REVIEW ARTICLE

OXIDATIVE STRESS IN CHRONIC VASCULAR DISEASE: FROM PREDICTION TO PREVENTION

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Abstract

This review article is intended to describe the strong relationship between oxidative stress and vascular disease. Reactive oxygen species (ROS) play an important role in the pathogenesis of vascular disease: oxidative stress is intimately linked to atherosclerosis, through oxidation of LDL and endothelial dysfunction, to diabetes, mainly through advanced glycation end-products (AGEs)/receptor for AGE (RAGE) axis impairment, **protein kinase C (PKC), aldose reductase (AR) and NADPH oxidase (NOX) dysfunction**, and to hypertension, through renin-angiotensin system (RAS) dysfunction. Several oxidative stress biomarkers have been proposed to detect oxidative stress levels and to improve our current understanding of the mechanisms underlying vascular disease. These biomarkers include ROS-generating and quenching molecules, and ROS-modified compounds, such as F_2-isoprostanes. An efficient therapeutic approach to vascular diseases cannot exclude evaluation and treatment of oxidative stress. In fact, oxidative stress represents an important target of several drugs and nutraceuticals, including antidiabetic agents, statins, renin-angiotensin system blockers, polyphenols and other antioxidants. A better understanding of the relations between atherosclerosis, diabetes, hypertension and ROS and the discovery of new oxidative stress targets will translate into consistent benefits for effective vascular disease treatment and prevention.

Keywords

Oxidative stress; isoprostanes; biomarkers; hypertension; diabetes; antioxidants.

Chemical compounds studied in this review

Superoxide (PubChem CID: 5359597); Peroxynitrite (PubChem CID: 104806); Hydroxide (PubChem CID: 961); NADP+ (PubChem CID: 5886); Nitric oxide (PubChem CID: 145068); Tetrahydrobiopterin (PubChem CID: 44257); Ascorbic acid (PubChem CID: 54670067); Alpha-Tocopherol (PubChem CID: 14985); 11-Dehydro-TXB2 (PubChem CID: 5280891); Angiotensin II (PubChem CID: 172198).

1. The long way to detect vascular oxidative stress: historical and innovative biomarkers

Reactive oxygen species (ROS) represent a family of molecules that includes free radicals, such as O_2^- (superoxide), $ONOO^-$ (peroxynitrite), HO^- (hydroxyl), and non-radicals, as H_2O_2 (hydrogen peroxide).

ROS are produced by aerobic cells, during the incomplete oxygen reduction process of the respiration, and they represent crucial protagonists of the oxidative stress, defined as an imbalance between ROS formation and elimination in favor of pro-oxidant processes. Focusing the attention on vascular oxidative stress, it has been demonstrated that, in the vascular wall, many enzymatic systems produce ROS, including **nicotinamide adenine dinucleotide phosphate** (NADPH) oxidase, mitochondrial enzymes, dysfunctional endothelial nitric oxide synthase (eNOS), and xanthine oxidase (XO). The vascular wall has also several antioxidant systems to contrast ROS generation: superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), paraoxonase (PONs), thioredoxin (TRX) peroxidase and heme oxygenase (HO) (Figure 1) (1).

Several pathogenetic elements related to cardiovascular (CV) disease are associated with oxidative stress in several clinical settings, and are thought to contribute to the development of vascular complications: endothelial dysfunction, inflammation, LDL oxidation, metabolic syndrome and insulin resistance are indeed strongly linked to ROS and oxidative stress (2). Among acquired diseases, a clinical paradigm of vascular oxidative stress-mediated disease is represented by ethanol consumption-induced hypertension, as showed by Passaglia et al in a recent issue of the *Journal* (3).

Moreover, ROS exert several physiological roles, such as defense from microbial agents, angiogenesis and response to fibrotic stimuli in lung injury (4). Thus, ROS appear to have a double role: they can mediate both physiologic events and cellular damage. Oxidative stress is quantified *in vivo* through the measurement of several molecules, some of which may serve as biomarkers. Biomarkers are indicators of physiological or pathological processes. An ideal marker is one in which

there is a specific, easily measurable increase that clearly orientates the physician towards a diagnosis or a predictable clinical outcome in such a way that it guides therapy. Biomarkers of oxidative stress are usually indirect indicators of oxidative status. It is possible to identify either molecules related to ROS generation or modified by interaction with ROS.

