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REVIEW ARTICLE

# Lung cancer biomarkers for the assessment of modified risk tobacco products: an oxidative stress perspective

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**Abstract**

Manufacturers have developed prototype cigarettes yielding reduced levels of some tobacco smoke toxicants, when tested using laboratory machine smoking under standardised conditions. For the scientific assessment of modified risk tobacco products, tests that offer objective, reproducible data, which can be obtained in a much shorter time than the requirements of conventional epidemiology are needed. In this review, we consider whether biomarkers of biological effect related to oxidative stress can be used in this role. Based on published data, urinary 8-oxo-7,8-dihydro-2-deoxyguanosine, thymidine glycol, $F_2$-isoprostanes, serum dehydroascorbic acid to ascorbic acid ratio and carotenoid concentrations show promise, while 4-hydroxynonenal requires further qualification.

**Keywords**

Antioxidant, cigarette, disease prediction, isoprostane, MRTP, smoking

**History**

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## Introduction, definitions and scope of review

The epidemiological link between smoking and lung cancer was first published over 60 years ago (Doll & Hill, 1950; Wynder & Graham, 1950) but, despite intensive research since that time, knowledge of the effect of tobacco smoke toxicants on the precise molecular steps and the host genetic influences required for the development of any one of this group of cancers remains elusive (Hahn & Weinberg, 2002). A summary outlining available mechanisms was provided recently (Department of Health and Human Services, 2010) and the feasibility of attempting to reduce smoking-associated lung cancer by modifying tobacco products has been highlighted (Institute of Medicine, 2001, 2012; Meier & Shelley, 2006).

Carcinoma of the lung is one of the most prevalent human solid cancers: in 2008, it accounted for around 12.7% of all new cancer incidence and 18.2% of all cancer mortality, or approximately 1.4 million deaths worldwide (Jemal et al., 2011). In male populations with long-term cigarette use, the proportion of lung cancer cases attributable to smoking approaches 90% (World Health Organization Classification of Tumours, 2004).

Reduced toxicant prototype (RTP) tobacco products are cigarettes that include technologies that reduce yields of certain smoke toxicants compared to conventional cigarettes (Bombick et al., 1998; Branton et al., 2011; Brown et al., 1997; Frost-Pineda et al., 2008; Liu et al., 2011a; McAdam et al., 2011; Russell, 1976; Sarkar et al., 2008; Smith et al., 1996). Modified risk tobacco products (MRTPs) have been defined as ''any tobacco product that is sold or distributed for use to reduce harm or the risk of tobacco-related disease'' (Institute of Medicine, 2012). It has been proposed that biomarkers are used as part of the overall approach to the scientific assessment of such products (Ashley et al., 2007; Gregg et al., 2006; Hatsukami et al., 2006; Institute of Medicine, 2012).

The Biomarkers Definitions Working Group defined a biomarker as ''a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention'' (Biomarkers Definitions Working Group, 2001). This definition was endorsed by a recent report from the US Institute of Medicine (IOM), which was commissioned by the US Food and Drug Administration (IOM, 2010).

In a broad sense, current cancer biomarkers, such as prostate specific antigen, the human oestrogen receptor and CA125 are used to distinguish patients with disease from disease-free individuals and as indicators of prognosis; however, these biomarkers are only detectable relatively late in the disease process, after clinical disease is evident, which is too late to be suitable for use in the scientific assessment of MRTPs.

For the scientific assessment of an MRTP, objective and reliable data on early biological effects could be generated in much shorter time frames than those obtained from conventional epidemiological studies. Some existing biomarkers of early biological effects, such as biomarkers of oxidative stress and of inflammation, appear to have suitable characteristics to suggest them as candidates for use in MRTP assessment. However, these biomarkers would have to be qualified for this purpose, before reliance could be placed on any data generated with them.

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The IOM defined qualification as an ‘‘evidentiary process of linking a biomarker with biological processes and clinical endpoints’’ (IOM, 2010). From theoretical and practical standpoints, we propose that the qualification of a biomarker linking an exposure with ‘‘biological processes’’ is distinct from one that would link exposures or biological processes to ‘‘clinical endpoints’’. Thus, we use the term ‘‘biomarkers of biological effects’’ for the former and ‘‘biomarkers of potential harm’’ for the latter. In this review, we discuss only biomarkers of biological effects. In addition to the definition offered by the IOM, we further suggest that to qualify for use in MRTP scientific assessment, biomarkers of biological effect should (1) be measurable in tissues or body fluids that can be obtained by non-invasive techniques; (2) show reversibility within a timeframe of less than 6 months after smoking cessation, as an indication of the effect that could be achieved with an suitable candidate MRTP and (3) have fully validated methods for their measurement, based on existing guidelines (Aggett et al., 2005; Chau et al., 2008; Food and Drug Administration, 2001; IOM, 2010, 2012; Lee et al., 2006), in order to reduce between laboratory differences in measurement, across studies. Thus, we propose that qualified biomarkers of biological effect would represent acute and sub-chronic response pathways to exposures and that for MRTP scientific assessment, alterations in their concentrations in the direction of those found in studies of smoking cessation would add to the overall ‘‘weight of evidence’’ to evaluate the potential of a MRTP to reduce risks.

In our terminology, we note that none of these biomarkers of biological effect are qualified as a predictive biomarker for a disease endpoint. We propose that they are only qualified against the type of exposure that could be anticipated by use of an MRTP compared to the use of a conventional cigarette. However, biomarkers of biological effect, such as biomarkers of oxidative stress and of inflammation, may measure the processes that have themselves been associated with disease endpoints and so alterations in these biomarkers may provide meaningful data for the scientific assessment of MRTPs.

Biomarkers can be measured in biofluids and excretions or assessed in recordings and images (IOM, 2001; Vasan, 2006). For MRTP assessment studies, relevant biomarkers of biological effect would be those that show early changes after exposure to smoke. Reversibility would not have to be measured as an absolute but could be given as a relative measurement from the time of initial exposure to a MRTP and it could also relate to objective changes that are reported by study participants via methods such as health questionnaires.

