning or smokeless tobacco products offers the possibility to consume nicotine with reduced risk to their health (Nutt *et al*. 2014, Hilton *et al*. 2016). What is already known from the plethora of existing data about toxicant exposure and subsequent health consequences of combustible tobacco consumption renders the use of clean nicotine in comparison to tobacco smoke a means to reduce harm (Cahill *et al*. 2013). However, the increased use by consumers of alternative, reduced risk nicotine products warrants confirmation and substantiation of the risk profile of this new product class. This substantiation requires the study of health outcomes in relation to product use in comparison to complete abstinence or continued smoking of conventional products. A number of biomarkers of biological effect and risk have been proposed to provide information about the risk profile of alternative tobacco products in the absence of, or not yet available, data of long-term use of the new nicotine-containing products. The objective of this publication is to report the results based on the analysis of data pertaining to a large number of biomarkers that have previously been used in studies investigating the effects of cigarette smoke and in studies where subjects have stopped smoking or switched to a reduced risk product (RRPs – products that present, are likely to present or have the potential to present less risk of harm to smokers who switch to these products versus continued smoking). A biomarker fit for purpose to substantiate reduced risk should be associated with the smoking of combustible cigarettes, decline following smoking cessation and/or smoking reduction and should be associated with at least one smoking-related disease (Biomarkers Definitions Working Group 2001, Strimbu and Tavel 2010).

Clinical significance

This review provides information related to a number of biomarkers that have been used to assess the effects of cigarette smoking. Many of them are used to monitor changes that occur following cessation (or switching to a modified risk product). To have meaningful studies with reliable outcomes, it is important to carefully select the biomarkers to be measured and the duration of the study. Here we provide evidence of biomarkers that do change following cessation and the time within which such a change occurs.

Methods

The PubMed database was searched for publications related to the biomarkers listed in Table 1. Three searches of the PubMed literature data base were conducted for English language articles within the time frame of 2008 to May 2017 for each of the biomarkers of interest.

Search 1: To identify literature that linked smoking, nicotine replacement or cessation to the biomarker using the search string: (biomarker) AND (smokers OR smoking OR nicotine replacement OR cessation) AND ("2008"[Date - Publication]: "2017"[Date - Publication]) AND English [Language]).

Search 2: To identify literature reporting an association between the biomarker and cardiovascular disease using the search string: (biomarker) AND ((cardiovascular disease) AND (association)) AND ("2008"[Date - Publication]: "2017"[Date - Publication]) AND English[Language]).

Search 3: To identify literature reporting an association between the biomarker and respiratory disease using the search string: (biomarker) AND ((respiratory disease) AND (association)) AND ("2008"[Date - Publication]: "2017"[Date - Publication]) AND English[Language]).

A set of specific criteria was applied to the identified studies to determine which papers should be selected for inclusion:

- Studies investigating the association of cigarette smoking, smoking cessation or nicotine replacement therapy (NRT) with the biomarker;
- Studies investigating the association of the biomarker with CVD or respiratory disease.

The following exclusion criteria were applied:

E1. Studies that used the measurement of the biomarker only for control purposes (e.g. results were adjusted for differences in the biomarker between populations, but the biomarker itself was not an endpoint);
E2. Studies involving dietary, pharmacological or exercise interventions in which the biomarker was an endpoint, but was lacking a clear link between the biomarker and either a disease of relevance (CVD, COPD, lung cancer) or smoking;
E3. Studies of passive or parental smoking;
E4. Disease not covered by the article;
E5. Studies focussing on a narrow population (e.g. pregnant women, individuals with a particular haplotype, individuals with a specific disease) or studies limited to human-derived tissue (e.g. retina, gums);
E6. Studies reporting results from *in vivo* animal experiments;
E7. Studies in which the biomarker was measured for toxicological or forensic purposes;
E8. Studies with no comparison between smoking and non-smoking/switching/cessation;
E9. The biomarker was not measured or was presented in a fashion that made the data unusable.

Table 1. List of biomarkers that were searched in the PubMed database and published between 2008 and 2017.

Biomarker	Abbreviation	Description
Respiratory biomarkers		
Forced expiratory volume in 1 s	FEV_1	Disease severity (COPD) (Aggarwal *et al.* 2006, Joo *et al.* 2013).
Biomarkers of oxidative stress		
8-iso-prostaglandin $F_{2\alpha}$	8-iso-PG $F_{2\alpha}$	Related to oxidative stress and increased in smokers (Borrill *et al.* 2008, Hoffmeyer *et al.* 2009, Inonu *et al.* 2012).
Thromboxane B2	11-DTXB2	Related to oxidative stress and CVD. Increased in smokers (Wennmalm *et al.* 1991, Eikelboom *et al.* 2002, Calapai *et al.* 2009, Frost-Pineda *et al.* 2011).
Biomarkers of inflammation		
C-reactive protein	CRP	Systemic inflammation and predictor of prognosis in COPD (Dahl *et al.* 2007, Mehrotra *et al.* 2010, Celli *et al.* 2012).
White blood cell count	WBC count	Systemic inflammation (Friedman *et al.* 1973, Yeung and Buncio 1984, Tell *et al.* 1985, Fernandez *et al.* 2012).
Soluble intercellular adhesion molecule-1	sICAM-1	Vascular inflammation. Raised in CVD (Ridker *et al.* 1998, Malik *et al.* 2001) and COPD (Riise *et al.* 1994, Lopez-Campos *et al.* 2012, Blidberg *et al.* 2013, Aaron *et al.* 2015).
Fibrinogen	FBG	Predictor of CVD (Kannel *et al.* 1987, Ernst and Resch 1993, Paramo *et al.* 2004) potential predictor of respiratory disease (Lock-Johansson *et al.* 2014).
Tumour necrosis factor α	TNFα	Related to inflammation in CVD (Skoog *et al.* 2002) and respiratory diseases (Barnes 2009) with levels increased in smokers (Bostrom *et al.* 1999, Tanni *et al.* 2010). May decrease after smoking cessation.
Myeloperoxidase	MPO	Several studies link MPO to arterial inflammation and CVD (Rudolph *et al.* 2008, Ikitimur and Karadag 2010, Lobbes *et al.* 2010, Anatoliotakis *et al.* 2013, Nussbaum *et al.* 2013) and smoking-related lung diseases (Dominguez-Rodriguez and Abreu-Gonzalez 2011, Park *et al.* 2013). Most studies show raised levels in smokers that do not fall following smoking cessation except after long periods of time (Andelid *et al.* 2007, Park *et al.* 2013).
Exhaled nitric oxide	FeNO	Related to airway inflammation (especially in asthma). Decreased in smoking (Kharitonov *et al.* 1995, Yates *et al.* 2001, Sundy *et al.* 2007, Zuiker *et al.* 2010) and increased following smoking cessation (Robbins *et al.* 1997, Hogman *et al.* 2002, Nadif *et al.* 2010), probably due to smoking-related inhibition of nitric oxide synthase.
Sputum polymorphonuclear leukocyte (PMN) content	sPMN	Related exclusively to airway inflammation, especially in COPD (Keatings *et al.* 1996, Tsoumakidou *et al.* 2003, Paone *et al.* 2011, Gupta and Singh 2013). Most studies show increased numbers in smokers (Swan *et al.* 1991, Keatings *et al.* 1996, Chalmers *et al.* 2001, Bouloukaki *et al.* 2009) with usually no reduction following cessation (Maestrelli *et al.* 1996, Paone *et al.* 2011).
Phospholipase A2	PLA2	Elevated levels are associated with increased risk of cardiovascular events (Lp-PLA(2) Studies Collaboration *et al.* 2010, Vittos *et al.* 2012). Levels are slightly increased in smokers (Persson *et al.* 2007, Tselepis *et al.* 2009, Fratta Pasini *et al.* 2013). Probably not directly implicated in smoking-related lung diseases.
Biomarkers of cardiovascular disease		
Albumin	ALB	Related to CVD. May be increased in smokers (Corradi *et al.* 1993, Metcalf *et al.* 1993, Pinto-Sietsma *et al.* 2000) and decreased after smoking cessation (Chase *et al.* 1991, Metcalf *et al.* 1993, Ikeda *et al.* 1997).
High and low-density lipoprotein-cholesterol	HDL-C and LDL-C	CVD.
Apolipoprotein A1	ApoA1	Related to CVD. Lowered in smokers (Craig *et al.* 1989) but increases after smoking cessation (Stubbe *et al.* 1982, Masarei *et al.* 1991, Iwaoka *et al.* 2014).
Oxysterols	OxS	Related to CVD via oxidative stress. May be increased in smokers (Mol *et al.* 1997).
Homocysteine	HCY	Raised levels are associated with recurrent CVD. Some studies suggest levels rise in smokers (Nygard *et al.* 1997, Nygard *et al.* 1998, Sobczak 2003, Sobczak *et al.* 2007) but it is not clear in all studies that levels fall following smoking cessation (Nygard *et al.* 1995, O'Callaghan *et al.* 2002, Bazzano *et al.* 2003).
P-Selectin	P-Sel	Directly involved in risk of CVD (Blann *et al.* 2003) and most studies show increased levels in smokers (Osterud *et al.* 1999, Ridker *et al.* 2001, Bermudez *et al.* 2002). Long-term smoking cessation may lead to lower levels (Ridker *et al.* 2001, Bermudez *et al.* 2002).
Thiocyanate	SCN	Related to CVD (Wang *et al.* 2007), some cancers (Wang *et al.* 2001) and respiratory diseases (Shiue 2015) but is predominantly used as a marker of exposure to cigarette smoke. Levels are increased in smokers and fall to non-smoker levels 3-6 weeks after smoking cessation.
Von Willebrand factor	vWF	Related to thrombosis and CVD (O'Callaghan *et al.* 2005) (especially in CVD patients) as well as respiratory disease (Bartholo *et al.* 2014, Holz *et al.* 2014). Only limited evidence that smoking cessation leads to a decrease in vWF levels.
Miscellaneous biomarkers		
Glycated haemoglobin	HbA1c	May indicate Increased risk of CVD (Eeg-Olofsson *et al.* 2010, Chamnan *et al.* 2013) especially in diabetics (Chamnan *et al.* 2013, Pacilli *et al.* 2013, Ohkuma *et al.* 2015). Increased in smokers (Vlassopoulos *et al.* 2013).
Blood carboxyhaemoglobin	COHb	Increased in smokers, reduced on smoking cessation or switching (Roethig *et al.* 2007, van Staden *et al.* 2013). Often used as a biomarker of exposure to carbon monoxide but is also associated with coronary artery disease and the pathogenesis of coronary atherosclerosis (Cohen *et al.* 1969, Whereat 1970).
Wheeze cough and sputum	W/C/S	Related to smoking-induced respiratory disease severity (Polley *et al.* 2008, Molassiotis *et al.* 2010b, Putcha *et al.* 2014, Vestbo 2014). Symptoms reduce after long-term smoking cessation (Viegi *et al.* 1988a, Toljamo *et al.* 2010, Bjerg *et al.* 2013).
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol	NNAL	Exposure marker that has been related to risk of lung cancer. Increased in smokers and is reduced following smoking cessation (Carmella *et al.* 2009, Goniewicz *et al.* 2009, Stepanov *et al.* 2009). Used primarily as a biomarker of exposure to NNK.

Table 2. Numbers of publications (2008–2017) and the reasons for their exclusion.

	Search number				Exclusion criteria										
Biomarker	1	2	3	Total	E1	E2	E3	E4	E5	E6	E7	E8	E9	Total	Used
Forced expiratory volume (FEV1)	1914	43	1215	2815	561	287	921	22	255	8	20	490	204	2768	47
C-reactive protein (CRP)	363	265	80	708	24	52	394	3	48	5	4	93	13	633	75
(Soluble intercellular adhesion molecule-(1) *sICAM-1*	80	185	31	292	3	27	144	3	29	12	1	28	10	257	35
White blood cell count (WBC)	416	370	145	931	275	90	250	4	29	13	37	46	18	762	168
Fibrinogen (FBG)	112	91	43	246	4	35	124	2	10	6	11		10	202	50
Carboxyhaemoglobin (COHb)	112	3	4	119	19	23	29	6	3	11	10	2	13	116	3
Glycated Haemoglobin (HbA1c)	306	265	58	935	14	45	446	1	14					516	113
High-density lipoprotein-cholesterol (HDL-C)	560	848	28	1436	371	245	175	8	156	8		113	53	1129	307
Low-density lipoprotein-cholesterol (LDL-C)	93	262	28	383	19	57	260		4	1		1		338	45
Oxysterols (OxS)	4	17	40	61	3	4	1		3	7		7		25	36
Apolipoprotein A1 (ApoA1)	19	28	24	71	6	1	27		7		3	4		47	24
8-*iso*-15(S)-Prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$/8-iso-$PGF_{2\alpha}$)	52	17	15	84	8	6	24	1	18	1	4	1		61	23
11-Dehydrothromboxane_B2 (11-DTXB2)	60	52	15	127	11	9	49		8	14		26		117	10
Albumin (ALB)	217	290	52	559	3	21	478		6	6	9	7	13	533	16
Fractional exhaled nitric oxide (FeNO)	61	6	34	101	2	18	28		21	7	9			75	26
Homocysteine (HCY)	113	113	18	244	14	45	113		22	3	3		1	201	43
Myeloperoxidase (MPO)	77	39	40	156	9	8	30	0	8	24		9	13	101	55
Phospholipase A2 (PLA2)	21	26	10	57	4	1	13		6		1			25	32
P-Selectin (P-Sel)	118	68	2	188	37	50	28		18	3		2		138	50
Sputum polymorphonuclear neutrophils (sPMN)	23	0	18	41	2	2	6		1			1		12	29
Thiocyanate (SCN)	25	10	18	53	4	3	6		3	8	1		2	27	26
Von Willibrand Factor (vWF)	18	53	53	124	7	22	35		7	5		2		78	46
Tumour necrosis factor α (TNFα)	212	143	69	424	14	15	155	1	27	66	3	48	47	376	42
Wheeze, cough, sputum (W/C/S)	277	29	175	481	3	28	223	4	16		4		2	280	35
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)	15	99	9	123	15			21	3	5		15		59	64

Table 3. Literature retrieved from monographs and review articles.