1.1 ROS-production markers

In vascular cells, platelets and inflammatory cells, O_2^- is synthesized by the enzymes NADPH oxidase, xanthine oxidase, lipoxygenase, uncoupled eNOS, inducible nitric oxide synthase (iNOS) and mitochondrial electron transport. Cells require a certain amount of O_2^- to maintain cellular homeostasis; however, when these O_2^--generating sources remain activated after a physiological stimulus has waned, the continued production of O_2^- alters cellular redox homeostasis resulting in increased oxidant stress. Cardiovascular risk factors may therefore trigger ROS generation thus playing a role in mediating oxidative stress in CV disease (CVD) (1).

NADPH represents a family of pro-oxidant enzymes, which are mainly defined by their membrane-bound NOX subunit into four main subtypes in CV system.

The endothelial source of NO, eNOS, displaying antioxidant, anti-inflammatory, and anti-atherogenic characteristics, is probably the most important enzyme in CV biology. In the presence of high levels of ROS, eNOS co-factor tetrahydrobiopterin (BH_4) is oxidized and this results in enzymatic uncoupling of eNOS, to generate O_2^- instead of NO. It has been shown that vascular but not plasma BH_4 is an important determinant of eNOS coupling, endothelium-dependent vasodilation, and superoxide production in human vessels (5).

Thus, although estimating the ratio of BH_4 to its oxidized forms (dihydrobiopterin and biopterin) may serve as a marker of systemic redox state, the complex regulation of BH_4 synthesis, induced by inflammation, limits its value as a biomarker (6).

The complexity of this regulation is also highlighted by the important effects of indirect strategies that restore vascular BH_4 bioavailability, using statins or folates, on vascular oxidative signaling, despite the inability of oral BH_4 administration to improve vascular redox state (7,8).

Thus, vascular BH_4 (and its oxidation status) may represent an excellent biomarker reflecting the overall vascular redox state and it may be critically involved in its regulation.

NADPH oxidase and uncoupled eNOS play a key role in ROS generation, but they are localized in the vessel wall and myocardium, thus making their quantitation very difficult to be measured. Recent studies showed that soluble NOX2-derived peptide (sNOX2-dp or gp91phox) is released from circulating cells into the serum and it has been correlated with NOX activity in the CV system. Thus sNOX2-dp has been used to measure NOX activity in a number of disease states, including microvascular obstruction post primary percutaneous intervention for myocardial infarction (9,10,11).

Serum NOX2 activity is increased in patients with obesity, hypercholesterolemia or metabolic syndrome. Consistently, this biochemical abnormality has been shown to be reversible with modulation of the underlying metabolic dysfunction, such as with successful weight loss in subjects with metabolic syndrome, or statins in hypercholesterolemic individuals (12,13). Myeloperosidase (MPO) is a heme enzyme, abundant in inflammatory cells, catalyzing ROS formation. Its level is correlated to CV risk markers, such as insulin resistance and endothelial dysfunction (14).

MPO function can be measured through peroxidase activity samples and MPO mass/concentration can be detected through ELISA plates. Several lines of evidence indicate that MPO is one of the most promising markers of CV oxidative stress (15).

In fact, MPO levels are increased in patients with unstable angina and with myocardial infarction as compared to controls and MPO represents an independent predictor of total mortality over a follow-up period of 5 years (16).

Circulating MPO levels have been positively correlated to abdominal subcutaneous and visceral adipose mass both in adults and in prepubertal obese children, with significant association with CV risk markers such as CRP, metalloproteinase-9, insulin resistance, and endothelial dysfunction (17).

Furthermore, higher MPO levels are associated with a rapid progression of atheroma in diabetic patients and with a lower benefit of statin therapy on disease progression (18).

A relationship between uric acid and redox state has also been described, with ROS production occurring as a consequence of the purine degradation process. In fact, xanthine oxidoreductase (XOR) catalyzes the conversion of hypoxanthine to uric acid in the purine degradation process, with production of ROS. Xanthine oxidase and XOR activity are upregulated in myocardial ischemia/reperfusion injury and in heart failure. A strong relationship between uric acid and redox state has been described and it may predict mortality in ischemic heart disease (19,20).

A recent study showed that serum uric acid is emerging as a marker for the natural progression of chronic heart failure mediated by cardiovascular remodeling (21).

1.2 Antioxidant markers

The antioxidants comprise enzymatic and non-enzymatic molecules. The non-enzymatic antioxidants include ascorbic acid (vitamin C) and α-tocopherol (vitamin E), histidine dipeptides and uric acid. Vitamin C and E inhibit LDL oxidation by ROS scavenging, and block lipid peroxidation, thus improving NO bioavailability (22).