Using this approach, we propose that useful biomarkers of biological effect for MRTP assessment should alter within a short time frame, generally less than 6 months. Those that alter in less than 2 weeks could be assessed in clinical confinement studies, which enable good control of product switching and of some confounding factors, such as diet and exercise; whereas those biomarkers which take longer than 2 weeks to change could only be investigated in typical lifestyle settings, with inevitable loss of control over exclusive product use and confounding factors related to diet, exercise and other lifestyle choices.

In this review, we comment on previously published candidate biomarkers and discuss biomarkers of biological effect related to oxidative stress, oxidatively generated damage to DNA, anti-oxidant capacity and lipid peroxidation; commenting on their potential utility in the scientific assessment of MRTPs. Pre-neoplastic lesions and cytological changes, which are currently used as risk markers for clinical endpoints and for assignment of therapy, fit our definition of biomarkers of potential harm and, therefore, are outside the scope of the current review.

## Previously published biomarker lists

Several groups have reviewed the availability of candidate biomarkers for the assessment of lung cancer risk in the context of tobacco products assessment.

### Institute of medicine

The candidate biomarkers proposed by the IOM (2001) were intended to show short-, medium- and long-term biological effects related to lung cancer, which are associated with exposure to tobacco smoke but not exclusively (Table 1). All the biomarkers, however, have important limitations that greatly reduce their usefulness in MRTP assessment studies; for example, conventional genetic toxicology assays (chromosome aberrations, micronucleus induction and sister chromatid exchanges) were criticised for their lack of specificity (IOM, 2001) and additional assay development work and investigations are required to identify clear mechanistic links to disease processes. Importantly, the IOM did not recommend any of these biomarkers or their group as definitive biomarkers for the assessment of MRTPs – defined by the authors as potential reduced-exposure products or PREPs (IOM, 2001).

### Life sciences research organisation

In 2007, the Life Sciences Research Organisation (LSRO) presented a list of biomarkers of biological changes associated with the use of MRTPs – termed, by them, potential reduced-risk tobacco products (Table 2) (Life Sciences Research Office, 2007). The LSRO suggested that multiple biomarkers should be assessed simultaneously to compare the risks of toxic effects related to smoking MRTPs versus conventional cigarettes, and that the following potential mutagenic and carcinogenic pulmonary effects be taken into account in study designs: genetic damage (panels of markers of chromosomal aberrations, mutations in genes encoding cell cycle, signal transduction, DNA repair and tumour suppressor proteins; urine mutagenicity and/or adduct formation); cytological changes in cells and tissues (assessed by cytology and pathology of sputum and/or biopsy samples and by imaging with spiral CT) and epigenetic alterations (DNA methylation). While chromosomal aberrations and the induction of micronuclei are included in the list, the assays to measure these biomarkers have poor specificity (Collins, 1998; IOM, 2001).

### Hatsukami et al.

Hatsukami et al. (2006) presented a review of candidate lung cancer biomarkers (Table 3). They included chemical

Table 1. IOM candidate biomarkers for lung cancer.

| Category and variables | Dose–response data | Associated with cessation or half-life | Target tissue assay available | Chemical specificity | Specific to tobacco | Related to a disease risk | Limitations | Strengths |
|---|---|---|---|---|---|---|---|---|
| Enzymatic induction | | | | | | | | |
| Aryl hydrocarbon hydroxylase | No | >30 d | Yes | Yes | No | Yes | Technically difficult to assess in large epidemiological studies. | Indicates acquired changes in susceptibility; related to DNA adduct levels. |
| CYP1A2 | No | NDA | Yes | Yes | No | Yes | Technically difficult to assess in large epidemiological studies. | Indicates acquired changes in susceptibility; related to DNA adduct levels. |
| DNA repair enzymes | NDA | Yes | Yes | NA | No | NDA | Technically difficult. | Indicates acquired changes in susceptibility; provides analysis of what is likely to be critical part of carcinogenesis. |
| Microarray assays for mRNA expression and proteomics | NDA | NDA | Yes | NA | No | NDA | Difficult to perform; relationship to disease risk is technically difficult to prove; requires extensive laboratory validation; RNA and protein microarray assays are expensive; large-scale studies are needed; refined bioinformatic analysis required. | Reflects integrated measure of multiple genotypes, provides complex data potentially usable for rapid identification of important risk factors. |
| Chromosomal alterations | | | | | | | | |
| Chromosomal aberrations | Yes | Yes | Yes | No | No | Yes | Very non-specific; relationship to target organ is not established; lack of specificity and wide overlap between smokers and non-smokers. | Can be done in blood as surrogate tissue. Similar lesions observed in cancer. Can be measured in persons without cancer. |
| Micronuclei | Yes | Yes | Yes | No | No | NDA | Lack of specificity. | Facile assay. |
| Sister chromatid exchanges | Yes | Yes | No | No | No | No | Very non-specific; relationship to target organ is not established; predictivity for disease risk not established. Association with cancer in case-control studies may have case bias. Wide overlap between smokers and non-smokers. | Easy to do in blood as surrogate tissue. Can be measured in people without cancer. |
| Loss of heterozygosity | Yes | Yes | Yes | No | No | NDA | Technically complex; relationship to cancer risk unknown. | Similar lesions observed in cancer. |
| Mutations in reporter genes (*HPRT1*, *GYPA*) | Yes | Yes | No | No | No | NDA | Relationship to target tissue or blood unknown. | Facile assay in blood. |
| Mutational load in target genes (*TP53*, *KRAS*) | NA | NDA | Yes | No | No | NDA | Very difficult to do in normal tissues. | Target gene specificity. |
| Mitochondrial mutations | | | | | | | | |
| Deletions, insertions | NDA | NDA | Yes | No | No | NDA | Relationship to disease not established. | Provides corroborative marker. |
| Epigenetic cancer effects | | | | | | | | |
| Whole genome methylation | NDA | NDA | Yes | No | No | No | Relationship to disease unknown. | Facile assay. |
| Hypermethylation of promoter regions | NDA | NDA | Yes | No | No | No | Technically difficult; relationship to risk unknown. | Similar lesions observed in cancers. |

NDA = no data available.
mRNA = messenger RNA.
NA = not applicable.

