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Review Article

# Evaluation of Enzymes Involved in the Production of Endogenous Reactive Oxygen Species and Role of Long-term Oxidative Stress in Pathobiology of Atherosclerosis with Common Oxidative Stress Markers

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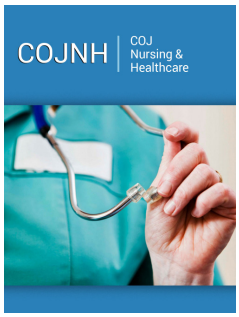
**ISSN: 2577-2007**

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Submission: March 27, 2020

**Published:** January 28, 2021

Volume 7 - Issue 1

**How to cite this article:** Leta Shiferaw Melaku. Evaluation of Enzymes Involved in the Production of Endogenous Reactive Oxygen Species and Role of Long-term Oxidative Stress in Pathobiology of Atherosclerosis with Common Oxidative Stress Markers. COJ Nurse Healthcare. 7(1). COJNH. 000652. 2021. DOI: 10.31031/COJNH.2021.07.000652



**Abstract**

Free radicals are molecules with an unpaired electron; thus, they are highly reactive. The human body deals with the pathological effects of free radicals by utilizing antioxidant system. The concentration and location of ROS are the main determinants of their effect. Although there are several sources of vascular free radicals, the enzyme NADPH oxidase is emerging as a strong candidate for the excessive ROS production. A multitude of studies provide evidence that an uncontrolled production of ROS is involved in the development and progression of cardiovascular disease like atherosclerosis. Atherosclerosis is characterized by the formation of intimal plaques. Monitoring and rapid detection of oxidative stress markers is necessary to combat the spread of various diseases.

**Keywords:** Free radicals; ROS; Antioxidant; Atherosclerosis; Oxidative stress markers

## Introduction

As the key life-supporting element, oxygen was independently discovered by Priestly et al. [1] & Scheele et al. [2]. Within a few years of these seminal findings, oxygen toxic side effects that did not support life were also discovered [3]. The good and bad facets of oxygen are played out by its unique molecular structure [4]. The structural configuration of oxygen is a diradical can accept four electrons and the resultant one-step tetravalent reduction results in the formation of water, with a concurrent production of ATP. Ironically, if these four electrons are added one at a time, partially reduced forms of oxygen or free radicals are produced [5-7]. Free radicals can be defined as reactive chemical species having a single unpaired electron in an outer orbit [8]. This unstable configuration creates energy that can initiate autocatalytic reactions so that molecules to which they react are themselves converted into free radicals [9]. Although ROS (reactive oxygen species) more common in biological systems [9], free radicals also include RNS (reactive nitrogen species) [10]. The endogenous sources of ROS are the mainly by-products formed in the cells of aerobic organisms within mitochondria [11].

Furthermore, certain enzyme, neutrophils, eosinophil's, macrophages, microsomes and peroxisomes are also sources of ROS [9,12-15]. It has been established that ROS can be both harmful and beneficial in biological systems depending on the environment [16,17]. At normal physiological levels, in phagocytic cells ROS plays a key role in cell-mediated immunity and microbiocidal activity [18,19]. In nonphagocytic cells, they are involved in a number of cellular signaling systems as well as in the induction or inhibition of cell proliferation [20-22]. In comparison, the rate of ROS production in nonphagocytic cells is only about one-third of that of phagocytic cells [23-26]. In contrast, at very high concentrations, ROS is often associated with the principle of oxidative stress [27]. The term oxidative stress is used to describe the condition of oxidative damage to a wide range of cellular structures as a result of an imbalance between free radical production and antioxidant defenses [28]. Short-term oxidative stress may occur in tissues injured by trauma, infection, heat injury, hypertoxia, toxins, and excessive exercise [29,30]. These harmful effects are balanced by the action of antioxidants [31]. However, in long-term oxidative stress, ROS have been implicated in the induction and complications of various cardiovascular diseases like atherosclerosis despite the presence of the cell's antioxidant defense system [32,33].