Vitamin C (ascorbic acid) is a chain-breaking antioxidant, scavenging ROS directly, and preventing the propagation of chain reactions that would otherwise lead to a reduction in protein glycation. Experimental evidence suggests that vitamin C in involved in the autonomic nervous regulation of blood pressure, by preventing DNA damage induced by renovascular hypertension, and by restoring hypertension-associated baroreflex dysfunction (23,24).

Consistent with findings in animal models, in humans, acute infusion of vitamin C has been associated with a significant reduction of blood pressure and sympathetic nerve activity (25).

In addition, vitamin C has been shown to decrease serum cellular oxidative stress in patients undergoing cardiac surgery, and to reduce the incidence of postoperative atrial fibrillation (26).

Vitamin C contributes to support endothelial cells by increasing the synthesis and deposition of type IV collagen in the basement membrane, promoting endothelial proliferation, inhibiting apoptosis, scavenging radicals and sparing endothelial cell-derived NO. However, the exact molecular mechanism on endothelial cells is still unknown (27).

A systematic review and meta-analysis of randomized controlled trials suggests that supplementation with either vitamin C or vitamin E alone improves endothelial function (28).

Consistently, a systematic review and meta-analysis of randomized controlled trials in T2DM subjects confirmed that prolonged antioxidant vitamin E and/or C supplementation may be effective to improve endothelial function in non-obese T2DM subjects (29).

Vitamin E is a term that encompasses a group of potent, lipid-soluble, chain-breaking antioxidants. Structural analyses have revealed that molecules having vitamin E antioxidant activity include four tocopherols (α, β, γ, δ) and four tocotrienols (α, β, γ, δ). Vitamin E reacts directly with peroxyl and superoxide radicals and singlet oxygen and protects membranes from lipid peroxidation (30).

Vitamin E has been associated with no significant improvement in lipid profile and the protective vascular mechanism of vitamin E is most probably via prevention of oxidation of LDL. This is demonstrated by increases in antioxidant gene expression and antioxidant enzymes in vitamin E-treated patients (31).

A deficiency in vitamin E is associated with increased peroxides and aldehydes in many tissues. Conversely, Vitamin E may ameliorate oxidative stress in diabetic patients and improve antioxidant defense system (32).

Moving from pathophysiological endpoints to hard, clinical endpoints, vitamin E or C supplementation showed no effect on the incidence of major cardiovascular events (33).

A systematic review of randomized controlled trials confirms that there is no evidence to support the prescription of vitamin and antioxidant supplements for the prevention of CVD (34).

A number of explanations have been raised to explain such discrepancy, including the occurrence of tocopherol-mediated lipid peroxidation (35).

Moreover, the kinetic data and physiological molar ratio of vitamin E to substrates show that the peroxyl radicals are the only radicals that vitamin E can scavenge to break chain propagation efficiently and that vitamin E is unable to act as a potent scavenger of hydroxyl, alkoxyl, nitrogen dioxide, and thiyl radicals *in vivo* (36).

Thus, the beneficial effect of vitamin E against the oxidation mediated by nonradical oxidants such as hypochlorite, singlet oxygen, ozone, and enzymes may be limited *in vivo*.

Finally, tocotrienols, the less abundant components of vitamin E compared to tocopherols, have molecular targets distinct from those of the tocopherols that may result in better preventive outcomes and new therapeutic opportunities (37).

Tocotrienol rich palm oil extract is more effective than pure tocotrienols at improving endothelium-dependent relaxation in the presence of oxidative stress. Indeed, tocomin and α-tocopherol restored endothelial function in the presence of oxidative stress but α-, δ-, and γ-tocotrienols were ineffective (38).

The enzymatic antioxidant include SOD, GPx, catalase, TRX, HO and PON. There are 3 forms of SODs and SOD3 is the predominant form in the vascular wall. It catalyzes the dismutation of superoxide into hydrogen peroxide and oxygen. **In ApoE mice, over-expression of SOD has been shown to retard the development of atherosclerosis (39).**

GPx has antioxidant effects by reducing free hydrogen peroxide to water. **In patients with coronary artery disease, the activity of red cell GPx isoform 1 was shown to have prognostic value in addition to that of traditional risk factors (40).**

Catalase promotes the degradation of hydrogen peroxide to oxygen and water. TRX is present in endothelial cells and vascular smooth muscle cells (VSMC) and it can scavenge ROS such as H_2O_2 and $ONOO^-$. HOs catalyze heme breakdown to biliverdin, then converted to bilirubin, a molecule with radical scavenging properties and able to inhibit Nox enzymes. PON enzymes have peroxidase-like activity and protect against lipoprotein oxidation (41).