Table 2. LSRO candidate biomarkers for lung cancer.

| Disease/biological process | Primary biomarker[a] | Secondary biomarker[b] | Tertiary biomarker[c] |
| --- | --- | --- | --- |
| Cytopathological changes | Squamous cell dysplasia | Hyperplasia and metaplasia for SCC, cytology and pathology (sputum, forceps and brush biopsy) for other histological tumour types, spiral CT | – |
| Genetic damage | – | Chromosomal aberration, micronuclei, aneuploidy, loss of heterozygosity, acquired genetic effects to specific targets, DNA adducts, urine mutagenicity | Gene array technologies |
| Epigenetic alterations | – | DNA methylation | – |
| Inflammation | – | – | Inflammatory markers in respiratory tract fluids or tissues |
| Oxidative stress | – | Oxidatively generated DNA products | Isoprostanes |
| Protein changes | – | Abnormal or elevated protein concentrations, inactivated or activated proteins/ enzymes/receptors, protein adducts | Proteomic technologies |

[a]Linked to clinical outcomes with strong evidence.
[b]Secondary biomarkers have been linked to clinical outcomes with moderate evidence.
[c]Tertiary biomarkers have been linked to clinical outcomes with preliminary evidence.
SCC = squamous cell carcinoma.
CT = computer tomography.

biomarkers that measure carcinogen exposure and some measure metabolic activation and binding to DNA or proteins via adducts. By contrast, cellular biomarkers are related to toxicant concentrations at the cellular level, and they measure effects that have been associated with pathological change related to cancer, including genetic damage and other cellular alterations. These authors presented the biomarkers in order of usefulness at the time (most to least) for tobacco product assessment and for them, the three most useful chemical markers were 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronide conjugate (NNAL-Gluc) in urine and aminobiphenyl/aromatic amine haemoglobin adducts. Of the cellular biomarkers, detection of mutagens in urine with *Salmonella typhimurium* strains has been the most widely applied. They also recommended the use of the sister chromatid exchange assay. All of these assays have limits as biomarkers for possible carcinogenic endpoints: simple exposure to a carcinogen does not show the outcome of metabolism or detoxification; urine mutagenicity does not mean that a mutagen was present at the target cell DNA and mutagens are not necessarily carcinogens (Ames et al., 1979; Gold et al., 1992); furthermore, mutations may be repaired or induce apoptosis (Czabotar et al., 2011; Liu et al., 2011b; Sahu et al., 2011). Sister chromatid exchange is a physiological process within lymphocytes and this activity correlates poorly with chemical carcinogenesis (Aitio et al., 1988). Nonetheless, all of these processes could be used as part of a ‘‘weight of evidence’’ approach to demonstrate overall reductions in biological activity for an MRTP but it is doubtful whether they would be sufficient to permit a claim of reduced carcinogenic potential for an MRTP with lower activity than a conventional cigarette.

These lists of biomarkers provide a useful starting point for the quest to obtain a definitive set of biomarkers of biological effect related to carcinogenic endpoints for use in MRTP assessment. Further exploration is required to identify additional biomarkers and other mechanisms that could prove to be more useful for this role. Below, we discuss some potential biomarkers of interest and their possible utility in MRTP studies.

## Oxidative stress in carcinogenesis

Reactive oxygen species (ROS) and similar oxidising species act directly on biomolecules, damaging lipids, proteins and, if they are present within a cell nucleus, DNA. In a situation where repeated and sustained intra-nuclear ROS are generated, DNA damage may become extensive, and extensive DNA damage generates genomic instability, which contributes to carcinogenesis (Charames & Bapat, 2003; Hanahan & Weinberg, 2000). Endogenously formed ROS, such as the hydroxyl radical ($HO^{\bullet}$), which is generated during physiological oxidative respiration, can lead to chemical alterations in purines and pyrimidines (Valavanidis et al., 2009; Valko et al., 2004) which, in turn, affect gene integrity. However, it is unlikely that $HO^{\bullet}$ generated in a remote cell compartment can diffuse into the cell nucleus, due to its extreme reactivity and it has been proposed that $H_2O_2$ serves as a diffusible latent form of $HO^{\bullet}$ that reacts with a metal ion in the vicinity of a DNA molecule to generate the oxidant species (Marnett, 2000). Others have suggested that lipid peroxidation products may also function as intermediates between endogenous metabolic products or xenobiotic agent-induced alterations and DNA effects (Voulgaridou et al., 2011). Any oxidative lesion that is not repaired can become a fixed mutation in a cell with replicative capability, which increases the risk of carcinogenesis (Clayson, 1994).

ROS may also act indirectly through the recruitment of inflammatory mediators that trigger a secondary oxidative response. Oxidative stress is thought to be involved in the initiation, promotion and progression phases of cancer, and its role in each of these phases is complex. Several diverse

Table 3. Cancer-related biomarkers reported by Hatsukami et al. (2006).

