Biomarkers of Oxidative Stress and the Relationship to Cigarette Smoking

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Abstract: Oxidative stress has been implicated in the development of smoking-related diseases such as lung cancer, cardiovascular disease and chronic obstructive pulmonary disease. Damage to biological tissues from interactions with free radicals found within the smoke and induced within cells by smoke exposure, is thought to contribute to smoking-related disease development. Many of these radical components are very short-lived *in vivo* due to their highly reactive nature and the highly efficient detoxification mechanisms possessed by the body to counteract their effects. Hence, biomarkers are needed to assess the extent of radical exposure and subsequent oxidative damage in humans. Oxidised lipids, proteins and DNA bases persist longer *in vivo*, and have been extensively investigated as surrogate measures of radical damage. Furthermore, assessment of the body's antioxidant defence mechanisms such as antioxidant enzyme activity and antioxidant compounds can help to understand the extent of radical exposure. This mini review critically evaluates various biomarkers falling into these categories and the consistency of their relationship with smoking status, as a preliminary evaluation of their usefulness in dissecting disease pathways in smokers. The future use of such biomarkers is also briefly discussed.

Keywords: 8-hydroxydeoxyguanosine, Antioxidant, Biomarker, Isoprostane, LDL cholesterol, Malondialdehyde, Oxidative Stress, Smoking.

INTRODUCTION

Cigarette smoke is a complex mixture of over 5000 different chemicals that partition between a gaseous and a particulate phase [1]. Interaction between the smoke constituents adds further complexity to the mixture. Much research has focused on lung cancer, diseases of the respiratory tract (e.g. chronic obstructive pulmonary disease) and diseases of the coronary and other vascular systems in the context of cigarette smoking. However, the relationship between specific tobacco smoke chemicals and mechanistic steps in these diseases remains unclear [2]. Oxidative stress is thought to be a contributing factor to the development of smoking-related diseases [3, 4].

The mechanistic link between exposure to radical species arising from the smoke itself or from intracellular sources as a result of cigarette smoke exposure has been discussed in depth earlier in this supplement (Fearon *et al.*). Briefly, radical components can arise within the body as a consequence of normal cellular respiration, exposure to radical-generating compounds and pathological conditions causing an imbalance in the pro/antioxidant homeostasis, both locally and/or systemically. Once radical components are formed, they are converted to other chemically more stable forms through reactions with cellular biomolecules such as lipids, proteins and DNA. Such interactions can damage these molecules, and if not repaired, could potentially lead to toxicity and have pathological consequences. The human body has well-developed defence mechanisms against radical species which maintain homeostasis by removing radical species, detoxifying oxidised molecules and repairing DNA. Thus, oxidative stress occurs when the levels of radicals or radical-generating compounds exceed the protective threshold offered by the body's defences.

Although unfavourable for the body, biomolecules damaged by interactions with radical species are an important source of biomarkers for the measurement of oxidative stress. Furthermore, measurement of the body's local and systemic antioxidant defences and repair mechanisms can also yield information on the extent of exposure to radical species. At the present time, a variety of biomarkers of oxidative stress are being utilised, and current efforts are focused on understanding sources of variability in subject populations and determining their relevance to disease processes. In parallel, there is a need to validate methodologies for the identification and accurate quantification of such biomarkers.

This review considers biomarkers of oxidative stress related to antioxidant levels and oxidative DNA, protein and lipid damage in the context of cigarette smoking. Sources of reference for this review were selected from biomarker studies in the past 20 years, which investigated biomarker levels and correlations with smoking status. Insight into how these biomarkers might be used in future studies of smokers is provided.

BIOMARKERS OF LIPID OXIDATION

An imbalance between levels of oxidants (increased) and antioxidants (reduced), which is responsible for oxidative stress, can promote the progression of many smoking-related diseases [6, 7]. Among the mechanisms of damage caused by reactive oxygen species (ROS), lipid peroxidation is probably the most extensively investigated process. Oxidation of cell membrane phospholipids produces a chain reaction, which targets the polyunsaturated acids and results in the formation of unstable lipid hydroperoxides and secondary carbonyl compounds such as aldehydic products [5]. Among the latter, malondialdehyde (MDA) has received most attention in the literature.