The measurement of the net antioxidant capacity of the serum activity of antioxidant enzymes such as catalase, GPx-1 and SOD is also very important and it has been quantified in plasma as measures of antioxidant capabilities. In a prospective study of patients with suspected coronary artery disease, erythrocyte GPx-1 and not SOD activity was inversely associated with the incidence of CV events after adjusting for CV risk factors (40).

1.3 ROS-modified compounds

All macromolecules, such as carbohydrates, protein, lipids and DNA, can be modified *in vivo* by ROS excess (figure 2).

1.3.1 *LOOHs*

Phospholipids, glycolipids and cholesterol, all polyunsaturated fatty acids (PUFAs), are important targets of ROS-mediated lipid peroxidation. LOOHs measurement is an optimal direct index of oxidative status. To quantify oxidized lipids, high performance liquid chromatography (HPLC) is the best technique for sensitivity and specificity. LOOHs measurement is utilized as a marker of peroxidative damage of membrane lipids and oxidative stress *in vivo* (42).

1.3.2 MDA

Malondialdehyde (MDA) is an aldehyde, which results from lipid peroxidation *in vivo,* as an arachidonate by-product. MDA is quantified in plasma with colorimetric assay based on thiobarbituric acid (TBA). This TBA reacting assay lacks specificity for MDA. MDA and in particular MDA-modified low-density lipoproteins represent biomarkers of oxidative stress and atherosclerosis (43,44).

1.3.3 AGE/RAGE system

Advanced glycation end products (AGEs) derive from glycoxidation and lipoxidation of proteins and amino acids (45). A link between increased circulating AGEs levels and insulin

resistance, endothelial dysfunction and CV risk has been described. AGEs values may be predictive of major adverse CV events. Thus, AGEs may represent a relevant marker of metabolic abnormalities and vascular risk (46).

Moreover, receptor for advanced glycation endproducts (RAGE) and its ligands are involved in the pathobiology of a great range of diseases, including diabetes mellitus, metabolic syndrome, atherothrombosis, immune/inflammatory conditions, aging, cancer and neurodegeneration (47,48).

Soluble forms of RAGE (sRAGE) and the splice variant endogenous secretory (es)RAGE have been found circulating in plasma and tissues. Evidence is mounting to support a role for both sRAGE and esRAGE as biomarkers or endogenous protection factors against RAGE-mediated pathogenesis.

Decreased circulating levels of sRAGE and/or esRAGE have been consistently reported in several clinical settings, such as diabetes, obesity, hypercholesterolemia, rheumatoid arthritis (49,50). In these clinical settings, sRAGE levels are inversely correlated with the urinary excretion of 8-iso-$PGF_{2\alpha}$ and with plasma asymmetric dimethylargine (ADMA), suggesting that the ligand-RAGE axis may bridge endothelial dysfunction with oxidative stress (45). Interestingly, specific treatment of the underlying disease, including antihyperglicemic agents, statins, anti TNF-α drugs may concurrently revert all biochemical abnormalities, an effect that has been recently reported for high-amount-high intensity aerobic exercise in low-to intermediate risk subjects (51).

Recent observations clearly show that among patients with familial combined hyperlipidemia and/or metabolic syndrome, decreased plasma esRAGE, in cluster with altered adipokine profile, and markers of oxidative stress and platelet/coagulative activation, identify those with non-alcoholic fatty liver disease (47).

Thus, sRAGE and esRAGE are emerging as biomarkers of RAGE activity, possibly providing supplementary information to improve vascular risk stratification (45).

A recent study has shown, for the first time, a strong association between increased AGEs levels and human carotid rupture-prone plaques. In particular, plaque concentrations of the specific AGEs Nε-(carboxymethyl)lysine (CML) and 5-hydro-5-methylimidazolone (MG-H1) were higher in rupture-prone plaques and were associated with inflammatory plaque markers, such as inflammatory atheromatous lesions. CML and MG-H1 predominantly localized in macrophages surrounding the necrotic core. Thus the glycation pathway may be a major player in the progression of stable to rupture-prone plaques and subsequent plaque rupture (52).