| Biomarker | Measurement | Relation to tobacco product use | | | |
|---|---|---|---|---|---|
| | | Difference: users versus non-users | Change with cessation | Dose response with use | Change with reduced use |
| Chemical biomarkers | | | | | |
| NNAL and NNAL glucuronides | Carcinogen (NNK) uptake | Yes | Yes | Yes | Yes |
| 3-Aminobiphenyl, 4-aminobiphenyl and other aromatic amine haemoglobin adducts | Carcinogen (aromatic amines) uptake plus metabolic activation | Yes | Yes | – | Yes |
| 1-Hydroxypyrene in urine | Carcinogen (PAH) uptake | Yes | Yes | | Yes |
| Trans, trans-muconic acid in urine | Carcinogen (benzene) uptake | Yes | – | – | – |
| *S*-phenylmercapturic acid | Carcinogen (benzene) uptake | Yes | – | – | – |
| Benzene and other volatile organic carcinogens in exhaled air | Volatile organic carcinogens | Yes | – | Yes | – |
| Ethylene oxide haemoglobin adducts | Carcinogen (ethylene oxide) uptake | Yes | – | – | – |
| Other N-terminal valine adducts in haemoglobin | Carcinogen uptake | Yes | – | – | – |
| Cadmium and other metals in blood and urine | Carcinogen uptake | In part[a] | – | – | – |
| Acetaldehyde-DNA and protein adducts | Carcinogen uptake | – | – | – | – |
| $F_2$-isoprostanes and oxidised proteins | Oxidatively generated damage, inflammation[c] | Yes | – | – | – |
| 8-Oxoguanine or 8-hydroxy-deoxyguanosine in DNA or urine[b] | Oxidatively generated damage, inflammation[c] | In part | – | – | – |
| Mercapturic acids of acrolein and related compounds in urine | Toxin uptake and metabolism | In part | – | – | – |
| Benzo(a)pyrene diol epoxide-DNA and haemoglobin adducts | Carcinogen (benzo[a]pyrene) uptake and metabolic activation | In part | – | – | – |
| NNK and NNN-DNA and haemoglobin adducts | Carcinogen (NNK/NNN) uptake and metabolic activation | In part | – | – | – |
| Apurinic sites in DNA | DNA damage | – | – | – | – |
| $^{32}$P-post-labelling of DNA | Carcinogen uptake and metabolic activation | Yes | Yes | – | – |
| Immunoassays for DNA damage | Carcinogen (mainly PAH) uptake and metabolic activation | Yes | Yes | – | – |
| Cellular biomarkers | | | | | |
| Urine mutagenicity | Mutagen uptake | Yes | Yes | Yes | Yes |
| Sister chromatid exchange in peripheral lymphocytes | DNA damage | Yes | Yes | Yes | – |
| Chromosomal aberrations and micronuclei frequency in lymphocytes | DNA damage | In part | – | – | – |
| Hypoxanthine-guanine phosphoribosyltransferase mutant frequency in cultured lymphocytes | Gene mutations | In part | – | – | – |
| Bronchial metaplasia and dysplasia, sputum atypia | Preneoplastic changes | In part | In part | – | – |
| Comet assay-DNA strand breaks | DNA damage | No consistent effect | – | – | – |
| Proteome differences | Effects on proteins | – | – | – | – |
| Promoter methylation | Effects on gene expression | – | – | – | – |
| Carcinoembryonic antigen | Inflammation[c] | In part | – | – | – |

[a]Some studies support change in biomarkers by smoking status.
[b]Published values may be unreliable owing to unrecognised artefact formation.
[c]Uncertainty exists over whether the biomarker is a measurement of inflammation.
NNAL = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol.
NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.
NNN = *N*-nitrosonornicotine.
PAH = polycyclic aromatic hydrocarbons.

mechanisms have been proposed to link oxidative stress with the development of lung cancer (Knaapen et al., 2006; Tudek et al., 2006).

Cigarette smoke particulate matter contains stable ROS with very long half-lives (Valavanidis et al., 2009) and some species are present in the gas phase of cigarette smoke (Pryor et al., 1985). These oxidative species may interact directly with tissues and cell membranes, leading to damage (Faux et al., 2009). Tissue damage results in the induction of inflammation, which in turn generates the release of further oxidative species and leads to an overall imbalance in the redox state (Asami et al., 1997). Oxidative stress has been implicated as a driving force behind smoking-related diseases, including lung cancer (Allavena et al., 2008; Smith et al., 2006) and, therefore, biomarkers of oxidative stress could be used as early indicators of a response to smoke exposure. Hatsukami et al. (2006) highlighted the biomarkers 8-oxoguanine, 8-oxo-7,8-dihydro-2-deoxyguanosine and the $F_2$-isoprostanes as being of importance in assessing the extent of smoking-related oxidative stress in the human body (discussed below).

## Biomarkers of oxidised DNA bases

Interactions between ROS and DNA can lead to the formation of oxidised DNA bases such as 8-oxo-7,8-dihydroguanine (8-oxoGua), which is able to induce base substitutions due to mispairing of 8-oxoGua with adenine (Cheng et al., 1992; Shibutani et al., 1991). Measurement of 8-oxoGua in DNA extracts has been problematic due to artefactual oxidation of guanine residues during sample preparation stages, however some improvements have been made to analytical protocols to reduce this (Evans et al., 2010). A recent prospective study by Loft et al. (2012) investigated the link between levels of urinary 8-oxo-Gua and the risk of lung cancer in 25 717 men and 27 972 women aged 50–64 years with 3–7 years follow-up. Overall, the incidence rate ratio (IRR) (95% confidence interval) of lung cancer was 1.06 (0.97–1.15) per doubling of 8-oxoGua excretion, however there was no significant effect of smoking on urinary 8-oxo-Gua levels. The association between lung cancer risk and 8-oxoGua excretion was significant among men [IRR: 1.17 (1.03–1.31)], never-smokers [IRR: 9.94 (1.04–94.7)] and former smokers [IRR: 1.19 (1.07–1.33)]. The authors concluded that the association between urinary 8-oxoGua excretion and lung cancer risk among former and never-smokers suggests that oxidative stress with damage to DNA is important in this group.

Urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) is a prominent form seen in free radical-induced oxidative lesions (Valavanidis et al., 2009; Valko et al., 2004). 8-OxodG can induce $G \rightarrow T$ transversions, which are among the most frequent mutations in human cancers (Pilger & Rudiger, 2006). An association between disease and raised concentrations of 8-oxodG has been described (Vineis & Husgafvel-Pursiainen, 2005). Cooke et al. (2006) reported elevated concentrations of 8-oxodG in a high proportion of cases of several pre-cancerous and cancerous conditions.