MALONDIALDEHYDE (MDA)

It has been demonstrated that smokers present with increased oxidative stress as documented by elevated levels of MDA. Altered levels of MDA in biological tissues (lung) and in other compartments (blood, urine, etc.) [7, 8] following cigarette smoke exposure has been well documented *in-vivo* [9]. Recent data show contrasting evidence for the use of MDA as a biomarker of smoking-related oxidative stress. A number of studies showed elevated MDA in the serum of adult smokers [10-12], whereas Zhang and coworkers reported that serum MDA was significantly lower in smokers compared to non-smokers [13]. Ermis and coworkers studied MDA levels in the sera of smoking, passive smoking and non-smoking mothers and reported no significant difference between smoking and non-smoking mothers [14, 15].

The following studies point out that antioxidant status could play a vital role in regulating/influencing the levels of MDA seen in biofluids. In the studies by Ermis and co-workers, mean MDA levels were slightly higher in smoking mothers than in the non-smoking group, although not statistically significant. In parallel, the activity of superoxide dismutase (SOD) was not significantly different and glutathione peroxidase (GPx) was significantly higher in the smoking group. Thus, the levels of MDA formation could have

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been limited by the elevated GPx activity providing pro/antioxidant balance to the system. Similar results and conclusions were reported by Chávez *et al.* [16]. In contrast, a study of young female smokers by Ozguner and coworkers reported that levels of MDA were elevated alongside both SOD and GPx in the respiratory tract [17].

In summary, MDA levels vary in different biological compartments and under different degrees of smoking status. The contrasting data could be due to several possibilities:

- Variation in the method used and production of artifactual derivatives
- Varying levels of antioxidants in the biological compartments of smokers, which in turn could impact on the amount of MDA produced (discussed later in this review).
- Dietary differences influencing the overall antioxidant status of study subjects and in turn, influencing MDA levels

One possibility to explain the conflicting data could be dietary influences on the overall antioxidant buffer in the tissue/biofluid of interest. Of the smoking- related MDA studies reported to date, few studies have actively controlled the diets of the study subjects with respect to antioxidant intake. Alberg presented and discussed a selection of studies which controlled dietary levels of specific antioxidants including ascorbic acid and carotenoids [18]. The studies presented in the review did not address total antioxidant capacity, and more recently, this issue was further investigated. Bloomer measured MDA levels and antioxidant capacity in young smokers and non-smokers (age 24 ± 4 years) and reported significantly elevated MDA levels in the smoking group. Antioxidant-reducing capacity was significantly lower for smokers compared to non-smokers, whereas no significant difference was noted for total glutathione or trolox equivalent antioxidant capacity (trolox equivalent antioxidant capacity measures the antioxidant capacity of a given substance as compared to trolox as the standard). There was no significant difference between each study group with respect to dietary intake of antioxidant vitamins (A, C and E). The author concluded that chronic smoking reduces serum antioxidant levels and increases oxidative stress independently of dietary intake of antioxidants in a young population [19]. Taken together, this evidence speaks against a possible impact of diet on antioxidant levels mentioned above, and similar studies in older smokers would be required to confirm this.

F_2 ISOPROSTANES (ISOP)

Usually, eicosanoids with biological activity such as leukotrienes, prostaglandins and thromboxane are derived from arachidonic acid by the action of lipoxygenases and cyclooxygenases, respectively [20].

In 1990, Morrow *et al.* reported the discovery of a group of novel prostaglandin F_2-like compounds which were formed *in vivo* by a non-enzymatic mechanism. These F_2 isoprostanes (ISOP) are eicosanoid molecules derived from the peroxidation of arachidonic acid found in biological membranes. They were linked to oxidative mechanisms when their formation was suppressed by antioxidant compounds in stored biological fluids [21, 22]. One of these novel compounds, 8-epi-prostaglandin $F_{2\alpha}$ (8epiPGF), was subsequently found to have biological activity as an agonist of the vascular thromboxane and endoperoxide receptors in rat smooth muscle causing vasoconstriction, and also an antagonist of these receptors on rat and human platelets by competitive inhibition [23]. The formation of the isoprostanes and the various isoforms are extensively described in a review by Janssen [24].

Since tobacco smoking is associated with oxidative stress and cigarette smoke is known to contain radical species [25, 26], groups began to look for evidence of elevated ISOP levels in the biological fluids of cigarette smokers. Morrow and co-workers measured the levels of ISOP in human plasma and their urinary metabolites in age-matched and sex-matched smokers and non-smokers. The mean levels of free and esterified ISOP in urine and plasma were significantly elevated in smokers compared to non-smokers. After 2 weeks of smoking abstinence, ISOP levels in plasma were significantly lower compared to those measured when the subjects still smoked [27]. Numerous other studies have also noted differences in ISOP levels between smokers and non-smokers, and these are listed in Table **1**.