1.3.4 F_2-Isoprostanes

F_2-isoprostanes are a series of prostaglandin-like products of the peroxidation of arachidonic acid catalized by free radicals. F_2-isoprostanes represent the most sensitive and reliable biomarkers of lipid peroxidation. The F_2-isoprostane more frequently quantified in plasma, urine and other biological samples is the 8-iso-prostaglandin (PG) $F_{2\alpha}$ (53,54). Differently from COX-derived prostaglandins, isoprostanes are formed from arachidonic acid *in situ* on lipids (Figure 3). To detect F_2-isoprostanes, mass spectrometry coupled to gas chromatography (GC/MS), RIA and ELISA have been used, with mass spectrometry representing the gold standard (55).

F_2-isoprostanes have many advantages in comparison with other quantitative markers of oxidative stress. In fact, they are specific products of peroxidation, chemically stable, formed *in vivo*. They also have the important property to be present in all normal tissues and biological fluids in detectable quantity (56).

Elevated urinary levels of 8-iso-$PGF_{2\alpha}$ have been reported in association with the most important cardiovascular disease (CVD) risk factors, such as cigarette smoking, diabetes mellitus, hypercholesterolemia, obesity, hyperhomocysteinemia and arterial hypertension (57-64).

The Framingham Heart Study evaluated the urinary levels of 8-iso-$PGF_{2\alpha}$ as a marker of systemic oxidative stress, in a cohort of 2828 subjects. In age- and sex-adjusted models, increased urinary 8-iso-$PGF_{2\alpha}$ levels were positively associated with female sex, hypertension treatment, smoking, diabetes, blood glucose, body mass index (BMI) and a history of CVD. In contrast, age and total cholesterol were negatively correlated with urinary 8-iso-$PGF_{2\alpha}$ levels. After adjustment for several covariates, decreasing age and total/HDL cholesterol ratio, sex, smoking, body mass index, blood glucose, and CVD remained associated with urinary 8-iso-$PGF_{2\alpha}$ levels. Thus, this study and subsequent evaluations on the Framingham cohort showed that smoking, diabetes and BMI, with particular reference to visceral adipose tissue, are highly associated with systemic oxidative stress (65,66).

The strong correlation between the elements of metabolic syndrome, in particular BMI, blood glucose and cholesterol, and F_2-isoprostanes levels, and lipid peroxidation suggests that the latter may be causally linked to the underlying metabolic abnormalities rather than to the attendant vascular disease. This relation is confirmed by the fact that all dietary and pharmacologic interventions that induce reductions in BMI, plasma glucose, cholesterol or homocysteine lead to reduction in F_2-isoprostanes levels, too, the average extent of which showed a remarkable good fitting with their linear relation as established under basal conditions (57,58,61,62,67-69). A diet rich in fruit and vegetables and poor in red meat is associated with lower plasma F_2-isoprostanes levels (70). F_2-isoprostanes are increased in patients with hypercholesterolemia with a linear correlation with LDL cholesterol content (49,61). In diabetes mellitus, 8-iso-$PGF_{2\alpha}$ is elevated and correlates with impaired glycemic control (57,58,67,71,72).

In addition, urinary isoprostanes may better identify CV risk in apparently healthy subjects and patients with CVD (73). Indeed, urinary F_2-isoprostanes predict CV mortality in postmenopausal women (74) and may help to predict vascular events in patients with atrial fibrillation (75).

Urinary 8-iso $PGF_{2\alpha}$ have been also proposed as a biochemical tool to help monitoring the effects of antioxidant therapy in cardiovascular risk reduction (54,61,63,76), including improved glycemic control, statins, weight loss, and antioxidant supplementation (54,63,76).

F_2-isoprostanes are also useful biomarkers in central nervous system diseases (Alzheimer, Parkinson, Huntington and amyotrophic lateral sclerosis). Moreover, they are elevated in patients with breast, gastric and colorectal cancer (77-79).

Furthermore, F_2-isoprostanes are not only biomarkers; they have numerous biological effects, playing a crucial role as pathophysiologic mediators of oxidant injury. 8-iso $PGF_{2\alpha}$ is an important vasoconstrictor and F_2-isoprostanes can partially activate the thromboxane receptor (TP) in a COX-independent manner (80).

Isoprostanes may also serve to propagate platelet activation by amplifying platelet response to subthreshold concentrations of common agonists via glycoprotein (Gp) IIb/IIIa activation. Enhanced isoprostane formation is not inhibited by low-dose aspirin treatment, either in diabetes (67,80,81) or in other clinical settings, such as in heart failure (82), consistent with the non-enzymatic nature of its formation in vivo.