DNA repair is achieved by excision of 8-oxodG, which is excreted into the urine as an intact molecule. Thus, urinary 8-oxodG, which can be collected non-invasively, has been used as a biomarker for oxidatively generated DNA damage (Loft & Poulsen, 1999). In some reports, concentrations of 8-oxodG in the urine of smokers were elevated compared with those in non-smokers (Kristenson et al., 2003; Pourcelot et al., 1999) and oxidatively generated DNA damage was increased by 50% in smoking subjects (Loft et al., 1992). By contrast, a meta-analysis of oxidative stress and suitability of urinary 8-oxodG as a biomarker showed higher concentrations of 8-oxodG in non-smokers (Barbato et al., 2010). Furthermore, smoking was deemed to have little effect on some pathways involved in DNA damage and the anti-oxidative defence system (Besaratinia et al., 2001). Measurement of 8-oxodG in peripheral blood lymphocytes has also yielded mixed results, with some reports showing higher concentrations in smokers than in non-smokers (Asami et al., 1996, 1997; Lodovici et al., 2005; Yao et al., 2004) and some showing no difference (Besaratinia et al., 2001; van Zeeland et al., 1999; Zwingmann et al., 1998).

There is still considerable debate over the exact origins of urinary 8-oxodG (Halliwell & Whiteman, 2004). Current thinking suggests that diet and cell turnover have a negligible effect on the presence of 8-oxodG in urine and that it is derived mainly from the 2′-deoxynucleotide pool as a result of the ‘‘sanitising’’ action of Nudix hydrolases (Evans et al., 2010). Overall, therefore, given these mixed findings across studies, the use of 8-oxodG in MRTP assessment is not straightforward but could generate meaningful data in combination with other urinary DNA repair biomarkers, if used in a controlled study such as a short-term clinical comparison.

Other repair products in urine arising from oxidatively generated DNA damage have been suggested as biomarkers (Lowe et al., 2009), although there is less published literature available for review. For instance, the oxidation of thymidine by $HO^{\bullet}$ generates 5,6-dihydroxy-5,6-dihydrothymidine (thymidine glycol). Unlike most thymidine products, which are not generally potent pre-mutagenic lesions, the presence of thymidine glycol notably distorts the conformation of the DNA molecule and, if a cell with this DNA damage enters a division cycle, then the lesion is lethal (Wallace, 2002). As thymidine glycol has been found to be ‘‘inefficient as a pre-mutagenic lesion’’ (Evans, 1993), the link between levels of urinary thymidine glycol and malignant disease is questionable. That said, the concentration of urinary thymidine glycol correlates well with exposure to dimethylated arsenic compounds (Yamanaka et al., 2003) and increased excretion was reported in kidney transplant recipients with ischaemia–reperfusion-induced oxidatively generated kidney DNA damage (Makropoulos et al., 2000; Thier et al., 1999). Furthermore, in our own studies, differences between groups of smokers, former smokers and never-smokers were observed (Lowe et al., 2009). Thymidine glycol, therefore, might be useful as a generic biomarker of oxidatively generated DNA damage to complement other biomarkers such as 8-oxodG for MRTP assessment studies.

## Comet assay

The comet assay is widely used to detect DNA damage caused by oxidative species, such as free radicals, and it has been researched extensively. Most data have indicated systemic oxidation, with circulatory lymphocytes being a common

target tissue for use with the assay (Faust et al., 2004). Attempts to use tissues more relevant to the study of lung carcinogenesis, such as buccal epithelial cells, have been subject to many technical problems. Briefly, Pinhal et al. (2006) reported that upon harvesting cells from the buccal mucosa, the cellular population is a mixture of buccal epithelial cells, buccal lymphocytes and other cell types. The lymphocyte fraction readily forms typical comets, as found with peripheral blood lymphocytes; however, buccal epithelial cells were much more resistant to lysis and gave rise to atypical comets which were not suitable for analysis (Pinhal et al., 2006). Initial comparisons of non-smokers and long-term smokers using buccal lymphocytes by the same group did not show any significant difference with respect to DNA damage (Pinhal et al., 2006). Recently, Szeto et al. (2012) reported optimised conditions for the assessment of DNA damage by the comet assay using buccal lymphocytes, and the new protocol demonstrated a dose-response with $H_2O_2$ treatment and the genoprotective effects of quercetin. This promising development opens the door for further work with DNA repair enzymes for the study of oxidatively generated DNA damage in buccal cells.

While recent studies on the buccal cell comet assay show promise, there is currently insufficient data in the literature to qualify this assay as being suitable to detect differences in oxidatively generated DNA damage between smokers of MRTPs and conventional cigarettes. Further studies in groups of smokers and former smokers and smoking cessation studies would be required for this qualification.

## Anti-oxidant status

Anti-oxidants protect the body from the harmful effects of free radical damage. Thus, the measurement of anti-oxidant levels in target tissues or biofluids might be a way to assess the extent of an oxidative insult. Anti-oxidants as biomarkers can be divided into the following groups: total anti-oxidant capacity (TAC), which indicates the oxidant-buffering potential of a tissue or biofluid; specific compounds (which can be absorbed from the diet or synthesised *in vivo*), precursors or metabolites, such as ascorbic acid, that scavenge free radicals; and enzyme activity, such as that of superoxide dismutase (SOD), which reflects conversion of free radicals into less toxic entities.

### Total anti-oxidant capacity

TAC involves enzymatic components (SOD, catalase and glutathione peroxidise (GPx) plus several other enzymes), endogenous small macromolecules (bilirubin, albumin, ceruloplasmin and ferritin) and molecules of dietary origin (ascorbic acid, α-tocopherol, β-carotene and polyphenols), and is generally decreased when oxidative stress is increased (Young, 2001). Importantly, variation in anti-oxidant levels has been associated with increased risk of developing cancer (Serafini et al., 2006; Stephens et al., 2009) and differs between smokers and non-smokers (Aycicek & Ipek, 2008; Bloomer, 2007; Buico et al., 2009). The use of TAC as a biomarker, however, may be criticised: *in vitro* and *in vivo* results are discordant (Somogyi et al., 2007); the results also vary across different TAC assays (Cao & Prior, 1998); and additionally, oxidation sources, targets and measurements differ across assays used in plasma (Somogyi et al., 2007). In most intervention trials, dietary supplementation did not alter TAC, which was possibly explained by the effect of endogenous anti-oxidants (Collins, 2005). Hence, the recommended approach is to measure individual anti-oxidants and markers of oxidatively generated damage in parallel with TAC (Young, 2001).