Additionally, Gopaul and co-workers demonstrated that ISOP can be formed from the copper-mediated oxidation of low density lipoprotein cholesterol and that 8epiPGF was the principal ISOP formed [37]. Increased ISOP levels have been detected in the exhaled breath condensate of smokers compared to non-smokers [34, 35] and also in smoking asthmatics compared to healthy smokers [38]. Elevated levels of ISOP have been detected in patients with chronic obstructive pulmonary disease [39] and lung cancer [40]. Given the evidence of how ISOP is generated, elevated levels of ISOP in the lungs, blood and urine of smokers, and the reductions in response to smoking cessation, it is clear that ISOP is a useful biomarker of smoking induced oxidative stress.

OXIDISED LOW DENSITY LIPOPROTEIN CHOLESTEROL

Oxidised low-density lipoprotein (oxLDL) is assumed to play an important role in the development of diseases of the cardiovascular system [41-47]. The chemical nature of oxLDL is complex. The LDL particle itself is made up of a lipid moiety and a protein moiety mainly consisting of apolipoprotein B-100. Both the lipid and protein moieties of low density lipoprotein (LDL) can be chemically modified by oxidative stress [43]. The oxidation of the protein moiety of LDL can form several products including protein-bound 3-nitrotyrosine (3-N-T), malondialdehyde-modified LDL [48], and 3-chlorotyrosine (3-Cl-T) [49, 50]. Elevations in plasma oxLDL levels (oxidised protein moiety) have been reported in most studies comparing smokers to non-smokers [19, 51-57]. However, a few studies showed no difference between these groups [58-60].

Oxidation of the lipid fraction of LDL can lead to formation of conjugated dienes by molecular rearrangement [61]. Conjugated dienes therefore can be utilised as biomarkers for the determination of oxidised LDL *in vivo* following extraction of the LDL particles from the blood sample [62]. Ahotupa and Asankari described various associations between diene-conjugated LDL (dcLDL) and various risk factors for cardiovascular disease including atherosclerotic burden, hypertension, arterial elasticity, obesity, hyperglycemia, and physical inactivity [62]. With respect to tobacco smoking, Mosca and co-workers reported significantly elevated dcLDL levels in coronary heart disease patients who were former smokers compared to patients who never smoked [55]. Brown and co-workers also reported significantly higher levels of conjugated dienes in the plasma of smokers compared to non-smokers. After 10 weeks of vitamin E supplementation, dcLDL levels significantly decreased in smokers approaching that seen in non-smokers [63].

Auto-antibodies in plasma can also be used as a biomarker for oxLDL formation [64], and they have been explored extensively as a biomarker of cardiovascular disease risk. Initially, it was assumed that high levels of circulating oxLDL are accompanied by high antibody titers. However, it has recently been shown that oxLDL and anti-oxLDL antibodies are inversely correlated [65], and that high anti-oxLDL titers are a predictor for lower cardiovascular disease risk [66].

Table 1. Levels of ISOP in Smokers and Non-Smokers

Biomatrix	N	Smokers	Non smokers	Units	Author
Urine	2828	240±145	148±100	ng/mmol creatinine	Keaney [28]
Urine	60	530±370	250±150	ng/mmol creatinine	Liang [29]
Urine	14	65±16	25±5	ng/mmol creatinine	Oguogho [30]
Urine	138	124±11	58±5	ng/mmol creatinine	Harman [31]
Urine	20	1.04±0.36	0.61±0.21	µg/mg creatinine	Lowe [32]
Urine	50 & 65	1.94, 0.69, 4.61*	1.03, 0.58, 2.17*	µg/mg creatinine	Zedler [33]
EBC	10	24.3±2.6	10.8±0.8	pg/ml	Montuschi [34]
EBC	18 & 10	49.9±2.9	8.9±4.0	pg/ml	Borril [35]
		Heavy smokers	**Moderate smokers**		
Urine	10	176.5±31	92.7±5	pmol/mmol creatinine	Reilly [36]
		Smokers	**After 2 weeks abstinence**		
Plasma (Free)	10	250±156	156±67	pmol/liter	Morrow [27]
Plasma (Esterified)	10	624±214	469±108	pmol/liter	Morrow [27]

All data were statistically significant ($p<0.05$) and presented as mean ± SD

* denotes median, minimum, maximum respectively

With respect to tobacco smoking, there are conflicting data in the literature on anti-oxLDL antibody titers. In a number of studies, smoking was found to increase the antibody titer [67-73], while no effects were reported in other studies [74-81] or a decrease in anti-oxLDL antibody titers with smoking was observed [82, 83].