The cause-and-effect relationship between oxidative stress and platelet activation is demonstrated by the linear relationship between the excretion rates of 8-iso-PGF_{2alpha} and the urinary thromboxane (TX) metabolite 11-dehydro-TXB_2, and by the down-regulation of these metabolites following improvement in metabolic control (antihyperglycemic drugs, statins, weight loss or insulin

sensitizers, folic acid) (57,58,61,62,67,68). These results suggest that the primary metabolic abnormality may trigger TX-dependent platelet activation mediated, at least in part, through enhanced lipid peroxidation (83).

Experimental and clinical data suggest that selected isoprostanes may represent important alternative activators of the TP when endogenous TXA_2 levels are low, e.g., in aspirin-treated individuals with CVD (80,84).

Whether enhanced oxidative stress *in vivo*, with lipid peroxidation and isoprostane formation, may be a source of less-than-optimal response to aspirin has been a matter of debate over the past years (85). Several studies reported that, in patients with stable CAD, redox-generated isoprostanes are associated with residual platelet activity, thus hypothesizing that isoprostane formation may affect COX-independent mechanisms of high on-aspirin platelet reactivity (86,87). In addition, urinary 8-iso-$PGF_{2\alpha}$ excretion correlates with and may predict residual, aspirin insensitive, thromboxane biosynthesis, as reflected by TX metabolite excretion in patients with type 2 diabetes (88) or with acute coronary syndromes (89).

In addition, in patients with risk factors for atherothrombosis, platelet isoprostane formation is enhanced through a platelet NADPH oxidase-dependent mechanism, which is poorly inhibited by aspirin treatment (90). Conversely, in diabetic but not in nondiabetic patients, aspirin enhances the platelet production of isoprostanes up to functionally relevant concentrations, thus enhancing platelet recruitment via GpIIb/IIIa activation. This effect is likely to mitigate the antiplatelet effect of aspirin and may account for its lower clinical efficacy in type 2 diabetes (91).

Thus, twenty-five years after discovery of isoprostanes, studies continue to increase our knowledge on these molecules, confirming the importance and their accuracy as biomarkers and mediators of oxidative stress.

2. Oxidative stress in chronic vascular disease

2.1 Oxidative stress in atherosclerosis

LDL oxidation is a widely studied phenomenon involved in the atherosclerosis-generating process. The oxidation of LDL to oxidized LDL (ox-LDL) takes place in areas of inflammation, mostly in the sub-endothelial space. LDL has many different types of particles, such as phospholipids, free cholesterol, triglycerides and cholesteryl esters. When polyunsaturated lipids undergo oxidation, several by-products, such as aldehydes and MDA, are formed. These by-products can react with apolipoprotein B-100 thus impairing its function. The result is the formation of minimally modified LDL, with pro-atherogenic properties. ROS-mediated LDL oxidation *in vivo* involves NADPH oxidase and ROS mitochondria. In patients with high level of cholesterol and coronary artery disease, NADPH oxidase appears to be the major source of O_2^- radical dot. The catalytic membrane subunit, gp91phox, also known as NOX2, is important for NADPH oxidase activation (12,13,92). Patients with hypercholesterolemia and obesity showed upregulation of circulating NOX2 (12,92). In obese patients, weight loss determined down-regulation of NOX2 along with amelioration of artery dysfunction (13).

LDLs may also indirectly enhance oxidative stress by angiotensin II via upregulation of the angiotensin receptor type 1 (AT1) (93). Both native LDL and oxLDL have been shown to increase $O2^-$radical dot, $ONOO^-$ and uncoupled eNOS generation (94). Oxidative stress has also an important influence on transcriptional pathways such as NF-kB and the transcription factor AP-1, involved in atherogenesis.

Oxidative stress does not act on atherosclerosis only in relation to LDL but also inhibits the cholesterol efflux function of HDL. Myeloperoxidase-induced chlorination of apoA-I, the most important proteic component of HDL, impairs the ability of apoA-I to enhance cholesterol efflux through ABCA1, the macrophage ATP-binding cassette transporter (95). The lecithin–cholesterol acyltransferase (LCAT) binding site on apoA-I is a strategic target for oxidative modification in atheroma, reducing LCAT activity, thus resulting in a dysfunctional form of HDL (96).

Endothelial dysfunction participates in the process of atherogenesis through oxidative stress generation. It consists in the reduction of NO availability in VSMC, with impairment of its effects on vasorelaxation. NO production derives from the eNOS of endothelial cells. This enzyme utilizes l-arginine and molecular oxygen as substrates to produce NO and l-citrulline. In this reaction NADPH is an electron donor and the presence of tetrahydrobiopterin (BH_4) is essential. Under physiological conditions BH_4 is "coupled" to eNOS to produce NO. ROS and more specifically $ONOO^-$ lead to "uncoupling" of the enzyme, leading to a dissociation of its complex and to production of O_2^-. These radicals react with NO thereby forming ONOO- which further oxidizes BH_4 to dihydrobiopterin (BH_2), thus creating a loop by further eNOS uncoupling.