	Monographs				Reviews			Both (Mongraphs + Reviews)		
Biomarker	Monographs	Articles	Excluded	Used	Reviews	Excluded	Used	Total (all)	Excluded (all)	Used
CRP	2	22	5	17	14	0	14	36	5	31
FBG	2	34	1	33	42	27	15	76	28	48
HbA1c	2	4	0	4	79	34	45	83	34	49
LDL-C	2	17	1	16	63	41	22	80	42	38
OxS	2	2	0	2	26	2	24	28	2	26
ApoA1	2	16	4	12	16	1	15	32	5	27
Alb	2	3	0	3	26	12	14	29	12	17
FeNO	2	16	0	16	30	15	15	46	15	31
HCY	2	4	1	3	15	0	15	19	1	18
MPO	2	11	2	9	23	6	17	34	8	26
PLA2	2	0			11	0	11	11	0	11
P-Sel	2	6	3	3	42	28	14	48	31	17
sPMN	2	16	4	12	10	2	8	26	6	20
SCN	2	10	0	10	16	4	12	26	4	22
vWF	2	6	0	6	28	8	20	34	8	26
TNFα	2	23	3	20	53	17	36	76	20	56
W/C/S	2	5	3	2	4	3	1	9	6	3

Monographs searched for biomarkers of effect and smoking were the US Surgeon General's report 2010 (Centers for Disease Control and Prevention *et al.* 2010) and the Life Sciences Research Office Report (Life Sciences Research Office 2007). The articles possibly extracted from reviews were not separately counted, but the reviews themselves were include or excluded.

The number of articles retrieved for each biomarker in each of the three searches and the numbers excluded according to the criteria (E1–E9 listed above) are presented in Table 2.

The information retrieved for each biomarker is presented in the Findings section as a summary of the biology, pathological role and an indication of the validity of the biomarker based on the information available in the literature, although it is important to note that for many of the biomarkers discussed below literature published prior to 2008 has been cited. This was done when it was not possible to reach a meaningful conclusion based on more recent data.

For some of the biomarkers, data were retrieved by searching monographs and reviews and extracting relevant literature from them. The number of articles found, and the numbers excluded for these biomarkers is presented in Table 3.

Findings

Respiratory biomarker

Forced expiratory volume in 1 s (FEV_1)

The respiratory disease that is most commonly associated with cigarette smoking is chronic obstructive pulmonary disease (Ford *et al.* 2013) (COPD) which is defined by a reduction in airflow that is not entirely reversible. Current diagnostic practice recommends that the obstruction be confirmed by the use of a post-bronchodilator forced expiratory volume in 1 s (FEV_1) to forced vital capacity (FVC) ratio of less than 0.7. (Vestbo *et al.* 2016) although it is accepted that this fixed ratio may lead to an underestimation of airflow obstruction in younger subjects and an over-diagnosis in the more elderly (partly attributable to

normal aging) (Cerveri *et al.* 2008, Runarsdottir *et al.* 2013, van Dijk *et al.* 2015, Woodruff *et al.* 2016).

The published data suggest that the link between cigarette smoking and annual decline in FEV_1 is moderate with one recent study showing a peak in the rate of decline of 53.8 ml per year in subjects with COPD GOLD grade 1 (Bhatt *et al.* 2016), and another showing that at age 45 years the estimated rate of FEV1 decline was larger in current smokers than former and never smokers (Mirabelli *et al.* 2016) and a reduction in FEV_1 is generally observed with increasing smoking history (Tommola *et al.* 2016). For each smoking history, however, there is considerable heterogeneity in FEV_1 response. Also among non-smokers, there is a subset with severe lung function impairment that will be diagnosed as COPD due to exposure to wood smoke, traffic pollutants, environmental tobacco smoke and occupational exposures etc. (Hu *et al.* 2010, Moreira *et al.* 2013, Diaz-Guzman and Mannino 2014, Pallasaho *et al.* 2014, Ramirez-Venegas *et al.* 2014).

Through years of regulatory practice, the FDA accepts the use of FEV_1 as a surrogate endpoint of disease status. All the articles in which smokers were compared to non-smokers showed that smoking decreased FEV_1 over time to a greater extent than the decrease attributable to aging.

The landmark lung health study between 1986 and 1994 (U.S./Canadian Lung Health study) (Anthonisen *et al.* 1994), involving almost 6000 subjects, showed that subjects who stopped smoking for 1 year had improved lung function.

The current literature search identified some studies reporting a short-term improvement in FEV_1 after smoking cessation. However, this was not consistently the case, probably due to the relatively large variability (Becklake 1986, Crapo and Jensen 2003) of this marker.

Several studies have shown that subclinical inflammatory changes in small airways exist years before the advanced stages of COPD (Cosio *et al.* 1978, Guy *et al.* 1994, Takizawa *et al.* 2000, Verbanck *et al.* 2004). Although relatively few longitudinal studies additional to the lung health study have assessed the effects of smoking cessation on inflammation in smokers without chronic respiratory symptoms, these studies demonstrated that the inflammatory response decreased rapidly within the first few months after smoking cessation. It seems likely that the favourable changes in lung function as assessed by FEV_1 in studies of less than 1-year duration may be best explained by a decrease in the inflammatory state of the lung following quitting smoking.

FEV_1, which is the accepted marker for COPD progression, will have its role in the assessment of novel risk reduced tobacco products although studies will need to be of fairly large size to account for the inherent variability of the measure. They will also need to be of longer duration. It may be that after two decades the time has come to reproduce the results of the lung health study since there have been many changes in the pattern of tobacco consumption and products on the market in the intervening years. Although lung function remains stable during the years between the age of 20–35, FEV_1 then declines due to the natural aging processes (with an accelerated decline after the age of 70 years) (Sharma and Goodwin 2006, Skloot 2017). Ethnicity and gender also affect spirometric measures of pulmonary function (Quanjer *et al.* 2012a, Coates *et al.* 2016, Mirabelli *et al.* 2016, LaVange *et al.* 2017)

The small airways have been proposed to be the major site of increased airway resistance in COPD (Hogg *et al.* 1968, Flenley 1988, Hogg *et al.* 2004, Burgel 2011, Hogg *et al.* 2013) inducing a slowing in the terminal portion of the spirogram interpreted as a reduction in the mean expiratory flow between 25 and 75% of the forced expiratory flow ($FEF_{25-75\%}$). Although it is thought not to contribute to clinical decision making (Quanjer *et al.* 2012b, 2014) several reports have shown that $FEF_{25-75\%}$ is reduced in smokers compared to non-smokers (Casale *et al.* 1991, Urrutia *et al.* 2005, Bird and Staines-Orozco 2016) with one study showing a dose–response between smoking and lower $FEF_{25-75\%}$ (Gold *et al.* 1996). In a study in which smokers were invited to switch to an e-cigarette and abstain from smoking, the $FEF_{25-75\%}$ increased significantly ($p = 0.034$) from 85.7% to 100.8% predicted over 52 weeks (Cibella *et al.* 2016). There are, however, several reasons why the $FEF_{25-75\%}$ may not be useful: it is dependent upon the FVC, it lacks the repeatability of FEV_1, it has a wide normal range (Quanjer *et al.* 2012b, Al Ghobain *et al.* 2014, Balasubramaniam *et al.* 2014, Gutierrez *et al.* 2014, Rufino *et al.* 2017) and it is reduced in the presence of proximal airway narrowing (Johns *et al.* 2014). These drawbacks severely limit the use of $FEF_{25-75\%}$ for diagnostic purposes, but might not preclude its use in aetiological studies where differences between groups may be valuable. However, without clear validation $FEF_{25-75\%}$ cannot be recommended as a biomarker for use in short-term smoking/cessation/switching studies.

Biomarkers of oxidative stress

In eukaryotic organisms, more than 90% of reactive oxygen species (ROS) are produced by the mitochondria, via the mitochondrial electron transport chain (Skulachev 2012, Sies 2014) although smaller amounts are also produced by electron transport chains in endoplasmic reticular (Brignac-Huber *et al.* 2011), nuclear (Vartanian and Gurevich 1989) and plasmatic (Luthje *et al.* 2013) membranes. When the levels of these free radicals become sufficient to overwhelm the antioxidant defence mechanisms then "oxidative stress" occurs. Oxidative stress and the effects it causes, such as lipid peroxidation, are involved in a number of smoking-related diseases including atherosclerosis, cancer, inflammation and COPD (Barrera 2012, McGuinness and Sapey 2017) and in diseases such as accelerated aging and depressive disorders (Maurya *et al.* 2016). Furthermore, the chronic inflammation associated with these diseases also induces oxidative stress (Thanan *et al.* 2014).

Cigarette smoke exposure elicits a series of adaptive cellular stress responses (Bialas *et al.* 2016, Jiang *et al.* 2017) and a number of biomarkers have been evaluated as surrogate measures for these responses. These are discussed below although it is unlikely that a single biomarker would be capable of

reflecting the complexity of cellular stress responses to exogenous stressors such as cigarette smoke in man.

8-Iso-15(S)-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$)

The isoprostanes are prostaglandin (PG)-like compounds that are formed from the peroxidation of the ubiquitous polyunsaturated fatty acid, arachidonic acid. Unlike PGs, however, which are formed via the action of the cyclooxygenase enzymes, isoprostanes are generated as a result of the free radical-mediated peroxidation of arachidonic acid (Morrow *et al.* 1990) although there is increasing evidence that they may be formed via the actions of PG endoperoxide synthase enzymes which are induced during inflammation (van't Erve *et al.* 2015, 2016)

The 2001 Institute of Medicine report (Stratton *et al.* 2001) suggests the measurement of one of the four positional/regioisomers of 8-iso-$PGF_{2\alpha}$ (also termed 8-epi-$PGF_{2\alpha}$), for the assessment of oxidative damage. For many years, 8-iso-$PGF_{2\alpha}$ has been regarded as the gold standard for the detection of oxidative stress (Morrow 2005, Ho *et al.* 2013a)

This literature search provided no evidence of a strong link between 8-iso-$PGF_{2\alpha}$ and smoking (e.g. urinary 8-iso-$PGF_{2\alpha}$ levels were 0.73 μg/g creatinine (95% CI, 0.61–0.87) in non-smokers and 0.86 μg/g creatinine (95% CI, 0.70–1.05) in smokers (Calapai *et al.* 2009) contrary to some earlier publications (Reilly *et al.* 1996) where smokers had a pre-cessation mean of 145.5 ± 24.9 compared to non-smokers 63.7 ± 5 pmol/mmol creatinine 8-iso-$PGF_{2\alpha}$ in urine. In heavy smokers', urinary 8-iso-$PGF_{2\alpha}$ levels fell to 114.6 ± 27.1 after 2 weeks of cessation. A 90-day study in Japanese subjects found that switching from menthol cigarettes (mCC) to a menthol tobacco heating system (mTHS) product resulted in a 12.7% lower urinary 8-iso-$PGF_{2\alpha}$ concentration compared to continued mCC users (Lüdicke *et al.* 2017). A recent meta-analysis of over 200 publications found that tobacco smoking caused a relatively small increase in free 8-iso-$PGF_{2\alpha}$ (van't Erve *et al.* 2017) which the authors note as interesting for a condition that has long been thought to have a high level of oxidative damage. Given that "for a biomarker of exposure to be accepted as a biomarker of risk or a surrogate endpoint of disease, there should be a strong biological rational as well as compelling data from clinical and epidemiologic studies"(Committee on Scientific Standards for Studies on Modified Risk Tobacco Products 2012) it would appear that although 8-iso-$PGF_{2\alpha}$, as a marker of oxidative stress (and a specific marker for lipid peroxidation), is valuable in establishing a diagnosis or assessing disease progression due to oxidative stress (Janssen 2001), it may not be suitable for the assessment of cigarette smoking cessation in the short term, i.e. less than 3 months' duration, since the current literature search provided no substantial evidence of a strong direct link between 8-iso-$PGF_{2\alpha}$ and smoking cessation except after long periods of time (Harman *et al.* 2003, Lowe *et al.* 2009).