BIOMARKERS OF OXIDATIVE DNA DAMAGE

Since DNA contains genetic information, the biological impact of damage caused by ROS, mostly in the form of $HO^{\bullet}$, can be profound. Damage to DNA by ROS may result in mutations, which are associated with carcinogenesis [84]. Cigarette smoking is known to trigger ROS production leading to oxidative stress [26]. Cigarette tar contains high concentrations of stable ROS with very long half-lives [85]. It has also been reported that ROS are present in the gas phase of tobacco smoke [86].

OXIDISED GUANINE BASES

Interactions between ROS and DNA can lead to the formation of oxidised DNA bases such as the oxidised guanosine: 8-hydroxydeoxyguanosine (8OHdG, 8-oxodG) [87]. 8-OHdG/8-oxodG is one of the predominant forms of free-radical induced oxidative lesion [85, 88]. This is of high biological relevance because 8-OHdG/8-oxodG possesses the ability to induce G-T transversions, which are amongst the most frequent mutations found in human cancers [89]. The link between disease and the presence of elevated 8-OHdG/8-oxodG is well documented. Cooke and colleagues reviewed measurements on the levels of 8-OHdG/8-oxodG in a large number of cancerous and pre-cancerous conditions [90] and found elevated levels in most cases.

8-OHdG/8-oxodG is repaired by excision from the DNA and eliminated in the urine as an intact molecule, where its concentration can be measured. This approach is advantageous as urine collection is non-invasive. Investigations aiming to quantify 8-OHdG/8-oxodG in smokers' urine tend to find increased levels in smokers compared to non-smokers [91, 92]. Based on multiple regression analysis, it has been suggested that smoking is associated with a 50% increase in oxidative DNA damage [93]. Investigations based on quantification of 8-OHdG/8-oxodG levels exclusively in peripheral blood lymphocytes (PBL) reported mixed results. Several investigators detected higher levels of 8-OHdG/8-oxodG in smokers compared to non-smokers [94-97]. In contrast, several studies did not show increased levels of 8-OHdG/8-oxodG in smokers compared to non-smokers [98-100]. A very recent meta-analysis by Barbato and co-workers, involving traffic air pollution, oxidative stress and suitability of urinary 8-OHdG/8-oxodG as a biomarker, concluded that higher levels of 8-OHdG/8-oxodG were found in non-smoking subjects compared to smokers [101]. Furthermore, one study concluded that cigarette smoking has a low impact upon certain pathways involved in DNA damage and the antioxidative defence system [102]. With this contrasting data, it is difficult to determine if the measurement of 8OHdG/8-oxodG would help an investigator form robust conclusions regarding smoking induced oxidative stress at the DNA level. It is unclear whether elevated levels of 8OHdG/8-oxodG reflect increased exposure to oxidant molecules, or a more efficient DNA excision repair system in the subject.

THE COMET ASSAY

The Comet assay or single cell gel electrophoresis assay (SCGE) is a widely used technique for measuring and analysing DNA breakage in individual mammalian cells which can be applied to both *in vitro* and *in vivo* systems. Cells exposed to a test agent are lysed to leave behind intact nuclei, containing the cellular DNA. Nuclei are then suspended in a thin agarose gel on a microscope slide. The tightly coiled cellular DNA is then allowed to unwind under alkaline conditions (>pH 13), electrophoresed and stained *in situ* with a fluorescent DNA-binding dye. During the electrophoresis, the damaged DNA strands (single strand breaks) are drawn away from the main nucleus as DNA is negatively charged. The broken strands of DNA travel further in the agarose gel than the unbroken strands. Eventually, the shape observed under a fluores-

cence microscope resembles that of a comet, with a dense 'head' of unbroken DNA and a 'tail' of fragmented DNA. The extent of DNA migration and the intensity of the tail correlate to the extent of DNA damage incurred by the cell.

The Comet assay can detect several different types of DNA damage. Of interest for the purpose of this review is the fact that it is able to detect different kinds of oxidised nucleotides if specific endonucleases such as formamido-pyrimidine-glycosylase (FPG), endonuclease III (Endo III) and 8-hydroxyguanine DNA-glycosylase (hOOG) are utilised. Detection of oxidative lesions is achieved by allowing the cells a period of time to repair non-oxidative single strand breaks. As oxidative lesions take longer to repair than non-oxidative lesions, these enzymes can be added to the DNA after this period of repair time, to induce new single strand breaks at sites of oxidation. The new single strand breaks are then detected by electrophoresis as mentioned previously. The FPG endonuclease excises 8-OHdG/8-oxodG and ring-opened purines resulting from oxidation, while endonuclease III excises oxidised pyrimidines. Finally, hOOG detects 8-OHdG/8-oxodG and methyl-fapy-guanine. The Comet assay combined with these enzymes has been shown to detect higher levels of 8-OHdG/8-oxodG in a number of diseases including diabetes, Alzheimer's disease and myocardial ischemia [103-106].