### Anti-oxidant compounds

Ascorbic acid and dehydroascorbic acid have been used as biomarkers of oxidative stress for many years. Ascorbic acid is a free radical scavenger and is involved in pathways that regenerate other anti-oxidants. Throughout plasma and tissues, it exists mainly in its reduced state. Oxidation by the semidehydroascorbyl radical produces dehydroascorbic acid, which is transported into, for example, erythrocytes and regenerated intracellularly to ascorbic acid. Thus, increased concentrations of dehydroascorbic acid suggest a redox imbalance and inadequate recycling capacity (Lykkesfeldt, 2007a), and ascorbic acid acts as a general biomarker of anti-oxidant status.

Comstock et al. (1997) reported on various anti-oxidant compounds in 258 patients with lung cancer and 515 matched healthy controls from the USA. They measured ascorbic acid in plasma, and α-carotene, β-carotene, cryptoxanthin, lutein and zeaxanthin, lycopene, α-tocopherol, selenium and peroxyl radical absorption capacity in serum or plasma. Concentrations of cryptoxanthin, β-carotene and lutein and zeaxanthin were significantly lower in lung cancer patients than in controls. Small differences, consistent with a protective action, were noted for α-carotene and ascorbic acid, but they were non-significant. From this study, endogenous β-carotene (cryptoxanthin, α-carotene and ascorbic acid might be also) appears to be a protective factor against lung cancer. The other compounds were not associated with lung cancer risk.

Whether dietary carotenoids have a protective effect against the development of lung cancer has been widely studied. Most data are from epidemiological studies, which show that α-carotene, β-carotene, lycopene, β-cryptoxanthin, retinol, lutein and zeaxanthin have protective effects (Holick et al., 2002; Ito et al., 2005; Michaud et al., 2000; Yuan et al., 2003). Intake via fruit and vegetables in a healthy diet without supplementation seems sufficient (Gallicchio et al., 2008; Wright et al., 2003), although effects seem to differ between men and women (Ito et al., 2005). Smokers have lower levels of circulating carotenoids than never-smokers and ex-smokers (Alberg, 2002) and Goodman et al. (2003) reported that healthy current smokers had lower mean levels of anti-oxidant compounds overall than did ex-smokers. No data are available, however, regarding the mechanisms underlying the lowered concentrations.

Cigarette smoking can affect the levels of some anti-oxidant compounds. Alberg (2002) reported that circulating concentrations of ascorbic acid and vitamin A precursors (carotenoids and cryptoxanthin) *in vivo* decreased with increasing numbers of cigarettes smoked per day. The inverse association between cigarettes per day and vitamin E levels,

however, was weak. Subsequent studies have confirmed these observations (Calikoglu et al., 2002; Lykkesfeldt et al., 2003).

Lykkesfeldt et al. (1997) reported an increase in the ratio of dehydroascorbic acid to ascorbic acid in smokers compared with that in non-smokers, and showed a significant inverse linear correlation between these two compounds in the plasma of smokers. Similarly, Chávez et al. (2007) reported raised concentrations of dehydroascorbic acid in smokers compared with those in non-smokers. Four weeks after smoking cessation, Polidori et al. (2003) noted that concentrations of ascorbic acid in plasma significantly increased. In female smokers, who received dietary supplements of 500 mg ascorbic acid and 400 IU Vitamin E for 15 months, levels of benzo(a)pyrene DNA adducts in leukocytes fell by 31% (Mooney et al., 2005).

The enzyme *mu* glutathione-*S*-transferase, which is encoded by *GSTM1*, has a role in the detoxication of benzo(a)pyrene and, therefore, helps to protect against oxidatively generated DNA effects. In women with the *GSTM1*-null genotype, adduct levels in leukocytes were lowered by 43% at 15 months (Mooney et al., 2005). By contrast, adduct concentrations did not differ from baseline in male smokers who received anti-oxidant supplementation (Mooney et al., 2005). In another study of dietary anti-oxidant supplementation, concentrations of 8-oxodG and concentrations of protein-bound carbonyls in peripheral blood decreased in smokers who consumed 200 IU vitamin E or 1.8 g red ginseng daily (Lee et al., 1998). Furthermore, Duthie et al. (1996) reported that daily supplementation with 100 mg ascorbic acid, 289 mg vitamin E and 25 mg β-carotene for 20 weeks was associated with significantly decreased endogenous oxidatively generated base damage in the lymphocyte DNA of smokers and non-smokers. In addition, *in vitro* tests showed increased resistance to oxidatively generated damage after challenge with $H_2O_2$ for all recipients of the dietary supplements.

On the basis of these studies, measurement of serum carotenoids as biomarkers for use in MRTP assessment studies seems warranted. However, attention should be paid to whether dietary intake of fruit and vegetables alters the effects and so studies in a controlled environment would appear to be most appropriate for initial assessment.

### Anti-oxidant enzymes

The activities of SOD, GPx and catalase are most frequently measured in the assessment of oxidative stress. The discovery of SOD greatly improved understanding of anti-oxidant defence systems, since it led to the theory of oxygen toxicity (Gregory & Fridovich, 1973). GPx is the main enzyme involved in the removal of peroxides in human tissue, and is highly specific for reduced glutathione (Chance et al., 1979). GPx reacts with $H_2O_2$ and other peroxides to catalyse the reduction of fatty acid hydroperoxides (Gaber et al., 2001). Glutathione reductase is the complementary enzyme to GPx and is involved in the regeneration of reduced glutathione. Measurement of glutathione reductase alongside GPx, therefore, provides information on the status of the entire glutathione anti-oxidant system.