## Enzymes involved in the production of endogenous reactive oxygen species

Although the importance of ROS in vascular pathophysiology is quietly clear, recently there has been particular interest in the enzyme sources in the blood vessel wall [34]. This is due to the fact that enzymes are the most common sources of the production of endogenous ROS such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals ($OH^-$) [35,36].

### A. Nicotinamide-Adenine Dinucleotide Phosphate-Oxidase

Several enzymes that have potential to produce ROS are now recognized [37] and perhaps the most important one is NADPH oxidase, which consists of five subunits: $p40^{PHOX}$, $p47^{PHOX}$, $p67^{PHOX}$, $p22^{PHOX}$ and Nox [38]. In resting cells, $p47^{PHOX}$ (47 kDa), $p67^{PHOX}$ (67 kDa) and $p40^{PHOX}$ (40 kDa) reside within the cytoplasm. On their stimulation, these polypeptides translocated to the inner face of the plasma membrane to form a fully active enzyme complex [39]. On the other hand, the plasma membrane contains two polypeptides, 22 kDa ($p22^{PHOX}$) and 91 kDa (gp91-phox), which together make up flavocytochrome b558 [37]. This heterodimer contains a FAD group and two haeme groups, which enables the transfer of electrons from cytosolic NADPH across the membrane to molecular oxygen. Therefore, in the regulation of cytoplasmic NADPH oxidase activity, two G-proteins are associated [19,40,41]. Rap copurifies with gp91-phox, its exact role is still obscure, and p21rac, which is involved in the activation of NADPH oxidase. In the inactive state, p21rac is in association with a GDP-dissociation inhibition factor, Rho-GDI, but on stimulation dissociation takes place, and p21rac translocates to the plasma membrane, where it aids in the activation of the NADPH oxidase complex [42]. Moreover, NADPH oxidase activity is also regulated by phosphorylation of NADPH oxidase components. Phorbol myristate acetate (PMA) is commonly used to stimulate NADPH oxidase activity in cells and its action probably being mediated by protein kinase C [43,44]. It is likely that other kinases too are involved [39,45]. The modulation of intracellular calcium ions is also commonly used to activate the oxidase, and again a kinase may be involved in mediation of the calcium signal, although here direct stimulation has been suggested in some cell types [46]. Cardiovascular NADPH oxidase isoforms are also induced by hormones, hemodynamic forces, local metabolic changes and natural forces such as wall stress [47,48]. For instance, Angiotensin II increases NADPH-driven $O_2^-$ production in cultured vascular smooth muscle cells and fibroblasts [49,50]. Thrombin, platelet-derived growth factor (PDGF), and TNF-α stimulate NADPH oxidase-dependent $O_2^-$ production in vascular smooth muscle cells [51]. Interleukin-1, TNF-α, and PDGF increases NADPH-dependent $O_2^-$ production in fibroblasts. Mechanical forces also stimulate NADPH oxidase activity in endothelial cells, and reoxygenation stimulates NADPH oxidase activity in cardiac myocytes [51-53].

### B. Xanthine Oxidase/xanthine oxidoreductase

This molybdenum-and iron-containing flavoprotein catalyses the oxidation of hypoxanthine to xanthine and then to uric acid and molecular oxygen is the oxidant, whose products include $O_2^-$ and $H_2O_2$ [46,54]. Unlike xanthine dehydrogenase, xanthine oxidases catalyse oxidation of uric acid xanthine in production of superoxide radicals [34]. In experimental animals with hypercholesterolemia, it is also capable of producing increased amounts of active radicals that directly leads to NO activity reduction [55]. Additional facts that support the role of xanthine oxidase in the process of atherogenesis are the following: 1) in patients with coronary syndrome the levels of this enzyme were found to be increased, the same applies to NAD(P)H; and 2) in young asymptomatic patients with familial hypercholesterolemia the increased activity of the enzyme is an early event [56]. It has been observed that in vessels of hypercholesterolemic patients, vasodilation is improved by the presence of allopurinol or oxypurinol, an inhibitor of the enzyme [34]. Therefore, this enzyme exists in plasma and endothelial cells but not in smooth muscle cells [34].