With respect to cigarette smoking, a variety of conclusions have been reported when applying endonucleases for detection of oxidised bases. One investigation displayed statistically significant FPG-sensitive sites in PBLs of smokers compared to non-smokers whereas it was shown to be considerably higher, but not statistically significant, in passive and ex-smokers compared to non-smokers [107]. In another study however, the FPG Comet assay did not reveal a significant difference between PBL damage in heavy smokers and non-smokers [108]. Oxidised DNA damage could also not be associated with smoking history in an Italian population of randomly selected subjects [109]. In contrast, smoking was shown to have a significant effect on DNA damage measured by the Endo III comet assay in a Greek population [110].

In vitro, endonuclease enzymes have been employed to examine whether mainstream cigarette smoke generates oxidised DNA bases in pulmonary cancer cells (NCL-H292) exposed to whole cigarette smoke [111]. The authors reported a significantly elevated presence of FPG-sensitive oxidative DNA lesions over negative control levels, but this was not observed with Endo III-sensitive oxidative DNA lesions [111]. Another investigation showed that hOGG1 appears to be more specific for 8-OHdG/8-oxodG and methyl-fapy-guanine lesions than FPG [112].

Over the last decade, the main focus has been on developing and refining the methodology to adapt it to measure DNA damage in buccal cells. This is of particular relevance for smoking-related oxidative stress, as the buccal mucosa is the initial entry point into the body for tobacco smoke. Since buccal cells are relatively simple to sample/obtain from human volunteers (using a mouthwash or mouth scraper) compared to primary lung cells, they can be used as a surrogate tissue for the assessment of oxidative DNA damage in the respiratory tract caused by direct exposure to tobacco smoke. However, development of the buccal cell comet assay has been hampered by technical difficulties such as a lack of adequate discrimination between buccal cells and other cell types such as leukocytes [113] and the lack of correlation between detection of DNA damage (comet shaped cells) and the presence of non-viable cells [114]. With respect to smoking, two investigations did not report any effect on DNA damage in buccal cells of smokers [114, 115]. In contrast, two other studies demonstrated higher levels of DNA damage in smokers when compared to non-smokers [116, 117].

To summarise, urinary analysis of the free radical induced oxidative lesion 8-OHdG/8-oxodG represent a convenient and well established approach to assess oxidative DNA damage in smoking studies, providing precautions are taken to limit the amount of artifactual chromatographic oxidation. The Comet assay combined with the enzyme FPG is regarded as the most reliable method for monitoring levels of 8-OHdG/8-oxodG and for assessing oxidative stress in general [118].

THYMIDINE GLYCOL

There are other repair products arising from oxidative DNA lesions which have been identified in urine and employed as biomarkers of oxidative DNA damage, although they have received less scientific attention. Such is the case with 5,6-dihydroxy-5,6-dihydrothymidine (thymidine glycol). Most of the thymidine products retain their ring structure, primarily pair with their cognate bases and are not potent premutagenic lesions. However, these lesions can significantly distort the DNA molecule and are sometimes lethal [119].

The oxidation of thymidine by $HO^{\cdot}$ generates formation of thymidine glycol. Thymidine glycol has been used as a biomarker of DNA oxidation damage providing good correlations to exposure to dimethylated arsenic [120]. An increased excretion of thymidine glycol was also observed after kidney transplantation and was explained by the ischemia-reperfusion-induced oxidative DNA damage in the kidney [121, 122]. Thymidine glycol has subsequently been measured by LC-MS-MS in the urine of smokers and non-smokers by Lowe *et al*. [32], and was reported to be significantly elevated in the smoking group.

BIOMARKERS OF OXIDATIVE STRESS INDUCED DAMAGE TO PROTEINS

Oxidation of proteins appears to play a causative role in many chronic diseases of ageing including cataractogenesis, rheumatoid arthritis, and various neurodegenerative diseases such as Alzheimer's disease [123, 124].