Contrary to the current literature search, one earlier publication has shown that urinary 8-iso-$PGF_{2\alpha}$ levels can decrease rapidly following smoking cessation (Morita *et al.* 2005). A small (27 healthy, smoking medical students) study that had no non-smoking controls; another study that compared circulating levels of 8-iso-$PGF_{2\alpha}$ in smokers and non-smokers (242 ± 147 and 103 ± 19 pmol/L, respectively) with levels falling to 156 ± 67 pmol/L after 2 weeks of smoking cessation (Morrow *et al.* 1995) and a very small study (heavy smokers $n = 5$; moderate smokers $n = 5$; non-smokers $n = 14$) that showed 8-iso-$PGF_{2\alpha}$ levels falling within 3 weeks of cessation but failing to reach levels seen in non-smokers (Reilly *et al.* 1996). Normal plasma levels of 8-iso-$PGF_{2\alpha}$ are affected by ethnic background with one study showing that West African immigrants had lower 8-iso-$PGF_{2\alpha}$ levels than US-born African Americans who themselves had lower levels than non-Hispanic white Americans (Annor *et al.* 2017). Similar differences have been reported for urinary levels of 8-iso-$PGF_{2\alpha}$ (Il'yasova *et al.* 2012)

Thromboxane B2 (TXB2)

A potentially important platelet-derived marker is thromboxane A2 (TXA2). TXA2 is generated in platelets from its precursor prostaglandin H2 by the action of the enzyme thromboxane synthase, which in turn is formed by the action of cyclooxygenase-1 on arachidonic acid (Moore 1982). Its importance is attributed to the fact that, amongst other actions, it is a vasoconstrictor, a potent hypertensive agent and it can initiate the release reaction which is followed by platelet aggregation (Gryglewski *et al.* 1978). The literature indicates that increased levels of TXA2 are associated with increased risk of atherogenesis (Dogne *et al.* 2005, Nakahata 2008). TXA2 is highly unstable; thus, its levels are measured using two metabolites related to thromboxane B2 (TXB2) which are 11-dehydro-TXB2 and 2,3-dinor-TXB2 (Catella *et al.* 1986). The literature search indicated that TXB2 may be a valid biomarker; therefore, a second literature search was conducted covering years prior to 2008. In this search, 14 cross-sectional studies were identified that compared levels of one or both of the TXB2 metabolites in smokers and non-smokers, and these are listed in Table 4.

As can be seen from Table 4, all 14 studies showed that one or both TXB2 metabolites were elevated in smokers compared to non-smokers. For 12 of these studies, the results were statistically significant at the 5% level. Study 10 (McAdam *et al.* 2005) did not provide a p value, while the difference reported by study 14 (Prasad *et al.* 2016) was found not to be statistically significant. Of particular interest are the results of study 13 (Frost-Pineda *et al.* 2011), the only large study, where the difference was statistically significant with $p < 0.0001$. Therefore, published evidence would appear to support the view that one or both TXB2 metabolites are elevated in smokers compared to non-smokers.

To assess the potential utility of TXB2 metabolites for the assessment of RRPs, it is also essential that their levels have been found to decline following smoking cessation. Only three studies were found that determined the levels of at least one of the TXB2 metabolites following smoking cessation and the results from these can be seen in Table 5.

Although only three studies were found, they all showed a statistically significant decrease in the two TXB2 metabolites following smoking cessation. These data support that either 2,3-dinor-TXB2 or 11-dehydro-TXB2 is a potentially useful

Table 4. Association of TXB2 metabolites with smoking status.

No.	Study	Sample size	Metabolite	Results
1	Nowak *et al.* (1987)	6 smokers, 6 non-smokers	2.3-dinor-TXB2	Levels higher in smokers than in non-smokers ($p < 0.05$).
2	Barrow *et al.* (1989)	30 smokers, 37 non-smokers	2,3-dinor-TXB2, 11-dehydro-TXB2	Levels of both metabolites higher in smokers than in non-smokers ($p < 0.001$).
3	Wennmalm *et al.* (1991)	73 smokers, 377 non-tobacco users	2,3-dinor-TXB2	Levels higher in smokers than in non-tobacco users ($p < 0.001$).
4	Uedelhoven *et al.* (1991)	13 smokers, 15 non-smokers	2,3-dinor-TXB2, 11-dehydro-TXB2	Levels of both metabolites higher in smokers compared to non-smokers ($p < 0.0001$ and $p < 0.005$, respectively).
5	Rangemark and Wennmalm, (1991)	13 female smokers, 13, female non-smokers	2,3-dinor-TXB2	Levels higher on day 3 ($p < 0.01$), day 10 ($p < 0.05$) and day 20 ($p < 0.05$) of the menstrual cycle in smokers compared to non-smokers.
6	Rangemark *et al.* (1992)	125 smokers, 125 non-smokers	2,3-dinor-TXB2	Levels higher in smokers compared to non-smokers ($p < 0.001$).
7	Dotevall *et al.* (1992)	18 smokers and 17 non-smokers	2,3-dinor-TXB2	Levels higher in smokers than in non-smokers ($p < 0.05$).
8	Rangemark and Wennmalm (1996)	23 smokers and 26 non-smokers	2,3-dinor-TXB2	Levels higher in smokers than in non-smokers ($p < 0.001$).
9	Weber *et al.* (2000)	10 smokers and 10 non-smokers	2,3-dinor-TXB2	Levels higher in smokers than in non-smokers ($p < 0.05$).
10	McAdam *et al.* (2005)	17 smokers and 15 non-smokers	11-dehydro-TXB2	Levels higher in smokers than in non-smokers (p value not given).
11	Ikonomidis *et al.* (2005)	30 smokers, 30 non-smokers all with active CAD	11-dehydro-TXB2	Levels higher in smokers than in non-smokers ($p < 0.01$).
12	Calapai *et al.* (2009)	20 smokers, 20 never smokers	2,3-dinor-TXB2, 11-dehydro-TXB2	Levels of 2.3-dinor-TXB2 higher in smokers compared to never smokers ($p < 0.01$), levels of 11-dehydro-TXB2 higher in smokers ($N = 18$) compared to never smokers ($N = 17$) ($p < 0.05$).
13	Frost-Pineda *et al.* (2011)	3346 smokers, 1051 non-smokers	11-dehydro-TXB2	Levels higher in smokers than in non-smokers ($p < 0.0001$).
14	Prasad *et al.* (2016)	40 smokers, 40 non-smokers	11-dehydro-TXB2	Levels higher in smokers than in non-smokers, but the difference was not statistically significant.

Table 5. Changes in levels of TXB2 metabolites following smoking cessation.

No	Study	Sample size	Study type	Study period	Metabolite	Results
1	Wennmalm *et al.* (1993)	43 smokers	Prospective	31–44 months	2,3-dinor-TXB2	Levels were significantly lower in individuals who stopped smoking ($N = 9$) compared to continuing smokers ($p < 0.002$).
2	Rangemark *et al.* (1993)	8 smokers	RCT [a]	3 days	2,3-dinor-TXB2, 11-dehydro-TXB2	Both metabolites were significantly lower at day 3 following smoking cessation (2,3-dinor-TXB2, $p < 0.02$; 11-dehydro-TXB2, $p = 0.02$).
3	Saareks *et al.* (2001)	60 smokers	RCT [a]	14 days	11-dehydro-TXB2	Levels had decreased to the levels measured in non-smokers ($p < 0.0001$).

[a]RCT: Randomized controlled trial.

biomarker for the evaluation of reduced risk products. This conclusion is substantially reinforced by two studies where 11-dehydro-TXB2 was used to evaluate such a product. One study (Roethig *et al.* 2008) evaluated the change in 11-dehydroxy-TXB2 levels in a 12-month randomized controlled trial comparing smokers who switched to a second-generation electrically heated cigarette smoking system ($N = 50$) with those who continued smoking conventional cigarettes ($N = 32$). There was a 20% decrease for those subjects who switched compared to a slight increase for those subjects who continued to smoke conventional cigarettes ($p = 0.003$). Martin Leroy *et al.* (2012) reported the results of a similar randomized controlled trial where 237 smokers were switched to an electrically heated cigarette smoking system and 79 subjects continued to smoke their own brand. In this 1-month study, there was a decrease of 10.2% in the median level of 11-dehydro-TXB2 in subjects who switched compared to a 2.9% decrease in those subjects who continued to smoke ($p < 0.001$).

The last requirement to qualify TXB2 metabolites as useful biomarkers in studies examining reduced risk products is that they are associated with at least one smoking-related disease. There is clear evidence that this is the case for CVD, since TXB2 metabolites are markers of platelet activation and aggregation (Sharma and Berger 2011). On the other hand, there is no evidence that TXB2 metabolites would be a useful marker for smoking-related respiratory diseases.

Biomarkers of inflammation

The 2014 U.S. Surgeon General's Report "The Health Consequences of Smoking: 50 Years of Progress" (US

Department of Health and Human Services 2014) identifies inflammation (and oxidative stress) among others, as key mechanisms underlying all major smoking-related diseases, and there is ample evidence showing that both mechanisms are intricately linked to each other and to disease development in smokers. Cigarette smoking is causally linked to the development of CVD contributing to endothelial injury and dysfunction, a pro-atherogenic lipid profile, chronic inflammation and an abnormally increased tendency toward coagulation. COPD is characterized by an abnormal inflammatory response and is linked to a number of sustained cellular stress responses including endoplasmic reticulum, hypoxic stress and autophagic stress (US Department of Health and Human Services 2014). Furthermore, most of the inflammatory processes identified in smokers with COPD can also be observed in smokers with lung cancer (Yanbaeva *et al.* 2007, Faux *et al.* 2009, Mons *et al.* 2016), suggesting a shared aetiology. Given the importance of inflammation in the development of smoking-related diseases (Hamilton and Rath 2015, Bozinovski *et al.* 2016, Zaynagetdinov *et al.* 2016) several inflammation-related biomarkers have been proposed.

C-reactive protein (CRP)

C-reactive protein (CRP) increases in response to a wide range of acute and chronic inflammatory conditions such as bacterial, viral or fungal infections as well as rheumatic and other inflammatory diseases, malignancies and tissue injury and necrosis. The literature searches demonstrated that CRP is a well-established biomarker of inflammation, suitable for both clinical purposes and epidemiological studies. The association between CRP and the risk for CVD has been documented in many large, prospective epidemiological studies, although some meta-analyses appear to de-emphasize the significance of CRP as a prognostic marker (Adukauskiene *et al.* 2016). Furthermore, CRP has also been reported to be predictive for respiratory diseases (Danesh *et al.* 2004, Pepys 2005, Lowe and Pepys 2006) and various types of cancer, particularly of lung cancer (Agarwal *et al.* 2013, Firouzjahi *et al.* 2013, Deng *et al.* 2014, Kawada 2015). Numerous studies have produced a large variation in what normal levels of CRP should be (Zhang *et al.* 2016, Zhu *et al.* 2016). CRP levels in smokers have been shown in one study to be 2.53 mg/L compared to 1.35 mg/L in non-smokers ($p < 0.0001$) (Tonstad and Cowan 2009) with levels falling within 5 years of smoking cessation but taking over 20 years to revert to levels of never smokers. Similar results were found in another study with never smokers having CRP levels of 1.13 mg/L while even light smokers had 1.87 mg/L ($p < 0.001$) (Wannamethee *et al.* 2005). At present, there is consensus that CRP is an indicator for acute and chronic inflammation processes rather than being directly involved in the pathophysiological processes of CVD, COPD or cancer. A major limitation of the suitability of CRP as a biomarker of effect for the evaluation of reduced risk tobacco products would be the very long period of time required to achieve non-smoker levels after switching (Lowe *et al.* 2001). CRP levels increase with increasing age (Wyczalkowska-Tomasik *et al.* 2016) and levels differ between male and female subjects (Woloshin and Schwartz 2005, Siemons *et al.* 2014). Ethnic background also affects this biomarker with percentage of subjects with levels >3 mg/L being 31, 40, 51 and 58% in white men, black men, white women and black women, respectively (Khera *et al.* 2005).