### C. Myeloperoxidase

It is produced by activated phagocytes and uses $H_2O_2$ for the production of more powerful oxidative substances [41]. This enzyme, through NAD(P)H, leads to the production of HOCl and its analogs (substances related to endothelial injuries due to the action of $H_2O_2$) [57]. It is considered to participate in both cell-mediated immunity and microbiocidal activity [19,40] as well as in the process of atheromatosis, which is by the induction of oxidative modifications in low- and high-density lipoproteins [58]. This hypothesis is consistent with the results of clinical trials, according to which the levels of this enzyme and its products are elevated in patients with coronary syndrome. In contrast to human lesions, these oxidative products are absent in experimental animals with apolipoprotein E and LDL-receptor deficiency. The three mechanisms through which myeloperoxidase participate in oxidative modifications are NO consumption, LDL oxidation, and reaction with L-arginine for the production of NO synthase inhibitors. All of these are dependent on $H_2O_2$ [34]. Immunohistochemical studies have proved the presence of myeloperoxidase and HOCl in atherosclerotic lesions [59]. Therefore, both these substances participate in the modification of LDL and in atherogenesis.

### D. Lipoxygenases

They are enzymes that catalyse the reaction of $O_2$ with the polyunsaturated lipid acids, creating a family of biologically active lipids, such as prostaglandins, thromboxanes and leukotrienes, which participate in inflammatory reactions and increase the permeability of vessels [34]. In experimental models, 15-lipoxygenase induces LDL oxidation by enzymatic and non-enzymatic reactions [60]. Experimental animals with an absence of the 15-lipoxygenase gene or reduced expression of 5-lipoxygenase are protected from lesions like those found in animals with apolipoprotein E and LDL-receptor deficiency. Clinical data demonstrate that various genotypes of 5-lipoxygenase promoter are found in patients with atherosclerotic lesions or inflammation [61]. Whether lipoxygenases participate in

atherogenesis through lipid oxidation or defensive modifications is under investigation.

## The Role of Long-Term Oxidative Stress in Pathobiology of Atherosclerosis

Atherosclerosis is a multifactorial disease that involves the interplay of genetic and environmental factors and characterized by accumulation of cholesterol, infiltration of macrophages, proliferation of smooth muscle cells, and accumulation of connective tissue components and formation of thrombus [62,63]. It is the single largest cause of death and disability in the world [64] and most studies have shown that it starts early in life [65,66]. For instance, this disease appears earliest in the aorta (during fetal life), while it appears in the coronary arteries in the second decade and in the cerebral arteries in the third decade [65,66]. Furthermore, the growth of the lesion is abluminal in early stages of the disease, and the progress may vary from total cessation in some cases to very rapid with intervening periods of relative quiescence [67]. Hence, distinct clinical manifestations are seen depending on the type of vascular bed affected by atherosclerosis because it reduces the perfusion of a tissue [68,69]. Coronary lesions lead to myocardial ischemia or infarction [63]. Similarly, transient ischemic attacks and stroke are seen in the cerebral circulation, whereas intermittent claudication occurs in the peripheral circulation. Infarction of the gut produces lesions in the splanchnic circulation, while renal artery lesions result in ischemia due to reduced renal perfusion and damage the renal parenchyma, leading to uremia and eventually renal failure [70].

Several risk factors such as smoking, blood cholesterol, diabetes, physical inactivity and arterial hypertension are seen to contribute to the genesis of atherosclerosis; however, none of these factors are sufficient to produce an atherosclerotic lesion by themselves [71,72]. Evidence suggests that risk factors increase the risk of production of ROS from the endothelial cells, the smooth muscle cells and the adventitial cells of vasculature [73]. These ROS then oxidize cellular biomolecules to cause the atherosclerosis [74-76] as follows: the identification of the gaseous free radical as a major signal transducer molecule and EDRF [77] suggests oxy reduction reactions are important effector steps for autocrine or paracrine regulation of vessel tone, permeability, and structure in physiological or pathological conditions [78,79]. Therefore, the first physiological alteration in the pathobiology the problem is the impairment of the endothelium, which is manifested by enhanced vascular constriction and depressed dilatation of the vascular endothelium as well as excessive production of ROS [80]. The excess ROS generation, which is mainly due to NADPH oxidase activation, initiate vascular membrane lipid peroxidation that leads to inflammation and production of TNF-α via NF-κB induction [81,82] and other factors such as vascular adhesion molecule-1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1), endothelial-selectin, TGF-β1, Matrix metalloproteinase 9 (MMP9), iNOS and Mn-SOD [83-85].