$HO^{\cdot}$ is the main ROS inducing deleterious effects in proteins. In addition, cigarette smoke contains metals that catalyse the direct oxidation of cellular proteins by smoke oxidants [125]. Alternatively, reactions between ROS and proteins can affect the turnover of proteins *in vivo* by increasing their proteolytic susceptibility [126]. Protein carbonyl measurements have been employed in a number of smoking related investigations. A study in Chinese smokers found that protein carbonyl levels were higher in smokers than in non-smokers although the increase was not statistically significantly [127]. In addition, reduced levels of protein carbonyls were found in the plasma of ex-smokers compared to current smokers [127]. Increased protein carbonyl measurements on chronic alcoholics who were also smokers supported the evidence of a synergistic effect between smoking and drinking [128].

ANTIOXIDANT STATUS AS A BIOMARKER OF OXIDATIVE STRESS

Antioxidants serve to protect the body from the harmful effects of free radical damage. As mentioned previously, oxidative damage can occur when antioxidant defences are overwhelmed in the target tissue. Thus, the measurement of antioxidant levels in target tissues/biofluids could serve as useful indicators of the extent of an oxidative insult.

Antioxidants as biomarkers can be broken up into the following types:

- Total antioxidant capacity, which measures the oxidant-buffering potential of a tissue/biofluid
- Specific compounds or their precursors/metabolites, for example ascorbic acid, which help to scavenge free radicals. Compounds can be absorbed in the diet or synthesised *in vivo*.

- Enzyme activity such as superoxide dismutase, which convert free radicals into less toxic entities.

TOTAL ANTIOXIDANT CAPACITY

The antioxidant system includes enzymatic components (superoxide dismutase, catalase, and glutathione peroxidase), a number of endogenous small macromolecules (bilirubin, albumin, ceruloplasmin, and ferritin) and an array of molecules of dietary origin (ascorbic acid, α-tocopherol, β-carotene, and polyphenols). The combination of all these components represents the total antioxidant capacity (TAC) of the extracellular fluid. Measuring TAC provides a more integrated and comprehensive biological picture compared to that obtained by the measurement of individual components, as it considers the cumulative effect of all antioxidants present in plasma and other body fluids. The various assays currently available for assessing TAC are: Trolux equivalent antioxidant capacity, oxygen radical absorbance capacity, total radical trapping potential and ferric-ion reducing ability (FRA) in plasma.

In general, TAC is decreased in conditions associated with oxidative stress [129]. The TAC value may also reflect the dietary profile of each individual [130, 131]. Importantly, variations in antioxidant levels can aid identification of disease risk in conditions such as cardiovascular disease, diabetes and cancer [132, 133]. With regard to smoking, TAC was shown to be different between smokers and non-smokers [19, 134-136].

There are also criticisms in relation to the use of TAC. For instance, it has to be considered that TAC measured *in vitro* bears no similarity to *in vivo* measurements and may not have direct implication *in vivo* [137]. The TAC assays also correlate poorly with each other [138]. Moreover, assays for TAC measurement in plasma differ in the type of oxidation source, target and measurement used to detect the oxidised product, thus providing a wide variety of results [137]. In most intervention trials, TAC failed to demonstrate an effect of the supplementation of antioxidants and this might be explained by the effect of endogenous antioxidants in addition to those of dietary origin [118]. Hence, it has been suggested that a better approach is to use a range of measurements of individual antioxidants and markers of oxidative damage, with TAC measured in parallel to complement this approach [129].

ANTIOXIDANT COMPOUNDS

The effects of cigarette smoking on the levels of antioxidant compounds such as vitamin C, vitamin A precursors (carotenoids and cryptoxanthine) and vitamin E has been well described in the review by Alberg [18]. Generally speaking, cigarette smoking lowers levels of circulating vitamin C and vitamin A precursors *in vivo*, with the number of cigarettes smoked per day negatively correlating with levels of these compounds. Levels of vitamin E were not associated with cigarettes per day or smoking status [18]. Since Alberg's review, subsequent studies have confirmed these observations [139, 140].