Reported anti-oxidant enzyme activity seems to have differed within and between biofluids and between groups of smokers. Catalase activity was raised in the plasma of smokers versus that in non-smokers (Zhang et al., 2007) but was lower in serum (Aycicek & Ipek, 2008). GPx activity in plasma was reported to be lower in smokers than in non-smokers in one study (Abou-Seif, 1996) but similar in another (Orhan et al., 2005). Concentrations of SOD and GPx have been lower in smokers than in non-smokers in some studies (Hulea et al., 1995; Kim et al., 2003) but higher in other studies (Ozguner et al., 2005). A weak correlation between SOD activity in plasma and the number of cigarettes smoked has been reported (Zhang et al., 2007). Pannuru et al. (2011) reported increased plasma and erythrocyte membrane lipid peroxidation and nitrite/nitrate levels in smokers compared with those in controls. The activities of SOD, catalase and GPx were also increased in erythrocyte lysate. In addition, Greabu et al. (2008) reported significantly decreased GPx activity in the saliva of smokers compared with that in non-smokers. Inconsistent results also have been found in various tissues. In bronchoalveolar cells Hilbert & Mohsenin (1996) reported increased activity of SOD, GPx and catalase, while DiSilvestro et al. (1998) reported decreased SOD activity in bronchoalveolar lavage fluid, and Harju et al. (2004) reported increased SOD expression and activity in the alveolar epithelium of smokers, compared with those in non-smokers.

This wide variation in activity makes it difficult to use anti-oxidant enzymes as biomarkers of oxidative stress in smoking studies. If used at all, other biomarkers of oxidative stress, such as anti-oxidant levels, TAC and $F_2$-isoprostanes, must always be measured at the same time to help interpret the data.

## Biomarkers of lipid peroxidation

Among the mechanisms of damage caused by ROS, lipid peroxidation is probably the most extensively investigated. Oxidation of cell membrane phospholipids results in the formation of unstable lipid hydroperoxides and secondary carbonyl compounds, such as aldehydic products (Liebler & Reed, 1999). The major aldehyde products of lipid peroxidation are 4-hydroxynonenal (4-HNE), acrolein, malondialdehyde (MDA) and crotonaldehyde. They are highly reactive molecules that can damage DNA by the formation of exocyclic adducts, which are promutagenic (Esterbauer et al., 1991; Voulgaridou et al., 2011). Acrolein and crotonaldehyde are constituents of combustible cigarette smoke (Gregg et al., 2004) and so measurements of biomarkers related to them would give results related both to exposure and to biological effects. This leaves MDA and 4-HNE as candidate biomarkers of biological effect to be considered for MRTP assessment.

### Malondialdehyde

Altered concentrations of MDA (Fahn et al., 1998; Lykkesfeldt, 2007b; Tanriverdi et al., 2006) and MDA DNA adduct levels (Munnia et al., 2006) have been reported in tissues and biofluids *in vivo* after exposure to cigarette smoke. Furthermore, MDA DNA adduct levels are raised in patients with lung cancer who smoked but not in those who did not smoke (Munnia et al., 2006). Bartsch et al. (1992) reported an inverse correlation between MDA concentrations and the

number of days the lung cancer patients had refrained from smoking, and that concentrations were higher in recent smokers with cancer than in those without cancer.

Studies of MDA concentrations in blood have shown conflicting evidence. Although several studies found increased concentrations of MDA in the serum of adult smokers (Durak et al., 2002; Isik et al., 2007; Kim et al., 2003), Zhang et al. (2007) reported that concentrations were significantly lower in smokers than in non-smokers. Ermis et al. (2004, 2005) studied MDA concentrations in the sera of mothers who smoked, those exposed to environmental tobacco smoke and those who had never smoked, and they reported no significant difference between the smokers and non-smokers. Anti-oxidant status might explain some degree of these inconsistent findings; for instance Ermis et al. (2004) reported marginally but non-significantly higher mean MDA concentrations and SOD activity in mothers who smoked than in those who did not, while GPx activity was significantly higher. The high GPx activity could have limited formation of MDA. Similar results and conclusions were reported by Chávez et al. (2007). By contrast, Ozguner et al. (2005) reported small but significant rises in MDA concentrations and in SOD and GPx activities in plasma of smokers. They suggested that this finding indicates inadequate anti-oxidant protection of the respiratory system.

Measurement of MDA in lung tissue or lung fluids would be most relevant in MRTP assessment studies, but measurement in serum and erythrocytes might be useful as an indication of systemic oxidative stress alongside other measures of anti-oxidant status. However, due to the conflicting data, if measurements of MDA are to be used for MRTP assessment, then they should be made in conjunction with those of other biomarkers of oxidative stress in the same tissue or biofluid and, even then, interpretation might not be straightforward.

## 4-Hydroxynonenal

4-HNE is a highly reactive molecule, considered to be one of the main generators of oxidative stress, formed by enzymatic and non-enzymatic pathways during lipid peroxidation (Voulgaridou et al., 2011). Exposure of human cell lines to 4-HNE induces DNA adduct formation in the human *p53* gene, at a hotspot that is associated with hepatocellular carcinoma (Hu et al., 2002), suggesting potential for its use in MRTP studies. Furthermore, Rahman et al. (2002) reported elevated levels of 4-HNE-modified protein in airway and alveolar epithelial cells, endothelial cells, and neutrophils in smokers and ex-smokers with COPD, compared to subjects without COPD, although Yagi et al. (2006) did not find differences between non-smokers, smokers and COPD patients, using tissue immunohistochemistry. Recently, a urinary assay for the mercapturic acid conjugate of 4-HNE has been developed and reductions following 12-weeks of smoking cessation reported (Kuiper et al., 2010). In other studies, these authors reported that vitamin C supplementation reduced the urinary 4-HNE secretion (Kuiper et al., 2011). Thus, biomarkers of 4-HNE show promise as biomarkers of biological effect in smokers but, clearly, further work is required to understand the differences observed between detection methods and to estimate the possibility of dietary confounding in non-clinical studies. Urinary biomarkers for 4-HNE offer a non-invasive route to progress studies of this potential biomarker, with regard to MRTP assessment.