Experimental evidence also supports crucial role for inflammatory reactions as a connection between risk factors for atherosclerotic disorder and pathophysiologic complexity of the disease [86]. TNF-α, which is one of inflammatory cytokines, is involved in commencement as well as development of atherosclerosis by inducing transcription factor nuclear factor-κB (NF-κB). In the process of atherosclerosis NF-κB induces the transcription of VCAM-1, ICAM-1, MCP-1, and E-selectin in smooth muscle/endothelial cells of the blood vessels [87]. TNF-α also depletes NO levels in the endothelium that leads to dysfunction of the endothelium [88,89]. In addition, TNF-α has been reported to cause apoptosis of the endothelial cells through dephosphorylation of protein kinase B (Akt) that leads feature endothelial damage [90,91]. Among the biomarkers of inflammation that is modulated by IL-6, IL-1 and TNF-α is C-reactive protein (CRP) [92] and evidence suggest that raised blood CRP level is an important predictor of CVD [93,94]. CRP is implicated in the advancement of atherosclerotic lesions by enhancing the production of VCAM-1, ICAM-1, selectins, and MCP-1 in the endothelium through induction of powerful constrictor of the vessels ET-1 and IL-6 [95,96].

Moreover, it ameliorates the synthesis of NO in the endothelium by depressing the transcription and translation of enzyme NO synthase [75]. It also plays a significant role in cooperating with the activities of other cytokines and factors. CRP induces the biochemical synthesis and physiological functions of PAI-1 in the endothelium [97]. PAI-1 is known to be actively involved in thrombosis during atherosclerosis process and inhibits destruction of the fibrin clot by suppressing plasminogen activation [98]. In atherosclerotic process, resistin, which exerts inflammatory reactions/vasoactive effects in the endothelium, also induces transcription of cellular factors such as VCAM-1 and MCP-1 [99]. Cells from endothelium exposed to resisting deplete the levels of TNF receptor-associated factor (TRAF-3) which is a well-known inhibitor of the endothelial activation [100]. It is also suggested that augmented resistin concentration causes a significant dysfunction of the endothelium through activation of endothelial system [101]. Furthermore, resistin exposure activates endothelial cells by increasing ET-1 release through induction of transcription of ET-1 indicating its role in the impairment of the endothelium [102]. Serum amyloid A (SAA) protein has also been implicated in the inflammatory reactions associated with atherosclerotic disorder and used as a biomarker for cardiac and vascular disorders as well as heart and vessels outcome [103].

The ROS also up-regulate atherosclerotic events such as cell infiltration, migration, adhesion and platelet activation [81,82,104] and facilitates the oxidation of low-density lipoprotein (LDL) and production of foam cells [105]. The transport of cholesterol regulated by ATP-binding cassette transporter A1 (ABCA1) and transport of oxidized LDL through CD36 regulate the excess of cholesterol ester in the macrophages, which result in formation of foam cells [105,106]. Apolipoprotein E as well as low-density

lipoprotein (LDL)-receptor knock out animals display speedy atherosclerotic lesions [107,108] and they also have sizeable counts of macrophages/T cells in their plaques [106]. The elevated concentrations of factors involved in inflammatory pathway, namely TNF-α, MCP-1, Cox-2, TGF-β1, iNOS, and Mn-SOD in ApoE-deficient atherosclerotic mice [61,109], also proves the vascular inflammation as an integral process in the atherosclerotic pathophysiology [106]. Apart from excess foam cells, growth of smooth muscle/endothelial cells, collagens, matrix metalloproteinases, fibronectin, and elastin are also responsible for plaque development [75,76,80,110]. Leptin also increases the cellular growth as well as migration of cells of the endothelium [111] and cells of smooth muscle [112]. It also directly augments concentrations of monocyte colony-stimulating factor (MCSF) [113], increases cholesterol levels in hyperglycemia [114], and promotes new blood vessel formation [115]. It also induces the synthesis of MCP-1 in the cells of the aortic endothelium [116] and enhances the aggregation of the platelets and vascular thrombus formation through leptin receptor pathways [117,118]. Leptin also up regulates ET-1 as well as NO synthase biosynthesis in the endothelial cells and augments generation of free radicals and oxidants [117,118] that causes oxidative stress [119]. Depending on the histological picture, the lesions are classified into six types [120,121]. Type I contain atherogenic lipoproteins and infiltrates mononuclear leukocytes. The intima makes adaptive changes such as thickening.