White blood cell count (WBC)

White blood cell count (WBC) is elevated in cigarette smokers (5–30% higher than non-smokers) (Asthana *et al.* 2010, Shiels *et al.* 2014, Kryfti *et al.* 2015) and has been proposed as a biomarker of cigarette smoke-related cardiovascular risk (Fernandez *et al.* 2012). Elevated peripheral WBC levels are also an important determinant of the level of pulmonary function (Korhonen *et al.* 2011). The literature provides confirmation that a fall in WBC count occurs rapidly following smoking cessation with one study indicating that a significant decrease in WBC count occurred after only 3 days of smoking cessation [from 8.73×10^9/L to 8.15×10^9/L ($p = 0.0001$) (Sparrow *et al.* 1984)]. In one cross-sectional study, the WBC count fell from 6.03×10^9/L to 5.64×10^9/L ($p = 0.00009$) within 1 year of smoking cessation whereas continuing smokers had WBC counts of 6.09×10^9/L (Roethig *et al.* 2010). The literature searches confirm that WBC count can be used as a fully validated biomarker of inflammation and thus diseases associated with it. There is a very large data set [e.g. (Higuchi *et al.* 2016) and a meta-analysis accessible on the authors web site (Holt 1987, Yanbaeva *et al.* 2007)] that establishes that cigarette smoking significantly increases WBC counts, and there is also a very large data set (Lee *et al.* 2014) showing that there is a strong association between the level of WBC count and various CVD endpoints, particularly coronary heart disease (CHD). There is also a reasonable amount of literature confirming an association of WBC count with the other two major smoking-related diseases – lung cancer (Danesh *et al.* 1998, Pearson *et al.* 2003, Madjid *et al.* 2004) and COPD (Allin *et al.* 2016). WBC numbers are generally higher in males than females (Ruggiero *et al.* 2007, Nilsson *et al.* 2009) and there are slight decreases in the elderly (>65 years). The NHANES study found that there were differences in the mean WBC count between racial groups, between sexes and among age groups within race-sex groups in adults between the ages of 25–74 years (McGrath *et al.* 1982). Although some recent studies find no effect of age on WBC (Kverneland *et al.* 2016) many support the differences seen in different races (Zezulka *et al.* 1987, Nalls *et al.* 2008, Lim *et al.* 2010).

Soluble intercellular adhesion molecule (sICAM-1)

Intercellular Adhesion Molecule (ICAM)-1 is a glycoprotein constitutively expressed on the surface of endothelial cells and leukocytes. ICAM-1 expression can be induced by pro-inflammatory cytokines, which are also thought to signal its shedding from the membrane so that it can be detected as soluble ICAM-1 (sICAM-1) in serum or plasma. Smokers have significantly higher plasma sICAM-1 levels (145.1 ng/mL) than non-smokers (110.3 ng/mL) ($p < 0.0001$), which was associated with endothelial dysfunction and clinical CVD (Nordskog *et al.* 2015). Studies have shown that ICAM-1 is

overexpressed in the airway epithelium of subjects with COPD (Gross *et al.* 2012) and that release of sICAM-1 by bronchial epithelial cells following smoke exposure is significantly elevated in COPD (Chan *et al.* 2008, Rusznak *et al.* 2000). sICAM-1 levels have been shown to decline within 4–6 weeks following cessation of cigarette smoking (Aaron *et al.* 2015). Although care needs to be taken when comparing results, due to the fact that the most frequently employed immunoassays depend on antibodies incapable of recognizing all population variants of the adhesion molecule (Halvorsen *et al.* 2007, Tsai *et al.* 2012), sICAM-1 is considered to be a useful and reliable clinical risk endpoint in the assessment of vascular inflammation (Abdi *et al.* 2014). As a consequence, it should also be valid for evaluating a decrease in CVD risk associated with the use of RRPs. There are differences in sICAM levels in subjects of different ethnic backgrounds with levels being lower in individuals of African origin compared to whites (Hwang *et al.* 1997, Lutsey *et al.* 2006, Miller and Cappuccio 2007). Although plasma ICAM levels fall in children between the ages of 9 and 16 years (Nash *et al.* 1996), recent work suggests that there are no age-related differences between 23 and 74 years of age (Deneva-Koycheva *et al.* 2011, Bottino *et al.* 2015) which contradicts earlier evidence in which a linear relationship between the plasma concentration of sICAM and age were found in a comparison between subjects <40 ($n = 52$) and subjects >55 ($n = 38$) years of age (Miles *et al.* 2001). There is evidence that higher levels of sICAM in older subjects is significantly associated with frailty (Lee *et al.* 2016) and whilst there is no difference between healthy males and females (Deneva-Koycheva *et al.* 2011), sICAM levels are lower in pre-menopausal women than in post-menopausal women (Nyberg *et al.* 2014).

Fibrinogen (FBG)

Fibrinogen (FBG) is an acute phase protein produced in the liver that increases quickly and markedly in the circulation in response to vascular injury, infection and inflammation. FBG plays an essential role in coagulation, mediating fibrin assembly and platelet recruitment to form a platelet plug (thus limiting bleeding and promoting wound healing). Serum FBG levels show a positive association with age (Drenos *et al.* 2007, Wang *et al.* 2017) but there are no differences between genders (Wang *et al.* 2017). Ethnic differences have been reported with South Asians having similar levels to Caucasians and Chinese which are higher than those seen in Japanese subjects (Ishikawa *et al.* 1997, Gijsberts *et al.* 2015). South African black people were found to have higher levels of FBG than white South Africans (Lammertyn *et al.* 2015). FBG has been shown to be involved in major smoking-related diseases, such as CVD (Witkowska 2005), respiratory diseases (Koenig 2003, Marano *et al.* 2015) and cancer (Shibata *et al.* 2013, Lock-Johansson *et al.* 2014). A limitation of FBG as a biomarker of effect for smoking-related diseases is, however, that not all studies have found significant differences in FBG levels between smokers and non-smokers. One study that did find a significant relationship was published in 2003 and reported an odds ratio of 2.29 (95% CI, 1.69–3.09) for clinically elevated FBG in smokers of $\geq$24 cigarettes/day compared to non-smokers (Everett *et al.* 2007). On the other hand, a more recent, albeit smaller study, found no significant difference ($p = 0.09$) (Bazzano *et al.* 2003). In another study, there was no reduction in FBG levels in subjects who refrained from smoking for 1 year (King *et al.* 2017) One study found no significant difference between smokers who continued to smoke conventional cigarettes compared to those who switched to an RRP ($p = 0.88$) (Nordskog *et al.* 2015). It is important to note that many studies have shown that the functionality of FBG as an acute phase protein in response to inflammatory conditions is better reflected in changes in CRP than in corresponding changes of FBG levels (Al Rifai *et al.* 2017). Although some studies have shown an association between increased levels of FBG, CRP and a variety of other inflammatory cytokines in COPD (Martin Leroy *et al.* 2012), the usefulness of these measurements as biomarkers in clinical and investigational settings will rely on further refinement to understand whether they are associated with specific aspects of disease activity (Tran and Kalhan 2008).

Tumour necrosis factor-α (TNF-α)

Tumour necrosis factor-α (TNF-α) is an important cytokine involved in inflammatory processes, cell survival, growth and differentiation as well as apoptosis and anti-apoptosis (Rosenberg and Kalhan, 2012). Since inflammation plays a crucial role in CVD, particularly coronary artery disease (CAD), TNF-α has been intensively investigated as a biomarker. It is persistently elevated in post-myocardial infarction (MI) patients and is an independent risk factor for secondary coronary events in these patients, independent of other known risk factors (Bellisarii *et al.* 2001, Barnes 2009, Zelova and Hosek 2013). Plasma TNF-α concentration is associated with the degree of early atherosclerosis and correlates with metabolic and cellular perturbations that are considered to be important for the atherosclerotic process (Ridker *et al.* 2000). TNF-α also plays an important role in orchestrating inflammatory airway diseases, and there is an association between COPD and TNF-α (Skoog *et al.* 2002). In one study, serum TNF-α concentrations were found to be significantly elevated in COPD patients (6.97 pg/mL) compared to controls (5.3 pg/mL) (Wu *et al.* 2015) and another reported that smokers who smoked more than one pack of cigarettes/day had higher levels of serum TNF-α than those smoking less than one pack/day (Petrescu *et al.* 2010). However, in one study, serum TNF-α was lower in smokers with COPD (Higashimoto *et al.* 2008) while another reported lower levels of TNF-α in EBC from healthy smokers compared to levels in subjects with COPD (Maskey-Warzechowska *et al.* 2017). TNF-α has been measured in lung-derived matrices such as sputum (Ji *et al.* 2014), bronchoalveolar lavage fluid (BALF) and exhaled breath condensate (EBC) (Keatings *et al.* 1996). A recent study measured increased levels of TNF-α in the sputum of smokers although there were no differences in serum levels of TNF-α between the two groups (Kleniewska *et al.* 2016). TNF-α has also been studied in EBC (Garey *et al.* 2004, Gessner *et al.* 2005, Kuban and Foret 2013) and serum (Carpagnano *et al.*

2007, Dalaveris *et al.* 2009) from patients with lung cancer. Because smoking has inflammatory effects and is an established risk factor for inflammation-related diseases such as COPD, CVD and certain types of cancer, it is reasonable to assume that smoking should be correlated with increased systemic and also respiratory tract-related TNF-α levels. The information retrieved from the literature searches appear to confirm this assumption in general. The available evidence is limited, however, and the effect of smoking on increasing TNF-α levels is weak [although it has been shown that soluble TNF-α levels are significantly ($p = 0.05$) higher in acute exacerbations of COPD than in stable COPD (Tan *et al.* 2017)]. Particularly, there is only very weak and partly contradictory evidence for a dose-response relationship between TNF-α and smoking. Thus, it may be concluded that TNF-α, although mechanistically involved in the pathogenesis of inflammation-related diseases such as COPD, CVD and cancer, demonstrates no convincing evidence that it will be a suitable cytokine for measuring effects of smoking cessation or switching to a potentially less harmful product. Serum concentrations of TNF-α do not appear to change with increasing age (Kim *et al.* 2011, Kleiner *et al.* 2013, Wyczalkowska-Tomasik *et al.* 2016) except in subjects with type 2 diabetes where increases are seen over time, especially in females above the age of 41 years (Fatima *et al.* 2017). TNF-α levels are also not affected by gender (Lee *et al.* 1993, Arican *et al.* 2005) except in Chinese subjects in which levels are higher in females than males in the age range 20–45 years (Yuan *et al.* 2015). No differences were found between blacks and whites in a study of 508 males and females over 35 years of age (Paalani *et al.* 2011).

Myeloperoxidase (MPO)

Myeloperoxidase (MPO) is a pro-oxidant haeme-enzyme that is stored in and released from neutrophilic granulocytes which are an essential part of the innate (non-specific) immune system in humans (Derin *et al.* 2008). It also metabolizes tobacco smoke procarcinogens such as benzo(a)pyrene and aromatic amines into highly reactive intermediates (Chevrier *et al.* 2003). In a number of studies, a significant association between MPO activity and CVD was observed (Nussbaum *et al.* 2013), and circulating MPO may play a role as a diagnostic and a prognostic tool for CVD patients (Rudolph *et al.* 2008, Ikitimur and Karadag 2010, Anatoliotakis *et al.* 2013). It can be involved in the development of CVD, in particular atherosclerosis, by several mechanisms, including oxidation of low-density lipoprotein (LDL) to ox-LDL (Nicholls and Hazen 2005, Schindhelm *et al.* 2009, Koeth *et al.* 2013), plaque destabilization (Delporte *et al.* 2013), overproduction of MPO at the site of arterial inflammation and impairment of nitric oxide (NO)-induced vascular relaxation (Niccoli *et al.* 2010). Overall, the literature search revealed that the role of MPO as a risk and clinical marker for CVD (Meuwese *et al.* 2007, Baldus *et al.* 2003, Brennan *et al.* 2003, Thukkani *et al.* 2003), respiratory diseases (COPD and emphysema (Sapey *et al.* 2008, Stone *et al.* 2012) and various types of cancer (Hoy *et al.* 2002) is well established. However, from the 156 publications retrieved in the three searches, there was limited evidence that smoking leads to a dose-dependent increase in MPO levels (measured as amount or activity of the enzyme) or a decrease upon long-term smoking cessation (Andelid *et al.* 2007, Rudolph *et al.* 2012). One study found no increases in MPO in smokers compared to never smokers (Levitzky *et al.* 2008). Overall, the information available does not provide strong evidence for the suitability of the measurement of the amount or activity of MPO as a biomarker of effect in smoking studies. There is little evidence to suggest that there are age or gender-related differences in plasma MPO levels in healthy subjects (Meuwese *et al.* 2007, Sbarouni *et al.* 2011, Guven *et al.* 2013) and there is also no difference between American Africans and Caucasians (Kubala *et al.* 2008). Serum MPO levels do not change with increasing age except in subjects with type 2 diabetes (Shankar *et al.* 2012).

Exhaled nitric oxide (FeNO)

The fraction of exhaled nitric oxide (FeNO) is a promising biomarker for the diagnosis, follow-up and guide to therapy in patients with asthma (Malinovschi *et al.* 2012, Godinho Netto *et al.* 2016) and other respiratory diseases. It can also be used to measure initial physiological changes in the upper and lower airways. The measurement of FeNO is of limited relevance for CVD, although smoking decreases NO generation in endothelial cells (Messner and Bernhard 2014) which leads to a reduced endothelial-dependent vasodilatation (EDV) (Barua *et al.* 2001). Moreover, NO insufficiency leads to thrombosis, because endothelial NO limits platelet activation, adhesion and aggregation (Barua *et al.* 2001). FeNO is decreased in heart diseases such as congestive heart failure (Sumino *et al.* 1998, Hare *et al.* 2002). The literature search revealed that there is considerable variation in the findings when FeNO is measured in subjects with COPD with one early publication stating that smokers had lower FeNO than non-smokers [an observation also reported recently (Xu *et al.* 2016)] and that after 8 weeks of smoking cessation, levels in the former smokers rose to those seen in non-smokers although the number of subjects was very small (Loscalzo 2001). Generally, FeNO levels are near normal in COPD except during exacerbations (Robbins *et al.* 1997) and have so far not been shown to be predictive of important health outcomes in COPD (Verleden *et al.* 1999, Kharitonov and Barnes 2002, Kharitonov and Barnes 2006) with the exception of a recent study that showed that completely abstaining from smoking for 52 weeks led to near normalization of FeNO (and a concomitant drop in exhaled carbon monoxide) (Campagna *et al.* 2016). While some interest has been focussed on measuring alveolar FeNO (Yoon and Sin 2011), the levels are not associated with disease severity or smoking status (Brindicci *et al.* 2005). A recent study has concluded that reference values for FeNO among respiratory healthy non-smokers should be outlined stratified for gender using individual reference values and a separate cut-off limit for current smokers (Toren *et al.* 2017). More work will be required before the value of FeNO measurement in COPD is fully understood. Overall, therefore, it can be concluded that currently FeNO appears not to be a suitable

biomarker for evaluating acute (Lappas *et al.* 2016) effects of smoking and cessation/switching.