Several recent studies have investigated the association between nutritional antioxidants, oxidative stress and events linked to atherosclerosis and cardiovascular disease. Bamonti and co-workers studied the effects of antioxidant supplementation (*via* a mixed fruit and vegetable powder concentrate) on the levels of free MDA in smokers and non-smokers. They found that, initially, levels of free MDA were elevated in the smoking group. After 30 days of supplementation, those levels normalised to that of the non-smoking group [141]. Kaehler and co-workers reported that the prothrombotic state observed in heavier smokers can be reversed with ascorbic acid infusion. This suggests that oxidative stress plays an important role in the impairment of fibrinolysis in smokers [142]. Another study reported that impaired monocyte migration in smokers was reversed by ascorbic acid supplementation [143]. Sugiura and co-workers analysed the associations of serum carotenoids with metabolic syndrome, as stratified by smoking routine, using a cross-sectional study. They concluded that elevated levels of carotenoids offered a protective effect against the development of metabolic syndrome, especially in the smoking subjects [144]. Furthermore, van Herpen-Broekmans and co-workers measured circulating antioxidant levels and correlated them with biomarkers of endothelial function and chronic inflammation (associated with atherosclerosis development). They reported that lutein and lycopene were inversely related to soluble intracellular adhesion molecule 1, beta-carotene was inversely related to leukocyte count and C-reactive protein, whereas vitamin C and alpha tocopherol were inversely and positively related to C-reactive protein, respectively. Zeaxanthin was also found to be inversely related to flow-mediated dilatation, a measure of endothelial integrity. Thus, there is strong evidence to suggest that high levels of circulating antioxidants provide protection against cardiovascular events by suppressing oxidative stress [145].

Numerous studies have shown increased oxidative stress in the lungs of COPD patients, and antioxidant supplementation has been proposed to counteract the oxidative stress associated with COPD development [146-148]. Calikoğlu and co-workers reported decreased vitamin C levels and elevated oxidative stress biomarkers in COPD [140]. Furthermore, two recent studies have investigated the effects of nutritional antioxidants on lung function decline. Guénégou and co-workers studied the effects of serum carotenoids and vitamin E on the rate of lung function decline as measured by forced expiratory volume over 1 second (FEV_1) over a period of 8 years in a population of 1194 French subjects between 20-44 years of age. They found that FEV_1 decline was increased in subjects with lower levels of beta carotene, especially so in subjects who smoked. No effect was reported for alpha carotene, vitamin E and vitamin A [149]. Keranis and co-workers also investigated the effects of an antioxidant-rich diet on FEV_1 in comparison to a diet where subjects were free to eat as they chose (free diet), in a population of COPD patients followed for three years. They concluded that the antioxidant-rich diet yielded more favourable lung function measurements than the free diet [150]. This indicates that oxidative stress in the lungs is a driver for COPD morbidity and that increasing antioxidant levels may improve lung function in COPD patients.

Measurement of nutritional antioxidant biomarkers is important for determining the overall oxidative stress burden on subjects, especially as some antioxidant compounds correlate well with the number of cigarettes smoked per day. Additionally, nutritional antioxidant levels could help explain some of the biological variability observed in levels of oxidative stress biomarkers like MDA and ISOP in study populations.

Subject intra- and inter-individual variability is an important factor to consider for data interpretation when measuring any biomarker. Most cross-sectional studies reported in the literature use a single sampling time point for antioxidant biomarkers, which could attenuate possible associations and correlations with health-related factors like smoking. This has been recently highlighted by Block and co-workers, who investigated levels of nutritional antioxidants in smokers and non-smokers at sampling points which were 2 to 4 weeks apart. They specifically analysed intra- and inter-individual subject variability and the number of measurements required to reduce attenuation of the correlation coefficients. The authors concluded that with the exception of ascorbic acid and MDA, the biomarkers measured required two measurements to limit the attenuation of the correlation coefficients to no lower than 90%. The group determined that for ascorbic acid and MDA, repeat measurements had little effect on correlation attenuation, thus a single measurement was sufficient for these biomarkers. Where only single measurements are possible, power calculations should be performed to select a large enough sample size to overcome biological variability [151].

ANTIOXIDANT ENZYME ACTIVITY

The main antioxidant enzyme activities measured to assess the extent of oxidative stress are those of superoxide dismutase, glutathione peroxidase and catalase. Occasionally, other enzyme activities including glutathione reductase and glutathione-S-transferase are also assessed. The discovery of superoxide dismutase (SOD) enzymes provided much of the basis of the knowledge of antioxidant defence systems, since it led to the postulation of the superoxide theory of oxygen toxicity [152], which proposed that $O_2^{\cdot -}$ is a major factor in O_2 toxicity and that the role of SOD was of scavenging $O_2^{\cdot -}$ by coupling and converting it to hydrogen peroxide (H_2O_2). The fact that SOD accelerates H_2O_2 generation commits this enzyme to work in conjunction with enzymes that eliminate H_2O_2 e.g. catalase and glutathione peroxidase (GPx). Nevertheless, its functionality is vital since a variety of degenerative processes are driven by oxidative stress arising from superoxide [153].