This is seen in most people at birth. Type II has layers of macrophages or foam cells with SMC infiltration from the media into the intima. The gross lesion is designated as a fatty streak and is unique to the disease. Type III is an intermediary stage between types II and IV, with scattered coarse lipid granules or particles that disrupt the integrity of the SMC. Type IV lesions are characterized by typical atheromas containing a large extracellular lipid core and the abluminal growing atherosclerotic lesion. Type V lesions have atheromas with large extracellular lipid cores and the developing fibrous caps. There is an increase in the collagen and (more often) SMC content. Type V lesions are further classified into the Vb and Vc subtypes. Vb are characterized by largely calcified lesions, whereas the Type Vc contain more fibrous connective tissue, little lipid and no calcium [121]. Type VI lesions have ruptured atherosclerotic plaque with subsequent fissure formation or hematomas in the arterial lumen. As the thrombogenic lipid core comes into contact with the blood, thrombosis occurs due to platelet aggregation [63]. Inflammatory reactions are not only involved in progression of human vascular plaques generation but also have important role in the rupture of internal arterial plaques [106]. Generally, several factors are implicated in the rupture of internal arterial plaques including cytokines, cyclooxygenase-2, matrix metalloproteinases, and tissue factors [76,122,123].

## Biologic Markers of Oxidative Modifications

Lipids, proteins, carbohydrates, and DNA are all susceptible to oxidative modifications of ROS [9,18]. Some modifications have direct functional effects, such as enzyme inhibition, with the remainder functionally silent indicators of increased ROS levels in the microenvironment [124]. The direct impact of the molecular modifications on the cell, organ and system's ability to adapt to the elevated levels of ROS is an important contributor to the plausibility and validity of the marker, and its likelihood of emerging as a robust prognostic tool. However, it is challenged by the high reactivity and short half-life of many oxidative products as well as their variable specificity [125].

## Lipids

Lipids are particularly susceptible targets of oxidation because of their abundant reactive double bonds [126]. Reactive oxygen species from the mitochondria, P450 enzymes, lipoxygenase and transition-metal catalysis are involved in lipid oxidation, or lipid peroxidation [127,128]. The ROS attack of the polyunsaturated fatty acids in the membrane and initiation of a self-propagating chain reaction results in altered fluidity and inactivation of critical membrane-bound receptors and enzymes [129]. Furthermore, the end-products of lipid peroxidation, such as the highly reactive secondary aldehyde products, isoketals from the isoprostane pathway, directly threaten the viability of tissues via their ability to covalently modify molecules that are critical to cell function [130]. Thus, lipid peroxidation is recognized as a crucial step in the pathogenesis of several CVD states including atherosclerosis [131,132].

The sensitivity of lipids to peroxidation, and its functional effects have made lipid peroxides good candidates as redox biomarkers [124]. The most frequently studied markers of lipid peroxidation are isoprostanes and malondialdehyde. However, the other markers include lipid hydroperoxides, fluorescent probes of lipid peroxidation and oxysterols [133,134]. Isoprostanes are prostaglandin-like substances that are produced independently of cyclooxygenase enzymes by ROS-induced peroxidation of arachidonic acid [135]. The most commonly measured members of the family are the F2-isoprostanes [134]. F2-isoprostanes are detectable in all biological fluids, reflecting baseline or 'physiological' levels of redox signalling [136]. They are substantially elevated in animal models of oxidant injury as well as human disease states characterized by elevated ROS [137]. They also increase in association with well-recognized risk factors such as cigarette smoking, hypercholesterolaemia, and diabetes mellitus [128]. Their causal role in human atherosclerosis is suggested by their effect to induce vasoconstriction [138], platelet aggregation [139], proliferation of VSMC [140] and their increased levels in atherosclerotic lesions [141].