Sputum neutrophils

Although the literature searches found no published papers on the associations between CVD/heart disease or cancer and sputum neutrophil numbers, several studies are available that show that numbers of sputum neutrophils are associated with respiratory diseases such as COPD (Brindicci *et al.* 2005), asthma (Tsoumakidou *et al.* 2003, Singh *et al.* 2010, Zuiker *et al.* 2015) and chronic fibrosis (Chaudhuri *et al.* 2014). Most studies report an increase in the number of sputum neutrophils, as would be expected as a consequence of airway exposure to tobacco smoke, in samples from smokers compared to non-smoking controls. This is not always the case, however, and other studies have reported no differences (Conese *et al.* 2014) or even a decrease (Swan *et al.* 1991, Zuiker *et al.* 2015). Likewise, some studies have found no change or an increase in the number of sputum neutrophils following smoking cessation [e.g. from 60.1% before smoking cessation to 80.1% 1 year later (Lensmar *et al.* 1998)]. In terms of determining inflammatory biomarkers, sputum is almost as suitable as BALF or lung biopsies (Willemse *et al.* 2005), and its collection is minimally invasive and relatively safe. Although the majority of the evidence suggests that sputum neutrophil numbers are elevated in smokers, this association has been investigated only in relatively small studies. The available evidence for a dose–response relationship is limited and inconsistent. Smoking cessation is not found to steadily decrease the sputum neutrophil levels. To the contrary, levels were found to remain elevated or even to increase after smoking cessation and to remain so even after several months or years (Keatings *et al.* 1996), suggesting that smoking-related airway inflammation persists for a considerable time period. Taken together, the information would suggest that even though neutrophils are mechanistically involved in the pathogenesis of inflammation-related airways diseases they are not a suitable biomarker of effect in smoking and smoking cessation studies. Age has been reported to have either no effect on sputum neutrophil numbers from healthy subjects (Belda *et al.* 2000, Spanevello *et al.* 2000, Busse *et al.* 2017) or to cause an increase as subjects get older (>50 years) (Thomas *et al.* 2004, Pignatti *et al.* 2011, Carpagnano *et al.* 2013, Simpson *et al.* 2013), with one showing a greater increase in females than males (Pignatti *et al.* 2011).

Lipoprotein-associated phospholipase A2 (Lp-PLA2)

Lipoprotein-associated phospholipase A2 (Lp-PLA2), also known as platelet-activating factor acetylhydrolase (PAF-AH), is a vascular-specific inflammatory enzyme, predominantly expressed by macrophages, lymphocytes and foam cells in atherosclerotic plaques. Over 80% of circulating Lp-PLA2 is associated with atherogenic LDL-cholesterol, while about 10% is associated with high-density lipoprotein-cholesterol (HDL-C) (Willemse *et al.* 2005). Circulating Lp-PLA2 is constitutively active and is upregulated by mediators of inflammation at the transcriptional level as a physiological response to inflammatory stimuli (Tsimihodimos *et al.* 2002, Caslake and Packard 2003, Racherla and Arora 2012, Vittos *et al.* 2012). A number of epidemiological studies have demonstrated that elevated levels of Lp-PLA2 are associated with increased risk of cardiovascular events independent of established risk factors (Stafforini 2009). Plasma Lp-PLA2 was shown to be independently associated with coronary heart disease (CHD) in a cross-section study on 531 Chinese subjects (Yang *et al.* 2017) and a meta-analysis of 15 studies with 30,857 subjects showed that Lp-PLA2 was independently associated with cardiovascular events, particularly in subjects with stable CHD not receiving Lp-PLA2 inhibitor therapy (Li *et al.* 2017). Plasma levels of Lp-PLA2 are also associated with the severity of CHD (Ge *et al.* 2016). Lp-PLA2 is not widely used as biomarker in clinical or epidemiological studies on respiratory diseases, although increased levels have been reported in BALF from patients with acute respiratory distress syndrome (Nakos *et al.* 2004, 2005). In one study, smoking cessation lowered the plasma Lp-PLA2 to levels similar to those seen in never smokers (Persson *et al.* 2007). Differences between smokers and non-smokers were investigated in a small number of studies, which in general showed significant differences between smokers and non-smokers (e.g. 256 and 191 ng/mL, respectively; $p < 0.001$; Persson *et al.* 2007). Moreover, one study has shown a significant dose–response relationship between smoking intensity and Lp-PLA2 blood levels (Tselepis *et al.* 2009). It is highly likely that the link between smoking and the elevation of Lp-PLA2 levels is linked to the inflammatory and oxidative stress processes caused by smoking (Tselepis *et al.* 2009). The searches revealed no systematic studies showing that smoking cessation leads to a decrease in Lp-PLA2 levels and no published data are available about the effect on circulating Lp-PLA2 levels following switching from conventional cigarettes to an RRP or other nicotine product. For this reason, Lp-PLA2 is not likely to be a suitable biomarker for use in smoking/cessation/switching studies until more data becomes available. It has been shown that Lp-PLA2 activity was lower in women compared with men and was lowest in black, intermediate in Hispanic and highest in white subjects (Brilakis *et al.* 2008).

CVD Biomarkers

Albumin (ALB). Albuminuria is not only a predictor of declining renal function but also is independently associated with adverse cardiovascular outcomes (Imaizumi *et al.* 1990). How smoking might actually damage the kidney and lead to (micro)albuminuria is not yet fully elucidated, although a number of potential mechanisms for the involvement of smoking in the renal pathophysiological processes have been discussed, including smoking-related increases in blood pressure leading to alterations in kidney haemodynamics (Stephen *et al.* 2014); smoking-related atherogenic effects (Metcalf *et al.* 1993, Orth 2002, Hogan *et al.* 2007); effects of chronic smoking on endothelial dysfunction leading to a faster decline in renal function (Hogan *et al.* 2007); carboxyhaemoglobin-induced renal hypoxia (Orth 2002); elevated

levels of nephrotoxic cadmium (Metcalf *et al.* 1993); smoke-induced formation of advanced glycation end products (AGE) that increase vascular permeability (Hogan *et al.* 2007); smoking-induced insulin resistance (Pinto-Sietsma *et al.* 2000) and smoke-induced disturbances in glycaemic control leading to increased glomerular permeability (Pinto-Sietsma *et al.* 2000). The published literature shows that there is some evidence that smoking leads to increased urinary albumin (ALB) levels [e.g. 4.6 mg/L in non-smokers and 5.4 mg/L in smokers ($p < 0.001$) (Metcalf *et al.* 1993, Al Rifai *et al.* 2017); 62 mg/24 h in non-smokers and 100.4 mg/24 h in smokers ($p < 0.01$) (Metcalf *et al.* 1993)]. Most of the studies available, however, were conducted with diabetic subjects. Effects of smoking on ALB excretion appears to be higher in patients with diabetes or renal damage or dysfunction than in healthy subjects. Nevertheless, in almost all studies that investigated the dose–response relationship between ALB levels and the smoking dose, a significant association was found (Corradi *et al.* 1993). Former smokers, who stopped smoking at least 1 year before, were reported to have ALB levels between current and never smokers (Metcalf *et al.* 1993, Ikeda *et al.* 1997, Pinto-Sietsma *et al.* 2000, Hogan *et al.* 2007). However, in no case have acute effects (observed within days or weeks) of smoking cessation on ALB excretion been reported in the literature.

As noted above, a major limitation in the evaluation of urinary ALB as a biomarker of effect for smoking-related diseases is the fact that most of the studies have been performed with diabetic subjects. More data for healthy subjects (whether smokers, non-smokers or ex-smokers) are required. Therefore, taken together, the information available does not strongly suggest the use of urinary ALB as a biomarker of effect for studies on smoking or smoking cessation, although this conclusion could change if and when more data become available for ALB excretion in healthy subjects as well as additional results after smoking cessation. Some studies have reported decreases in serum ALB concentrations with increasing age (Greenblatt 1979, Cooper and Gardner 1989, Veering *et al.* 1990, Salive *et al.* 1992) although the decreases could be attributed to poorer nutritional status, inflammation or external loss (Kaysen and Levin 2002). Others have reported that hypoalbuminaemia is not a consequence of normal aging (Campion *et al.* 1988) and that age does not change the rate of synthesis or concentrations of ALB (Fu and Nair 1998). Ethnicity affects ALB levels in healthy subjects with differences between males and females (Tembe *et al.* 2014, Lim *et al.* 2015).

Lipids

Lipids are essential for a number of cellular processes including synthesis of cellular membranes, formation of lipoprotein particles (complex particles composed of multiple proteins), signal transduction and energy storage among others and the tight regulation of their metabolism contributes to the maintenance of cellular homeostasis. LDL is one of the five major groups of lipoprotein which are, from least dense to most dense, chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), LDL and HDL. All of these lipoproteins can carry cholesterol, and the vast majority of studies indirectly measure their levels by determining the level of bound cholesterol (e.g. LDL-C, HDL-C). Because of the association between LDL-C and CVD, in particular atherosclerotic CVD, it is often referred to as "bad cholesterol", as it can transport lipid molecules into artery walls, attract macrophages, and thus drive atherosclerosis (Metcalf *et al.* 1993, Freedman *et al.* 2005, Voulgari *et al.* 2011). A possible mechanism by which smoking might increase LDL-C is by increasing the level of free fatty acids (FFA) in blood, which in turn stimulates the hepatic synthesis and excretion of cholesterol. It has also been suggested that smoking may alter the expression of genes influencing the LDL particle diameter and thus increase the atherogenic potency of LDL-C (Otokozawa *et al.* 2010, Ference and Mahajan 2013, Imes and Austin 2013). The literature searches revealed that in general but not always smoking increased LDL-C levels, but there is conflicting evidence with respect to the effects of smoking cessation with some studies reporting lowering of levels (Hozawa *et al.* 2006) and others reporting no change (Allen *et al.* 1994, Ludviksdottir *et al.* 1999). Overall, the information available does not provide strong evidence for the suitability of LDL-C as a biomarker of effect in cessation or switching studies.

HDL has been suggested to fulfil a number of biological functions, the most important of which is reverse cholesterol transfer (RCT) which involves removal of cholesterol from the plasma, macrophages and other cells for transfer (primarily) to the liver and eventual removed from the organism. The fact that cholesterol bound to HDL (HDL-C) is in the process of being removed from the organism is why HDL-C has been universally regarded as "good cholesterol." Other functions hypothesized for HDL are: anti-oxidative activity, anti-inflammatory activity, anti-apoptotic activity, vasodilatory effects and inhibition of platelet aggregation (Freeman *et al.* 1993, Hatsukami *et al.* 2005, Hozawa *et al.* 2006). Whilst the association of HDL-C with various CVD endpoints has been well-known for many years its association with respiratory disease such as COPD is inconsistent with some reports showing association (Kontush and Chapman 2006) and others showing no association (Cirillo *et al.* 2002). A recent study found that adults with low HDL-C (and high triglyceride), particularly if they had diabetes, were at increased risk of incident CHD and stroke (Lee *et al.* 2017). Although a limited number of studies were retrieved when searching for HDL and cancer, all the studies identified showed a decreased level of HDL-C in lung cancer compared to some type of control or an association of low levels of HDL-C with the risk of lung cancer (Garcia-Larsen *et al.* 2014, Sato *et al.* 2014, Wei *et al.* 2014). There was overwhelming evidence that following smoking cessation, HDL increased and could revert to normal levels within 4–8 weeks (Kucharska-Newton *et al.* 2008, Ahn *et al.* 2009). In one study, 12 months of smoking cessation increased HDL-C from 42.4 to 44.7 mg/dL ($p < 0.001$) (Forey *et al.* 2013) and another reported a 9.3% increase in HDL-C one year after smoking cessation (Mandraffino *et al.* 2017). Two studies were found that determined the comparison of levels of HDL-C in subjects who switched to a second-generation electrically heated cigarette smoking system. Roethig

et al. (2008) reported an increase of 5 mg/dL for HDL-C after 12 months in smokers who switched, compared to an increase of only 1 mg/dL for those who continued to smoke conventional cigarettes ($p = 0.008$). In a similar study, Martin Leroy *et al.* (2012) reported an increase in HDL-C after 1 month following switching from 59.0 to 63.9 mg/dL ($p < 0.0001$). Despite the widespread use of the decrease of HDL-C as a marker for the increase in CVD and vice versa, there is an issue with HDL-C as a biomarker. Recent studies have shown that levels of HDL-C are not causally related to CVD (Hovingh *et al.* 2015, Pownall and Gotto 2016). During the past 5 years, several inhibitors of cholesteryl ester transport protein (CETP) have been evaluated as a possible approach to lower the risk of CVD. CETP is known to transfer cholesterol from HDL to VLDL, thus inhibition of CETP would be expected to increase the amount of cholesterol bound to HDL and, in turn, the level of HDL-C. Three potential CETP inhibitors have now been evaluated in phase 3 trials. As anticipated, in all three trials levels of HDL-C were indeed significantly elevated. However, none of the three compounds showed any effect with respect to reducing the risk of CVD (Voight *et al.* 2012). The most likely explanation is that while the amount of cholesterol bound to HDL increased, the actual amount of HDL did not. In conclusion, therefore, the published data would qualify HDL-C as a valid biomarker for use in smoking/cessation/switching studies. A Finnish study reported that HDL-C levels increased with advancing age with a more pronounced effect in females but with no ethnic differences (Thelle *et al.* 1982) and more recent studies have shown differences in HDL-C in American Africans, Latinos and whites (Bauman *et al.* 1998) and between Hispanics, non-Hispanic whites and American Mexicans (Chang *et al.* 2011). It has also been suggested that aging alters HDL composition which may lead to functional impairment or the onset or progression of CVD (Holzer *et al.* 2013).