In this regard, 5-methyltetrahydrofolate (5-MTHF) improves endothelial function and vascular superoxide production by preventing eNOS coupling. Perivascular adipose tissue also represents a regulator of vascular oxidative stress, exerting paracrine and endocrine effects on the arterial wall with prevention of NADPH-oxidase activity on eNOS coupling.

2.2 Oxidative stress in hypertension

Oxidative stress has been demonstrated to have a role in the pathophysiology of several cardiovascular risk factors, such as hypertension (97) (Figure 4). In particular, superoxide anion is a critical determinant of NO biosynthesis and bioavailability; it represents a vasoconstrictor and can modify endothelial function. NOS and its endothelial isoform eNOS represent a fundamental source of superoxide (97). eNOS generates superoxide, especially in response to atherogenic stimuli of NOS uncoupling, in which NO production is decreased and NOS-dependent superoxide production is increased (98). Thus, eNOS represents a peroxynitrite generator and it can lead to a sensible increase in oxidative stress, since peroxynitrite formed by the NO-superoxide reaction has deleterious effects on vascular function through oxidation of proteins and lipids (99). $ONOO^-$ preferentially activates PGH synthase while inhibiting prostacyclin synthase, thus shifting the balance between TXA_2 and prostacyclin towards enhanced TXA_2 production, leading to increased VSMC vasoconstriction and platelet aggregation (100). A decrease in NO bioavailability and an increase in oxidative stress have been found in patients with hypertension (101).

Inhibition of the renin-angiotensin-aldosterone system (RAS) is a cornerstone in the treatment of patients with hypertension: a possible explanation for this beneficial effect is the decrease of oxidative stress and ROS signaling (102). Several lines of evidence have underlined that angiotensin II (ANG II) has a crucial role in NADPH generation of ROS and activation of reduction-oxidation signaling cascades (103). ANG II elicits its effects through the angiotensin receptors AT1 and AT2. AT1 receptor leads to vasoconstriction and angiogenesis, instead AT2 receptor stimulates vasodilatation and anti-angiogenesis, thus representing pharmacological targets to maintain a normal balance between the vasodilator agent NO and ROS (104).

2.3 Oxidative stress and hypertension related to chronic ethanol intake

A clinical paradigm of oxidative-stress mediated hypertension and vascular disease is represented by the effects of chronic ethanol intake. In the general population, increased blood pressure is significantly correlated with high ethanol intake, but the mechanisms through which ethanol increases blood pressure are not completely known (105). A link between excessive ethanol intake and hypertension was suggested one hundred years ago (106).

Several mechanisms have been implicated in the relationship between alcohol and hypertension: increasing sympathetic nervous system activity, enhanced intracellular Ca^{2+} in VSMC, enhanced activity of the RAS system and endothelial dysfunction (107-109).

In rat models ethanol induces aortic vascular smooth muscle cell proliferation by increasing homocysteine and oxidized-low-density lipoprotein (110). In rats, chronic ethanol intake induces aortic inflammation and oxidative endothelial injury, reduces nitric oxide bioavailability in the vasculature, and alters responsiveness of mesenteric vasculature, inducing hypertension (111-113).

In a rat model, a time-course correlation has been described between vascular changes, in term of response to vasoconstrictor agents, and autonomic changes, contributing to the development of hypertension during ethanol ingestion. Intake of increasing ethanol concentrations for four weeks induces increasing blood pressure, which can be observed as early as after the first week of treatment. Thus, increased vascular responsiveness to vasoconstrictor agents may be a link for development and maintenance of the progressive hypertension induced by ethanol consumption (114).

Furthermore, renin-angiotensin system and ANG II have been implicated in the pathogenesis of alcohol-dependent hypertension: chronic ethanol consumption, in fact, increases plasma renin activity (PRA) and ANG II levels (115). Ethanol may alter endothelial signaling via AT1-receptor,

without changing systemic blood pressure. Stress and ethanol associated may also alter endothelial signaling via AT2-receptor and thereby increase blood pressure (116).