Malondialdehyde (MDA), generated via peroxidation of polyunsaturated fatty acids, is also widely used to examine redox state [142]. Malondialdehyde-induced generation of lysine-lysine cross-links in apolipoprotein B fractions of oxidized low-density lipoprotein (OxLDL) has been proposed to play a role in atherogenesis via impairing the action of macrophages [143].

Numerous studies have demonstrated the elevation of MDA in association with smoking and diabetes in both animals and humans. Malondialdehyde quantification therefore remains a useful biomarker in clinical research [124]. Another product of lipid peroxidation, 4-hydroxynonenal (4-HNE) appears to be particularly important for the regulation of vascular redox state in humans [144]. 4-hydroxynonenal is produced from the reaction of $OH^-$ with lipid structures, and is highly reactive with proteins, giving rise to a wide range of protein adducts [124]. Recent evidence suggests that 4-HNE produced in the vascular wall may exert paracrine effects on the neighbouring perivascular adipose tissue, leading to the activation of peroxisome proliferator-activated receptor-γ signalling in this fat depot [145]. As a result, perivascular fat releases the antioxidant adipokine, adiponectin, which exerts a paracrine effect back onto the vascular wall, reducing NADPH oxidase activity [145], and improving eNOS coupling. 4-Hydroxynonenal thus restores the balance between NO and $O_2^-$ in the vascular endothelium [146].

This cascade also underlines the complexities of regulation of vascular redox state in humans, involving multiple intravascular feedback loops in addition to communication signals with other tissues, which host either pro- or antioxidant mechanisms depending on the underlying diseases state [124]. It also highlights that the oxidation products (used also as clinical biomarkers) may not always be 'simple by-products' of oxidation with no biological effects, but might play an active role in the regulation of vascular redox state, e.g., as rescue signals released from the vascular wall.

## Protein

The direct, mostly reversible, functional effects of oxidative posttranslational modifications, like tyrosine nitration, protein carbonylation and S-glutathionylation, on many cellular proteins suggest proteins could be strong candidates for assessment of cellular redox haemostasis [147]. The nitration of protein tyrosines, which occurs through two predominant pathways, peroxynitrite and haeme peroxidase-dependent nitration [147,148] with steric effects, resulting in altered protein function, which is an important consequence of increased ROS. Many proteins including fibrinogen, plasmin, Apo A-I in the plasma, Apo B, Mn-superoxide dismutase (SOD) in the vessel wall, and creatine kinase (isoenzyme MM) as well as sarco/endoplasmic reticulum $Ca_2^+$-ATPase (SERCA) in the myocardium undergo nitration, with important functional effects [147].

Both free circulating 3-nitrotyrosine (3-$NO_2$-Tyr), which possibly reflects the turnover of nitrated proteins with the modified amino acid not recycled for de novo protein synthesis, and total protein 3-$NO_2$-Tyr, measured by hydrolyzing the protein fraction of the biological sample to its constituent amino acids, have been examined as biomarkers [149]. Although not used in clinical practice, the 3-$NO_2$-Tyr has achieved a number of intermediate milestones, including demonstration of the levels as independent predictors of cardiovascular risk [150]. Protein carbonyls can be formed by the oxidation of a few amino acid side chains via the addition of aldehydes such as those generated from lipid peroxidation [151]. Carbonyl compounds are widely used markers of severe protein oxidation [152]. As a marker of oxidative damage to proteins, carbonyls have been shown to accumulate during aging, ischaemia/reperfusion [152], diabetes, and obesity [153].