Apolipoprotein A1 (ApoA1)

ApoA1 is the major HDL apoprotein which belongs to the group of reverse cholesterol transport proteins having anti-atherogenic properties (Schwartz *et al.* 2012, Shinkai 2012, McLain *et al.* 2016) as well as exerting innate protective effects in systemic inflammation (Tall 1998) and having anti-oxidant properties through its thiol groups (Sharifov *et al.* 2013). Thus, as would be expected, ApoA1 is inversely associated with the risk of CVD. The role of ApoA1 in preventing CVD has been underlined by observations that autoimmunity to the protein is associated with an increased risk of CVD (Szadkowski and Myers 2008). Importantly, ApoA1-mimetics have seen therapeutic success for treating CVD (Vuilleumier *et al.* 2004, Teixeira *et al.* 2012, Vuilleumier *et al.* 2014). Smoking (amongst other factors) is correlated with an unfavourable apolipoprotein profile (Frondelius *et al.* 2017). There are a number of mechanisms by which smoking decreases the degree of functionally of intact ApoA1. Smoking promotes the MPO-mediated nitration and chlorination of tyrosine-192 in ApoA1 (Hovingh *et al.* 2010, Smith 2010, Imaizumi *et al.* 2011, Gordon and Davidson 2012, Leman *et al.* 2014) and also enhances the reaction of lipid peroxidation products with lysine-226 in ApoA1 (Hadfield *et al.* 2013). Acrolein and other oxidants and toxicants in smoke can cause oxidation of the thiol groups in ApoA1 (Zheng *et al.* 2004, Shao *et al.* 2005, Shao 2012). The literature search did not identify many studies comparing ApoA1 levels in smokers and non-smokers [one was identified in which quantification was by LC-MC and found reduced levels in smokers compared to non-smokers (Wang *et al.* 2015)], but a number of older studies have found significant differences (Szadkowski and Myers 2008). The literature search did find a small study that investigated the effect of smoking cessation on ApoA1 levels. Quitting smoking for 12 weeks significantly increased the ApoA1 levels in smokers from 151.7 to 158.6 mg/dL ($p < 0.01$) (Dedonder-Decoopman *et al.* 1980, Gnasso *et al.* 1984, Haffner *et al.* 1985). Overall, the data on differences between smokers and non-smokers, dose–response relationships and smoking cessation suggest that the inverse association of the serum or plasma ApoA1 levels with smoking is plausible. The number of relevant studies is limited, however, and to date no study has investigated the use of ApoA1 with respect to switching from a conventional cigarette to an RRP. Therefore, until further data are available, it is reasonable to continue to use HDL-C as the preferred lipid-associated biomarker of effect. Plasma levels of ApoA1 were not affected by age or gender in a Finnish study (Sirnio *et al.* 2017) but were found to decrease by 1.21 μg/mL per decade between the ages of 56–105 years in an Australian study (Muenchhoff *et al.* 2017). In a study in the Chinese Han population, ApoA1 levels were reported to increase between the ages of 18 and 50 years (Lu *et al.* 2012).

Oxysterols (OxS)

Oxidized cholesterol species (oxysterols, OxS) play important roles in many biological processes, including cholesterol homeostasis (Iwaoka *et al.* 2014). Compared to other biomarkers of effect linked to CVD and related diseases, the evidence for a predictive power of OxS is rather limited. In particular, oxysterols do not appear to have any advantages over other biomarkers for oxidative stress, inflammation or risk of atherogenic plaque formation. Nevertheless, there is growing evidence in the literature for the involvement of OxS in the pathogenesis of atherosclerosis (Bjorkhem 2002, Bjorkhem *et al.* 2002, Olsen *et al.* 2012). One report was retrieved from the literature that showed an association between respiratory disease and blood levels of 25-hydroxycholesterol in humans (Sugiura *et al.* 2012) but little published information is available for the association between smoking and OxS blood levels. The most probable mechanism by which smoking could elevate OxS levels is by oxidative stress and stimulation of inflammatory processes. Although mechanistically involved in the pathogenesis of many age-related diseases (Leoni *et al.* 2013), there is no convincing evidence that OxS would be suitable biomarkers of risk for these diseases in general or for use in smoking cessation or switching studies designed to provide information about the risk profile of alternative reduced risk tobacco products. Multiple sterols have been shown to vary

significantly by sex, age and ethnicity (Diczfalusy *et al.* 2008, Stiles *et al.* 2014).

Homocysteine (HCY)

Homocysteine (HCY) is a sulfhydryl-containing non-protein amino acid which has an additional methylene bridge compared to cysteine. It is biosynthesized from the amino acid methionine to which it can also be recycled in a process that involves folate and various B-vitamins (B6 and B12). When the upstream or downstream metabolic pathway of HCY is blocked or delayed for various reasons, it leads to the accumulation of HCY, causing hyperhomocysteinaemia which, although causing no symptoms, affects the interior lining of blood vessels, increasing the risk of atherosclerosis (Sun *et al.* 2017); increases the risk of deep vein thrombosis (Kamat *et al.* 2010, Ekim *et al.* 2015) and pulmonary embolism (Cellai *et al.* 2014); may be related to Alzheimer's disease (Sharma and Lipincott 2017) and other types of dementia (Xie *et al.* 2017) and may be associated with pre-eclampsia in pregnancy (Sayyah-Melli *et al.* 2016, Wadhwani *et al.* 2016). Thus, hyperhomocysteinaemia is associated with various diseases, in particular CVD (Olkkonen 2012, Lathe *et al.* 2014). A small number of older publications (Andersson *et al.* 2001, Seemungal *et al.* 2007, Fimognari *et al.* 2009), and one more recent (Khan *et al.* 2016) have investigated increased homocysteine levels in COPD. Associations between HCY and cancer were found to be inconsistent. Circulating HCY levels increase with age and are higher in men than women in all age groups (Marti-Carvajal *et al.* 2013), and levels may increase with chronic vitamin B12 deficiency, for example in vegetarians (Nygard *et al.* 1995). Smoking may increase plasma HCY levels by a number of mechanisms including: increase in reactive oxygen species levels) (Obersby *et al.* 2013); inactivation of enzymes involved in the metabolism of HCY (De Bree *et al.* 2002, Hu *et al.* 2005, Baccarelli *et al.* 2007); a reduced intake of dietary vitamins/folate by smokers (De Bree *et al.* 2002, Sobczak 2003, Baccarelli *et al.* 2007); and raised levels of the free (sulfhydryl) form of HCY in smokers induced by components of tobacco smoke (Subar *et al.* 1990, Sobczak 2003, Hu *et al.* 2005, McEligot *et al.* 2006, Baccarelli *et al.* 2007, Vardavas *et al.* 2008). Data from the US National Health and Nutrition Examination Survey (NHANES) study (Sobczak 2003) showed HCY levels of 11.04 μmol/L compared to 9.45 and 9.29 μmol/L in smokers, former smokers and never smokers, respectively ($p < 0.001$ for current smokers versus never smokers). A study in a Chinese aged population (with stable CVD) found circulating homocysteine values of 16.8, 14.6 and 14.1 μmol/L smokers, former smokers (at least 3 months smoking abstinence) and never smokers, respectively ($p = 0.004$) (Bazzano *et al.* 2003). Because present knowledge does not supply strong evidence for a relationship between HCY and CVD (or other diseases) (Chen *et al.* 2015), and since no acute effects of smoking cessation on HCY concentrations in blood have been reported in the literature it would appear that the information currently available does not suggest the use of HCY as a biomarker of effect for use in smoking cessation/switching studies. Plasma HCY levels are affected by age, sex and ethnicity (El-Sammak *et al.* 2004, Golbahar *et al.* 2004, Strassburg *et al.* 2004, Refsum *et al.* 2006).

P-selectin (P-sel)

P-Selectin (P-Sel), a component of the membrane of the α-granules of platelets and also of Weibel-Palade bodies, together with E- and L-selectin, is a member of the selectin protein family of adhesion molecules. The expression of membrane-bound P-Sel is a suitable indicator for platelet activation *in vivo*, a process which is involved in several pathophysiological processes such as atherosclerosis, thrombotic diseases and cancer (Chen *et al.* 2015). P-Sel participates in the very early inflammatory processes of leukocyte rolling, tethering, attachment and transmigration across the vascular endothelium, thus initiating events in atherothrombosis (Blann *et al.* 2003, Geng *et al.* 2004, Kappelmayer *et al.* 2004, Merten and Thiagarajan 2004, Chen and Geng 2006, Antonopoulos *et al.* 2014). There are few reports on a direct involvement or association of P-Sel in the development of respiratory diseases. The occurrence of pulmonary embolism as a complicating event in deep venous thrombosis might be regarded as an indirect involvement of P-Sel in pulmonary disease (Merten and Thiagarajan 2004, Chen and Geng 2006). In many, but not all, studies there were statistically significantly higher P-Sel levels and expression activities in smokers compared to non-smokers (Antonopoulos *et al.* 2014). The literature search identified only a single study that investigated levels of P-Sel following smoking cessation and which reported no significant decrease in P-Sel levels following three weeks of smoking cessation (Bermudez *et al.* 2002, Butkiewicz *et al.* 2006, Neubauer *et al.* 2009, Lupia *et al.* 2010). On the other hand, one older study was found which did report a statistically significant decrease in P-Sel following 6 weeks of smoking cessation (Lupia *et al.* 2010). In summary, data providing evidence validating the role of P-Sel in smoking cessation is sparse and would need to be expanded before it can be recommended for assessment of novel tobacco products. Circulating P-selectin levels have been shown to vary between sexes, ethnic groups and with age (Lee *et al.* 2008, Bielinski *et al.* 2015).

Thiocyanate (SCN)

Thiocyanate (SCN), also known as rhodanide, is a pseudohalogen anion and is the main metabolite and detoxification product of cyanide. SCN is also involved in the myeloperoxidase system, which catalyzes the formation of hypochlorite, hypobromite and hypothiocyanite ($OSCN^-$) by oxidizing chloride (Cl^-), bromide (Br^-) and SCN^-, respectively, in the presence of hydrogen peroxide (H_2O_2). The formation of $OSCN^-$ depends on the plasma thiocyanate levels and is the driving force in determining the extent of thiol oxidation with a direct influence on the redox balance in plasma. SCN is thus an important mediator of inflammation-induced damage to proteins, for example in smokers, who exhibit significantly elevated SCN blood levels (Blann *et al.* 1997b). Mean serum/plasma SCN concentrations for smokers and non-smokers have been reported to be predominantly in the

range of 90–180 and 40–75 μmol/L, respectively, with corresponding mean concentration ranges in urine of 150–560 and 75–285 μmol/L. Levels in saliva were in the ranges 2500–3300 and 1200–1300 μmol/L (Morgan *et al.* 2011). Urinary SCN was reported to be associated with active smoking in a dose-related manner subjects over 20 years of age (Jain 2016). A recent study showed that salivary SCN was increased in smokers compared to non-smokers and the increase was related to the number of cigarettes smoked (and correlated with an increase in the number of extranuclear micronuclei) (Baldawa *et al.* 2016) and another reported salivary SCN concentrations of 0.8 ± 0.3 mM in control subjects compared to 2.3 ± 0.8 mM in smokers (Hegde *et al.* 2016). The much higher salivary SCN concentrations are the result of the active secretion of thiocyanate by the salivary glands (Scherer 2006). Another important biochemical property of SCN is its ability to catalyze the endogenous formation of carcinogenic nitrosamines from secondary amines and nitrite as precursors (Tsuda and Kurashima 1991), and smokers are at higher risk for this reaction due to their elevated SCN levels in body fluids, particularly in saliva (Bartsch *et al.* 1989, Tsuda and Kurashima 1991, Tricker 1997). The role of SCN as a substrate of MPO and the MPO-catalyzed oxidation of thiocyanate with subsequent lipoprotein carbamylation might implicate it in the pathogenesis of atherosclerosis, particularly in smokers (Bartsch *et al.* 1989). SCN levels in urine have been shown to correlate with emphysema, chronic bronchitis, cancer, wheezing and coughing (Wang *et al.* 2007), although this correlation may be a function of SCN acting as a biomarker of exposure for smoking. Following smoking cessation, SCN concentrations fall to those of non-smokers within 3–6 weeks. Although cyanide and SCN may come from other sources (mainly via dietary intake), making it potentially a non-specific biomarker of exposure to tobacco smoke, there are clear differences between smokers and non-smokers making SCN a suitable biomarker for use in smoking studies. New instrument-free analytical systems for measuring thiocyanate levels in saliva have been used to estimate non-smokers as having 0.28–0.87 mM and smokers to have 0.78–4.28 mM (Pena-Pereira *et al.* 2016). Measured plasma SCN levels are higher in females than in males (Foss and Lund-Larsen 1986, Zil and Rahman 2006) and urinary thiocyanate content was higher in non-Hispanic whites than non-Hispanic blacks who had higher levels than American Mexicans (Jain 2016).