## $F_2$-isoprostanes

Prostaglandin $F_2$-like compounds formed *in vivo* by a non-enzymatic mechanism were first described by Morrow et al. (1990a,b). These eicosanoid molecules are derived from the peroxidation of arachidonic acid and are found in biological membranes. The formation of the $F_2$-isoprostanes and the various isoforms was reviewed thoroughly by Janssen (2001).

Various groups have measured $F_2$-isoprostane concentrations in the biofluids of smokers. The mean concentrations of free and esterified $F_2$-isoprostanes in the urine and plasma of smokers were significantly raised compared with those in non-smokers. Concentrations in plasma are significantly decreased in smokers 2 weeks after they have stopped smoking compared with those in the plasma of participants who continued to smoke (Morrow et al., 1995). Frost-Pineda et al. (2011) reported that concentrations of 8-epi-PGF2$\alpha$ (a specific $F_2$-Isoprostane) were 42% higher in adult smokers then non-smokers in a large cross-sectional study ($p < 0.0001$). Multiple step-wise regression models showed that body mass index (BMI) and age were the most important factors in a model which included the number of cigarettes smoked per day, however, in another model including urinary nicotine metabolites, BMI and urinary nicotine metabolites were the most important factors and accounted for 23% of the variability in the data (Frost-Pineda et al., 2011). Higher concentrations of $F_2$-isoprostanes have been found in the exhaled breath condensate of smokers compared to that of non-smokers (Borrill et al., 2008; Montuschi et al., 2000), as well as in patients with lung cancer (Dalaveris et al., 2009). Furthermore, Epplein et al. (2009) noted that the risk of lung cancer was doubled in men with $F_2$-isoprostane concentrations in urine in the second and third tertiles, independent of smoking status. Numerous other studies have been conducted and the most relevant results are summarised in Table 4.

In view of the non-enzymatic generation of $F_2$-isoprostanes, the tissues and biofluids in which they can be measured, and the reversal of changes in concentration after smoking cessation, these compounds should prove to be useful biomarkers of oxidative stress for MRTP assessment studies.

## Conclusions

In this short review, we have considered biomarkers related to oxidative stress because this set of biological effects are associated with disease endpoints, including cancer and specifically lung cancer. With regard to oxidatively generated damage to DNA, the biomarkers 8-oxodG and thymidine glycol have been detected in differing concentrations in the biofluids of groups of smokers and former smokers and therefore hold promise for use in MRTP assessment. With regard to overall anti-oxidant status, the TAC generates conflicting data but some individual chemical groups (i.e. the ratio of serum dehydroascorbic acid to ascorbic acid and the

Table 4. Concentrations of $F_2$-isoprostanes in biofluids from groups of smokers and non-smokers.

| Study | Biomatrix | Number of people | $F_2$-isoprostane concentration: Smokers | $F_2$-isoprostane concentration: Non-smokers |
|---|---|---|---|---|
| Keaney et al. (2003) | Urine | 2828 | 240 ± 145 ng/mmol creatinine | 148 ± 100 ng/mmol creatinine |
| Liang et al. (2003) | Urine | 60 | 530 ± 370 ng/mmol creatinine | 250 ± 150 ng/mmol creatinine |
| Oguogho et al. (2000) | Urine | 14 | 65 ± 16 ng/mmol creatinine | 25 ± 5 ng/mmol creatinine |
| Harman et al. (2003) | Urine | 138 | 124 ± 11 ng/mmol creatinine | 58 ± 5 ng/mmol creatinine |
| Lowe et al. (2009) | Urine | 20 | 1.04 ± 0.36 μg/mmol creatinine | 0.61 ± 0.21 μg/mmol creatinine |
| Zedler et al. (2006) | Urine | 115 | Median 1.94 (range 0.69–4.61) μg/mmol creatinine | Median 1.03 (range 0.58–2.17) μg/mmol creatinine |
| Montuschi et al. (2000) | Exhaled breath condensate | 10 | 24.3 ± 2.6 pg/ml | 10.8 ± 0.8 pg/ml |
| Borrill et al. (2008) | Exhaled breath condensate | 28 | 49.9 ± 2.9 pg/ml | 8.9 ± 4.0 pg/ml |
| Reilly et al. (1996) | Urine | 10 | 176.5 ± 31.0 pmol/mmol creatinine[a] | |
| Reilly et al. (1996) | Urine | 10 | 92.7 ± 5.0 pmol/mmol creatinine[b] | |
| Morrow et al. (1995) | Plasma (free) | 10 | 250 ± 156 pmol/l | 156 ± 67 pmol/l[c] |
| Morrow et al. (1995) | Plasma (esterified) | 10 | 469 ± 108 pmol/l | 624 ± 214 pmol/l |
| Frost-Pineda et al. (2011) | Urine | 1044 non-smokers 3322 smokers | 1890 ± 1053 ng/24 h | 1331 ± 754 ng/24 h[d] |

All data are statistically significant ($p < 0.05$) and are presented as mean ± SD unless stated otherwise.
[a]Heavy smokers.
[b]Moderate smokers.
[c]After 2 weeks' abstinence.
[d]$p < 0.0001$.

concentration of serum carotenoids) were reported to show differences between groups of smokers and on smoking cessation. However, in non-clinical settings, the need to design studies to avoid dietary confounding with the use of these biomarkers is imperative. With regard to lipid peroxidation, elevated urinary $F_2$-isoprostanes were associated with increased risk of lung cancer and differences are evident between groups of smokers and on smoking cessation. Hence, urinary $F_2$-isoprostanes offer the potential to generate data relevant to lipid peroxidation, endogenous ROS generation and lung cancer risk in MRTP studies. Other biomarkers, such as 4-HNE, have many suitable characteristics but require to be qualified (e.g. by showing reproducible, altered concentrations in smoking cessation studies) before use in MRTP assessment studies.

## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and contributed equally to the writing of this article.

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