GPx is considered the major peroxide-removing enzyme found in human tissue and is highly specific for reduced glutathione (GSH) rather than H_2O_2 [154]. GPx reacts with H_2O_2 as well as with peroxides, catalysing the reduction of fatty acid hydroperoxides [155]. As a consequence of oxidised glutathione production by GPx, a complementary enzyme is needed to regenerate GSH, namely glutathione reductase (GR). GR is sometimes measured alongside GPx to provide additional information regarding the status of the entire glutathione antioxidant system.

With respect to cigarette smoking, antioxidant enzyme activity was found to be variable between smoking subjects. Ermis and co-workers reported elevated serum GPx activity in smoking mothers and their newborn infants compared to non-smoking mothers and their newborn infants. The same group showed that serum SOD activity was not significantly different between mothers or infants [14]. Zhang and co-workers measured plasma SOD, GPx and catalase activity in a cohort of smokers and non-smokers. Of the three enzymes, only catalase activity was significantly elevated compared to non-smokers. However, they reported a weak positive correlation between SOD activity and the number of cigarettes smoked, and that catalase activity correlated with GPx and SOD activity. Interestingly, MDA was measured in parallel, and levels were significantly lower in the smoking group, possibly indicating that the antioxidant defences in the smokers were sufficient to prevent excessive lipid peroxidation, and hence increased MDA levels [13]. In other studies, serum SOD and GPx activities were lower [10, 156] or higher [157], and serum catalase was lower [158] in smokers compared to non-smokers, respectively. Plasma GPx activity was lower [159] or showed no significant difference [160] in smokers compared to non-smokers respectively. In the respiratory tract, increased SOD, GPx and catalase activities were reported in bronchoalveolar cells [161], decreased SOD activity in bronchoalveolar lavage fluid [162] and increased SOD expression and activity in the alveolar epithelium [163] of smokers compared to non-smokers, respectively.

In erythrocytes, SOD, GPx and catalase activities were increased in smokers [159], and GPx was decreased with SOD and catalase showing no change in comparison to non-smokers [164]. Orhan and co-workers reported significantly decreased SOD and GPx activities in smokers, while there was no significant difference in catalase activity compared to non-smokers [160]. Yildiz and co-workers reported significantly lower SOD and catalase in smokers, whereas GPx was not significantly different compared to non-smokers [165]. Lastly, Greabu and co-workers reported significantly decreased GPx activity in the saliva of smokers compared to non-smokers [166]. In summary, antioxidant enzyme activity is highly variable in various biological compartments, and hence, is not a clear-cut biomarker of oxidative stress in smoking studies. Measurements of antioxidant enzyme activity should be accompanied by other biomarkers of oxidative stress to help interpret the data.

FUTURE USE OF OXIDATIVE STRESS BIOMARKERS

There is a need to fully characterise the role of oxidative stress in smoking-related disease, in order to utlilise biomarkers of oxidative stress as tools for the health assessment of tobacco products. Novel tobacco products developed for their harm reduction potential are beginning to emerge in the market as alternatives for consumers who choose to continue to smoke. Biomarkers of oxidative stress could play a role in the health assessments of such products. Currently, an epidemiological approach is considered the best method to assess the health effects of conventional and novel tobacco products. However, due to the long latency of smoking-related diseases, an epidemiological approach is not well suited to provide early feedback regarding the efficacy of such products, to regulators and industry alike. Shorter term longitudinal studies utilising biomarkers as surrogate end points for disease in cohorts of smoking volunteers switching from a conventional to a novel product would help to give an insight into the early biological effects of novel tobacco product use.

Oxidative stress biomarker profiles which change in a consistent manner in response to smoking cessation would be promising candidates to use in such studies. Furthermore, as measurable changes can be seen in some biomarkers of oxidative stress after a number of weeks, these longitudinal studies should be easier to manage, cost less than larger scale epidemiological studies and allow for refinement of the product for further assessment.

Although the shorter term insight is welcome, all candidate biomarkers must still be qualified as fit-for-purpose tools for the assessment of disease risk. Such qualification can only come from epidemiological studies designed specifically for that purpose and from scientific consensus and regulatory acceptance.

CONCLUSION

Smoking-induced oxidative stress is likely to play a role in the development of smoking related diseases such as lung cancer, chronic obstructive pulmonary disease and cardiovascular diseases. The estimation of oxidative stress levels in the tissues and biofluids of smoking subjects could help to further understand the link between smoke exposure and disease. The biomarkers discussed in this review are useful indicators of oxidative stress and a panel based approach is recommended as opposed to the measurement of a single biomarker. Furthermore, extensive information regarding diet, lifestyle, health status, smoking history and smoke exposure data for each of the subjects used in the study, should be collected to assist with data interpretation and biological relevance.

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