Von Willebrand factor (vWF)

Von Willebrand factor (vWF) is a blood glycoprotein of importance for maintaining haemostasis. Deficiency in, or a defect of, vWF leads to diseases such as von Willebrand disease (the most common hereditary blood coagulation abnormality), thrombotic thrombocytopenic purpura (TTP, Moschcowitz syndrome), gastrointestinal bleeding (Heyde's syndrome) and possibly haemolytic-uraemic syndrome (HUS). More recently vWF has been found to be involved in several pathologic processes beyond haemostasis, such as angiogenesis, cell proliferation, inflammation, and tumour cell survival (Shiue 2015). Increased plasma levels of vWF (which are presumed to arise from adverse changes to the endothelium, the major site of vWF biosynthesis) are associated with thrombosis, cardiovascular, neoplastic and connective tissue diseases (Lenting *et al.* 2012). Several studies in initially healthy subjects showed rather weak associations between vWF levels and CVD risk that did not always reach statistical significance. This association becomes much stronger in patients with pre-existing vascular disease, particularly survivors of myocardial infarction (MI) and patients with type 1 and 2 diabetes (Sadler 1998, Franchini and Lippi 2007, Spiel *et al.* 2008), and the association between vWF levels and cardiovascular risk was found to be stronger in smokers compared to non-smokers (Lip and Blann 1995, Blann *et al.* 1997a, Whincup *et al.* 2002, Blann 2006, Vischer 2006, Franchini and Mannucci 2008, Frankel *et al.* 2008, Spiel *et al.* 2008, Paulinska *et al.* 2009, van Galen *et al.* 2012, Lenting *et al.* 2013, Sonneveld *et al.* 2015). Evidence for an association between elevated blood levels of vWF and respiratory diseases is rather limited, and no significant correlation between serum vWF levels and subsequent decline in FEV_1 was found, indicating that vWF is not a suitable predictor for increased risk of accelerated decline in FEV_1 (O'Callaghan *et al.* 2005). On the other hand, elevated levels of vWF have been reported to have some predictive and prognostic value for cancer in general (Chambers *et al.* 1999) and non-small cell lung cancer in particular (Blann *et al.* 1997a). Some studies provide evidence that smoking is associated with an increase of vWF blood levels [e.g. 102.4, 100.2 and 99.0 IU/dL in smokers, ex-smokers and never smokers, respectively ($p < 0.05$ comparing smokers to never smokers) (Martini *et al.* 2005), and 114 and 60 IU/dL in smokers and non-smokers, respectively ($p = 0.03$) (Kumari *et al.* 2000)]. One large prospective study found that vWF levels were not associated with smoking status based on a univariate analysis, but did report a small but statistically significant increase in vWF levels for current smokers versus never smokers ($p < 0.05$) using multiple logistic regression (Al-Awadhi *et al.* 2008). There is limited evidence for a dose-response relationship between smoking and vWF levels. In some studies, a rapid increase of serum vWF was found when samples were drawn immediately following the smoking of two cigarettes in smokers who had abstained from tobacco overnight (Conlan *et al.* 1993). Only limited information is available about the effect of smoking cessation on blood vWF concentrations but the sparse data available suggest that vWF levels may decline within weeks after smoking cessation [e.g. from 114.3 to 97.4 IU/dL after 6 weeks ($p = 0.01$) (Blann and McCollum 1993, Blann *et al.* 1998)]. There does appear to be some evidence for the association of vWF levels with smoking status and with smoking cessation. Data from the two switching studies that have been cited previously do not, however, support a utility for vWF as a biomarker. Martin Leroy *et al.* (Blann *et al.* 1997c) did indeed find a statistically significant decrease in vWF levels after one month for smokers who switched to an RRP, but they reported virtually the same difference for subjects who continued to smoke conventional cigarettes. Roethig *et al.* (Martin Leroy *et al.* 2012), however, did not find a significant difference in smokers who switched to an RRP ($p = 0.37$). Published data shows that vWF levels are

higher in females than males (Payne *et al.* 2014, Zhou *et al.* 2014), with higher levels in African Americans than Caucasians (Payne *et al.* 2014). Several studies also show that levels of vWF increase with age (Ishikawa *et al.* 1997, Cohen *et al.* 2012, Cowman *et al.* 2015).

Miscellaneous biomarkers

Glycated haemoglobin (HbA1c)

Glycated haemoglobin (HbA1c) is a long-term marker of blood glucose levels, reflecting the average glucose plasma concentrations over approximately 2 months prior to the test (Roethig *et al.* 2008) and is the "gold standard" for the diagnosis, therapeutic monitoring and prognosis of patients with diabetes mellitus. In epidemiological studies (Miao Jonasson *et al.* 2012), significant associations between HbA1c and CVD, cardiovascular (CV) death or total mortality were observed, although it has to be borne in mind that by far the largest number of studies on the associations between HbA1c and diseases were conducted with diabetic patients of both type 1 and type 2 diabetes. There is almost no evidence for an association between HbA1c and respiratory disease. A meta-analysis of 14 observational studies with 98,978 participants (with diabetes) found that smokers had significantly higher HbA1c levels than non-smokers (Kar *et al.* 2016). Only sparse information is available on the dose-response relationship between HbA1c and the smoking dose, determined for example by daily cigarette consumption (CPD), pack-years or suitable biomarkers of exposure such as cotinine in body fluids. The data that are available (Boeing *et al.* 2000, Anan *et al.* 2006, Kotani *et al.* 2010), however, show moderate but statistically significant associations with smoking and in this respect would qualify HbA1c as a possible biomarker of effect [e.g. 5.11 and 4.99% in smokers and non-smokers respectively ($p < 0.001$) (Gulliford and Ukoumunne 2001, Schwab *et al.* 2008, Clair *et al.* 2011, Vlassopoulos *et al.* 2013, Ohkuma *et al.* 2015), 4.86 and 4.67% in smokers and non-smokers respectively ($p < 0.05$) (Simon *et al.* 1989) and 8.6 and 7.6% ($p = 0.002$) in smokers and non-smokers respectively (Nilsson *et al.* 1995)]. Since the half-life of HbA1c is determined by the lifespan of red blood cells (~4 months), it is obvious that changes in HbA1c levels can only be expected in long-term observations (months–years). There is general agreement in the results retrieved that HbA1c levels in ex-smokers (after smoking cessation periods of 3 months or more) are close to those of never smokers (Baggio *et al.* 2002), which would generally qualify HbA1c as a suitable *long-term* (months–years) biomarker of effect in studies with large populations (e.g. post-market studies). In subjects with normal glucose tolerance, HbA1c levels rise by about 0.1% per decade (slightly less in those with impaired glucose tolerance or impaired fasting glucose) (Davidson and Schriger 2010). Ethnic background also affects HbA1c with non-Hispanic blacks having higher levels than Mexican Americans who have higher levels than non-Hispanic whites (age range 40–75 years) (Boltri *et al.* 2005, Davidson and Schriger 2010). Whilst HbA1c is the current "gold standard" for the diagnosis of diabetes it should not be forgotten that the value is dependent upon the life span of circulating red blood cells leading to the suggestion that in children a correction factor should be employed (An *et al.* 2016) and that in the elderly, HbA1c is actually unsuitable for diagnosing diabetes (Wu *et al.* 2017).

Carboxyhaemoglobin (COHb)

Carbon monoxide (CO) is produced by the incomplete combustion of organic material, including tobacco [in which it has been described as the most toxic component of burning cigarettes (Gulliford and Ukoumunne 2001, Hokanson *et al.* 2006, Clair *et al.* 2011, Voulgari *et al.* 2011, Choi *et al.* 2013, Gerber *et al.* 2013, Vlassopoulos *et al.* 2013, Ohkuma *et al.* 2015)]. CO binds to haemoglobin (about 200 times more strongly than oxygen) to form carboxyhaemoglobin (COHb), and the resulting lowered oxygen binding and transport means that less oxygen is available at peripheral tissue sites leading to hypoxia. COHb may be considered a biomarker of exposure and biological effect, the latter because the saturation of haemoglobin with CO is a direct measure for the biological effect of CO, namely, impairment of the oxygen supply of organs.

Cigarette smoking is known to elevate both respiratory and blood CO levels and, therefore, raising the COHb [e.g. 5.5% in smokers compared to 0.7% in non-smokers (Leone 2015)] levels which are associated with increased numbers of cardiac events, including fatal and non-fatal myocardial infarction. Lowering of COHb rapidly leads to an increase in the respiratory indices of vital capacity (VC), forced expiratory flow 25–75% (FEF_{25-75}) and FEV_1 (MacNee *et al.* 1989). The levels of COHb (in smokers and non-smokers) correlate with CVD risk (Hedblad *et al.* 2006, Pezzuto *et al.* 2013). Following smoking cessation, the levels of COHb decline quickly (Lind *et al.* 2004, Hedblad *et al.* 2005, 2006, Lüdicke *et al.* 2016) as its half-life is between one and four hours in blood with its elimination being mainly dependent on ventilation rate and level of physical activity (Roethig *et al.* 2007, Tricker *et al.* 2012, van Staden *et al.* 2013). Although the literature search provided no evidence of a *direct* link between COHb levels and disease, COHb should be considered for use in smoking/cessation/switching studies as it reflects exposure to carbon monoxide which is associated with respiratory, cardiovascular and inflammatory diseases. Carbon monoxide in blood, as assessed by the COHb%, is an accepted indicator of the cardiovascular risk in smokers. The biological, epidemiological and clinical trial information is in line with the induced effects observed upon smoking cessation, suggesting that a decrease in COHb mediates some of the reduced CVD risk observed after smoking cessation.

Wheeze, cough and sputum (W/C/S)

In general, wheeze, cough and sputum (W/C/S) are common symptoms among individuals with COPD (Scherer 2006), while cough is significantly associated with a number of other chronic respiratory diseases such as chronic bronchitis, bronchiectasis and asthma. Cough, in particular smoker's cough, is an unequivocal sign of chronic bronchitis, which is

itself a well-established risk factor for COPD and associated with poorer prognosis and higher mortality in patients with this disease (Putcha *et al.* 2014). It has been reported that chronic bronchitis, defined as cough and phlegm production on most days for 3 months or more in adults <50 years old (but not in subjects >50 years) represents an early marker of susceptibility to effects of cigarette smoking on COPD mortality (Vestbo 2014), while persistent wheeze, chronic cough and chronic phlegm together with dyspnoea were found to have strong predictive value for a decline in FEV_1 and were, therefore, suggested to be suitable *diagnostic* tools for therapeutic intervention (Guerra *et al.* 2009). Cough (most frequently productive cough) as well as wheeze and breathlessness are typical symptoms occurring in all stages and types of lung cancer and have some predictive value for the mortality in this disease (Sherman *et al.* 1992). It has been suggested that smoker's cough is a symptom for a disease (chronic bronchitis) rather than a mere biomarker of an upcoming respiratory disorder (Vollmer *et al.* 1989, Lange *et al.* 2003, Molassiotis *et al.* 2010a), and that it can be considered as a life threatening condition (Vestbo 2014). In general, there is a reduction in the respiratory symptoms of chronic cough, chronic phlegm production, wheezing and shortness of breath following smoking cessation (Gillissen 2011). Recent studies suggest that a single exposure to an e-cigarette (nicotine vapour) can enhance the cough reflex which is suppressed in otherwise healthy cigarette smokers (Dicpinigaitis *et al.* 2016).

Overall, while W/C/S symptoms are suitable clinical biomarkers for the management of respiratory diseases, their suitability as biomarkers of effect for use in smoking/cessation/switching studies is limited, mainly due to the fact that long-term studies (lasting at least for months–years) are required to obtain valid data.