Protein S-glutathionylation, the formation of a mixed disulphide bond between the reactive cysteine residue and the abundant glutathione is an excellent candidate for oxidative signalling due to its stability and reversibility (124). By conferring a 305 Da negatively charged adduct, it exerts steric effects on proteins similar to phosphorylation [154,155]. S-glutathionylation of critical cysteines plays a particularly important role in the cell membrane, mediating redox regulation of eNOS [156], the ryanodine receptor [157], SERCA [154], and the $Na^+$-$K^+$ pump [158], to name a few. In contrast to these, S-glutathionylation can also occur in non-critical cysteines without functional or regulatory effects [124]. Thus measuring 'total S-glutathionylated proteins' in serum, in a manner similar to that applied to protein nitrosylation, faces problems of both not representing S-glutathionylation at target tissues, as well as accounting for the subpopulation of 'silently' S-glutathionylated proteins. However, S-glutathionylation of the $Na^+$-$K^+$ pump in erythrocytes, which closely parallels in the myocardium in both animals and patients with heart failure [159], suggesting its biological validity as a circulatory marker in heart failure.

## Advanced glycation end products

Advanced glycation end products are a class of molecules resulting from modifications of proteins or lipids that become non-enzymatically glycated and oxidized after contact with aldose sugars [124]. They form in vivo in hyperglycaemic environments and during the ageing process, and mediate vascular disease in diabetes [160]. Because of their severe instability, most of the advanced glycation end products are difficult to correctly analyse and are not practical for measurement as biomarkers in cardiovascular disease [124].

## DNA

Reactive oxygen species can also mediate damage to all components of the DNA molecule, the purine and pyrimidine bases, as well as the deoxyribose backbone. Free radical induced damage to DNA in vivo can result in deleterious biological consequences such as the initiation and promotion of cancer [39]. One of the most abundant products of cellular DNA damage, 8-hydroxy-2′-deoxyguanosine (8-OHdG) can be detected by HPLC, and has been used as a redox biomarker, particularly in cancer research [124]. The levels of 8-OHdG have been found to be elevated in patients with CAD [161] and may also be useful for risk stratification in patients with subclinical cardiovascular disease, as shown for carotid atherosclerosis in a small study of haemodialysis patients [162].

Methodologies incorporating the technique of gas chromatography/mass spectrometry (GC/MS) have been also developed in recent years for measurement of free radical induced DNA damage. The use of GC/MS with selected-ion monitoring (SIM) facilitates unequivocal identification and quantitation of a large number of products of all four DNA bases produced in DNA by reactions with hydroxyl radical, hydrated electron, and H atom. Hydroxyl radical induced DNA-protein cross-links in mammalian chromatin, and products of the sugar moiety in DNA are also unequivocally identified [39]. The sensitivity and selectivity of the GC/MS-SIM technique enables the measurement of DNA base products even in isolated mammalian chromatin without the necessity of first isolating DNA, and despite the presence of histones. Recent studies revealed the usefulness of the GC/MS technique for chemical determination of free radical induced DNA damage in DNA as well as in mammalian chromatin under a vast variety of conditions of free radical production [163,164].

## Conclusion

Free radicals play a dual role as both toxic and beneficial compounds, since they can be either harmful or helpful to the body. Many data support the notion that ROS released from nicotinamide adenine dinucleotide phosphate oxidase, myeloperoxidase, xanthine oxidase, lipoxygenase, nitric oxide synthase. When an overload of free radicals cannot gradually be destroyed, their accumulation in the body generates a phenomenon called oxidative stress. This process plays a major part in the development of various cardiovascular diseases such as atherosclerosis. ROS are key mediators of signaling pathways that underlie vascular inflammation in atherogenesis, starting from the initiation of fatty streak development, through lesion progression, to ultimate plaque rupture. Plaque rupture and thrombosis result in the acute clinical complications of myocardial infarction and stroke. Moreover, increased vascular production of ROS in atherosclerosis and common conditions predisposing to atherosclerosis such as hypercholesterolemia, hypertension, diabetes, and smoking likely contributes to development and progression of atherosclerosis by oxidative modification of LDL and by promoting endothelial dysfunction enhancing vascular inflammatory responses. Despite the biological plausibility of redox biomarkers as important adjuncts in diagnostic and prognostic armamentarium, their validation for clinical application has been slow and none have yet reached clinical use. It is likely that such pursuits will lead to a better understanding of these biological phenomena, and hopefully will provide new opportunities for therapeutic interventions.

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