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) is one of seven known tobacco-specific nitrosamines (TSNA). Although levels of NNAL are relatively low in cigarette smoke, they are relatively much higher in smokers since NNAL is the main metabolite of NNK (4-(methynitrosoamino)-1-(3-pyridyl)-1-butanone) (Viegi *et al.* 1988b, Kanner *et al.* 1999). NNK is present in smoke at much higher levels than is NNAL and is metabolized to NNAL in the liver and lungs. Of the seven known TSNAs, NNK is one of the most potent carcinogens in cigarette smoke (Hecht *et al.* 2013). Since NNAL is the principal NNK metabolite in human lung (Hecht 2008), NNAL could be considered an important biomarker (Richter *et al.* 2009) of both exposure and effect (particularly given that NNK can only be detected in very low levels in body fluids sampled from smokers).

The literature searches did not identify any studies that attempted to determine if there was a specific correlation of NNAL levels and risk of a CVD endpoint, although one study had investigated the association of total NNAL levels with various biomarkers for CVD (Wei *et al.* 2016). In addition, only one study was identified that concerned NNAL and non-malignant respiratory disease (asthma) (Liu *et al.* 2011). Most of the studies identified have investigated a link between NNAL levels and cancer [primarily lung cancer since NNK, the precursor of NNAL, has been reported to cause lung cancer in all animal species irrespective of the route of administration (Ho *et al.* 2013b)].

Almost all of the studies identified in the searches demonstrated a good correlation between NNAL and other markers of cigarette exposure, e.g. nicotine (Hecht 1998) and cigarette consumption (Xia *et al.* 2011, Benowitz *et al.* 2012, Agaku *et al.* 2014). Most studies have shown a relatively slow rate of decline of NNAL following smoking cessation with half-lives of 8–23 days (Hertsgaard *et al.* 2008, Calapai *et al.* 2009).

It is important to note that a recent study investigated levels of total NNAL (free NNAL and NNAL glucuronides) in smokers of a large number of different e-cigarette brands compared to smokers of conventional cigarettes (Carmella *et al.* 2009, Goniewicz *et al.* 2009, Stepanov *et al.* 2009). Although this was a relatively small study (28 e-cigarette users, 17 conventional cigarette smokers), the results were highly significant, with a geometric mean of total NNAL of 0.02 pmol/mL in e-cigarette users and a geometric mean of 1.48 pmol/mL in conventional cigarette users ($p < 0.0001$) suggesting that e-cigarette users are exposed to far less total NNAL, and thus NNK in smoke, than are conventional smokers.

The earliest study that investigated a link between NNAL and lung cancer in humans was published in 2009 (Hecht *et al.* 2015) This study used a nested case control design to assess the association between total NNAL and lung cancer in 100 smoking lung cancer cases and 100 smoking controls drawn from the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial (PLCO). NNAL was the only factor statistically significantly associated with the risk of lung cancer following adjustment for other factors associated with lung cancer including age at randomization, family history of lung cancer, cotinine levels and years of cigarette smoking. The OR for the association of NNAL with lung cancer for a unit standard deviation increase (40 fmol/mL of serum) was 1.57 (95% CI, 1.08–2.28).

In the same year, the first of two papers that investigated the association of total NNAL levels with lung cancer risk using two Far Eastern cohorts was published (Church *et al.* 2009). The first cohort was the Shanghai Cohort study, which consisted of 18,244 men enrolled from 1 January 1986 to 30 September 1989. The second was the Singapore Chinese Health study, which consisted of 63,247 men and women enrolled between 1 April 1993 and 31 December 1998. Once again, a nested case control design was used. A total of 259 cases of smoking-related lung cancer diagnosed by March 1997 were matched to an equal number of controls in the Shanghai study, while the Singapore study consisted of 99 cases and an equal number of controls. Adjusted [age, year of interview, year of sample collection, study location, number of cigarettes per day (cpd), number of years smoking and cotinine levels] odds ratios (ORs) were calculated for each tertile of total NNAL and compared to the first tertile as reference. For both studies, combined the OR for the second total NNAL tertile was 1.43 (95% CI, 0.86–2.37), while for the third tertile OR =2.11 (95% CI, 1.25–3.54).

Table 6. Summary of findings for each biomarker investigated with respect to its relationship to smoking, smoking cessation and two smoking-related disease groups.

Biomarker	Change caused by smoking	Relationship to smoking	Relationship to cessation	Disease related to the biomarker		Time to normalise
				Resp	CVD	
Forced expiratory volume (FEV_1)	↓	+++	++	+++	−	Months
Iso-prostaglandin (8-iso-$PGF_{2\alpha}$)	↑	+	+/−	+/−	++	Weeks
Thromboxane B2 (11-DTXB2)	↑	++	+	−	++	Days–weeks
C-Reactive protein (CRP)	↑	++	+/−		+++	Years
White blood cell count (WBC)	↑	++	++	++	+++	Weeks–months
Soluble intracellular adhesion molecule (s-ICAM-1)	↑	++	+	++	++	Weeks
Fibrinogen (FBG)	↓↑	+/−	+/−	++	++	–
Tumour necrosis factor (TNFα)	↑↓	+	+/−	++	++	–
Myeloperoxidase (MPO)	↑↓	+/−	+/−	+++	+++	–
Exhaled nitric oxide (FeNO)	↓	+	+	++	−	–
Sputum Neutrophils	↑	+	+/−	++	−	No change
Phospholipase A2 (Lp-PLA2)	↑	+/−	−	−	++	Months
Albumin (ALB)	↑	+	+	−	+	Months–years
Low-density lipoprotein-cholesterol (LDL-C)	↓↑	++	+/−	−	+++	–
High density lipoprotein-cholesterol (HDL-C)	↓	+++	+++	+/−	+++	Months
Apolipoprotein A1 (ApoA1)	↓	+	+	−	++	Weeks–months
Oxysterols (OxS)	↑?	−	−	−	++	–
Homocysteine (HCY)	↑	−	−	−	+/−	Months
P-Selectin (P-Sel)	↑	+	+/−	−	+	Weeks–months
Thiocyanate (SCN)	↑	++	+	+	++	Weeks
Von Willebrand factor (vWF)	↑	+	+	−	+	Weeks
Glycated Haemoglobin (HbA1c)	↑	++	++	−	++	Weeks–months
Carboxyhaemoglobin (COHb)	↑	+++	+++	+	+++	Days
Wheeze, cough and sputum (W/C/S)	↑	+++	++	+++	−	Months–years
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)	↓	+++	+++	(cancer)	−	Weeks–months

Resp: respiratory diseases; CVD: cardiovascular diseases; arrows indicate increase or decrease in biomarker levels caused by smoking; time to normalize is an indication of how long following smoking cessation the biomarker returns to normal levels (for some biomarkers this is not clear from the literature).

The second paper, published in 2011, focussed solely on the results obtained for the Shanghai cohort study (Yuan *et al.* 2009). This paper presented results on a larger number of individuals than did the previous publication, in that additional cases of lung cancer had been diagnosed. As a consequence, data were available for 476 cases and controls. The results for total NNAL, adjusted for the same covariates used above, were OR (second tertile) = 1.67 (95% CI, 1.13–2.47) and OR (third tertile) = 1.98 (95% CI, 1.32–2.98), results that are clearly comparable to the results published in the earlier paper. One last result of this paper should be noted. Of five factors that were investigated in this study in a multiple factors model, including cigarettes/day, years smoking and total cotinine, NNAL had the weakest association with lung cancer risk (OR =1.28; 95% CI, 1.02–1.59). The authors hypothesize that this may be because of the low levels of TSNA in Chinese cigarettes.

It has been known for some time that changes in TSNA levels in smoke are widely different depending on the country in which the cigarettes are manufactured. This is particularly true for Chinese cigarettes and US cigarettes, with a difference of somewhat more than 10-fold (Yuan *et al.* 2011). However, no real comparison of risk associated with smoking as a function of TSNA smoke levels has yet been published although this point was recently addressed by Canadian investigators (Wu *et al.* 2005) who conducted a study in which total NNAL levels were measured in 507 Canadian smokers. The mean level of total NNAL in this sample was 71.2 pg/mL (95%CI, 63.1–80.4), considerably less than the level in a large American study by Xia *et al.* which reported total NNAL levels of 299 pg/mL (95% CI, 253–353) in 1373 smokers (Czoli and Hammond 2015). The authors question, however, "… whether the differences between US and Canadian cigarettes translate into meaningful differences in risk for these populations."

There is a clear association between NNAL and the smoking of conventional cigarettes and an unequivocal decline in NNAL following smoking cessation as well as switching to electronic cigarettes, making it a valid biomarker of exposure suitable for use in smoking cessation/switching studies. The situation is less clear with respect to the utility of NNAL levels as a biomarker of effect. As can be seen from the above results, the association of NNAL levels with lung cancer risk appears to be weak. As has been pointed out, however, the levels of NNK, the source of NNAL, are very low in Chinese cigarettes, and much of the data presented were obtained from Chinese smokers. However, the relationship of a sizable difference in total NNAL levels in American and Canadian smokers has been questioned as a predictor of risk. In a meta-analysis based on 40 US and 22 Chinese studies it was found that among ever smokers (i.e. current and former smokers combined) lung cancer mortality (per 100,000), in the 70–74 year age range, was 286.7 in the US and 315.6 in China (Lee and Forey 2013). It could be expected that if TSNA *was* a causal factor, levels should be similar in Chinese and US cigarettes. In contrast, TSNA levels have been found to be substantially higher in US than in Chines cigarettes (Ashley *et al.* 2003). Nevertheless, until a definitive study has been conducted, such as a comparison of age adjusted lung cancer rates in Chinese and American smokers, it seems best to err on the side of safety and assume that NNAL is also a biomarker of risk.

Summary and conclusion

Table 6 summarizes the interpretations of this literature review on these 25 potential biomarkers and outlines their relationship to smoking, smoking cessation and respiratory and cardiovascular disease (graded by the authors based on the literature findings).

An ideal biomarker for use in smoking/cessation/switching studies would be one that was associated with the effect of smoking cigarettes, was associated with at least one smoking-related disease and declined following cessation/smoking reduction or switching to a reduced risk product. It is clear from analysing the information retrieved from the literature that few of the biomarkers investigated fulfil all of these criteria. It is also clear that one single biomarker is unlikely to provide all the information needed. Useful biomarkers should consistently relate to a disease, represent biologically real pathways and change as the pathology changes. Many of the studies alluded to in this review might have failed to find high sensitivity and high specificity biomarkers due to the sometimes small number of subjects, various analysis techniques or data interpretation (Xia *et al.* 2011). It is unfortunate that highly standardized assays for biomarker identification and analysis are not frequently found, and although thousands of publications have been written, only a small number of successfully validated biomarkers have entered clinical use (Issaq *et al.* 2011, Poste 2011). A further complication is that many of the reviewed biomarkers reflect a complex network of multiple interacting molecular pathways coupled with their adaptive feedback and cross-talk loops which masks the chance of the biomarker to reflect responses of the system as a whole. For many reasons the relationship between markers and outcomes are neither unique nor simple (Drucker and Krapfenbauer 2013) and can be modified by many factors. Choosing a panel of biomarkers that will be able to reflect changes that might be expected within the duration of the study and associated with more than one smoking-related disease would seem an optimal approach. Epidemiological data being available for more than half a century, the chronic disease risks of cigarette smoking are well understood, as are the reductions in risk achieved over time by smoking cessation. In contrast, the chronic disease risk assessment of switching from cigarettes to novel modified risk tobacco products must rest on biomarker data obtained in pre-market studies. In spite of the uncertainty implied by this restriction to indirect evidence, not making such products available to consumers is not a realistic regulatory strategy. The reason is that this null option would not be precautionary but rather would entail doing harm, by restricting cigarette smokers who do not quit using tobacco to consume products of a category with well-known and considerable disease risks.

The risk estimation problem with analysing biomarker data is to quantify the degree of effective dose reduction that is achieved by cigarette smokers switching to a novel modified risk tobacco product, based on effects observed in measurements which are indicative of, but are not directly quantifying, risk reduction. Rather than attempting a direct estimation of the degree of risk reduction achieved by switching to modified risk tobacco products as compared to continued smoking, a re-parametrization of the estimation problem has recently been undertaken (Martin *et al.* 2017), making two reasonable assumptions. The first is on the informativeness of a comprehensive set of biomarkers measured in multiple clinical studies with regard to predicting chronic disease risks to be halfway between 0 and 100%. The second assumption is that, for each biomarker to contribute to a reduction in the effective dose, the minimal change upon switching from smoking to a novel product must be at least half of that observed upon smoking cessation.

Sensitivity analyses based on variations of these conservative parameter settings as well as on the omission of individual biomarkers from the estimation supported the robustness of the risk assessment. Future epidemiological health outcomes data can be used for assessing the model predictions and for quantifying the predictive accuracy of the observed biomarker effects.

Finally, limiting the period of the search (2008–2017) was intended to provide recent evidence supporting the use or not of the biomarkers of interest. However, for some biomarkers (e.g. TXB_2), the recent literature provided little information and in these cases, we have resorted to using publications made prior to 2008.

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