Recently, in a rat model, chronic ethanol intake has been shown to increase blood pressure, induce vascular oxidative stress and decrease NO bioavailability through AT1-dependent mechanisms. Thus, blood ethanol levels consistent with those found in the bloodstream of humans after moderate ethanol consumption, activated the systemic RAS, increased the vascular generation of O_2^- and decreased NO bioavailability in the vasculature. Ethanol-induced hypertension and increased systemic and vascular oxidative stress were prevented by losartan, further suggesting that AT1 activation plays a key role in these responses (3). This set of evidence opens new pharmacological horizons for novel targeting molecules to contrast ethanol long-term vascular dysfunction and cardiovascular damage.

2.4 Oxidative stress in diabetes mellitus and metabolic syndrome

Oxidative stress is deeply intertwined with insulin resistance and plays a crucial role in the pathogenesis of vascular complications of diabetes (69). Oxidative stress is at the same time cause and consequence in diabetes pathophysiology (117). The enhanced oxidative stress in subjects with T2DM and metabolic syndrome has been associated with hyperglycemia, insulin resistance, hyperinsulinemia and dyslipidemia, leading to mitochondrial superoxide overproduction in endothelial cells (118).

High glucose serum levels promote cellular ROS production through different pathways.

Deleterious effects of ROS stem from interactions with various ion transport proteins such as ion channels and pumps, primarily altering Ca^{2+} homeostasis and inducing cell dysfunction. A

correlation between higher intracellular H_2O_2 levels, oxidative damage and alterations in intracellular Ca^{2+} homeostasis has been reported, possibly due to modification of the ionic control in lymphocytes of T2DM patients (119).

The mitochondrial electron-transport chain acts as a primary $O2^-$ radical producer. Overproduction of mitochondria-derived $O2^-$ radical dot induces protein kinase C (PKC) activity and formation of AGEs (50). PKC and AGEs can increase NADPH oxidase functionality and inhibit eNOS activity (120). AGEs contribute to diabetic vascular complications by engaging RAGE, as previously mentioned.

The inactivation of two critical anti-atherosclerotic enzymes, eNOS and prostacyclin synthase, is another important mediator of oxidative stress in T2DM (121,122). Through these pathways, increased intracellular ROS promote angiogenesis, inflammation and epigenetic changes that allow long-term expression of infiammatory genes after glycemia is normalized, the so-called “memory of hyperglycemia” (107). Finally, oxidative stress in diabetes mellitus may play a fundamental role for the less-than-expected response to aspirin observed in diabetic patients, as reviewed elsewhere (85).

Insulin resistance (IR) is one of the most important pathogenetic elements of diabetes. A robust body of evidence suggests that oxidative stress is a potent mediator of IR in endothelial cells. Specifically, Insulin resistance reduces arterial prostacyclin synthase and eNOS activities by increasing endothelial fatty acid oxidation (123).

However, the molecular machinery responsible for ROS-induced endothelial IR is still partially uncharacterized. The mitochondrial adaptor p66Shc is emerging as a major determinant of endothelial dysfunction via mitochondrial ROS generation and translation of oxidative signals into apoptosis. The clinical relevance of p66Shc is related to its upregulation in peripheral blood mononuclear cells from insulin resistant subjects with T2DM (124). There is convincing evidence that endothelial activation of p66Shc may contribute to the pathogenesis of IR and to the increased

vascular risk in obesity and T2DM, and that selective targeting of p66Shc may restore endothelial insulin sensitivity and prevent adverse cardiometabolic phenotypes (125,126).

Sirtuin family, particularly SIRT1, is highly expressed in the vasculature and plays a critical role in the regulation of vascular function, protecting against vascular senescence and age-related vascular diseases. SIRT1 has been involved in the processes of aging, metabolism, and tolerance to oxidative stress through its ability to deacetylate several substrates, including histones, transcription factors and coregulators. p66Shc expression is negatively regulated by SIRT1, at the transcriptional level, through epigenetic chromatin modification. In fact, SIRT1 represses p66Shc transcription at the chromatin level, through decreased binding of acetylated histone H3 to the p66Shc promoter region, a result of the direct inhibitory role of SIRT1 on p66Shc expression (127).

In addition to AGE/RAGE system, multiple circulating biomarkers have been associated with vascular dysfunction in diabetes, including inflammation-associated biomarkers. Changes in the expression of adhesion molecules, pro-inflammatory molecules, and alterations in their regulation are deeply intertwined with ongoing oxidative stress, and have been observed since the early phases of the disease (128).

Activation of inflammatory processes in diabetes, as showed on the basis of the increased levels of the pro-inflammatory cytokines CRP, fibrinogen, IL-6, IL-1 and TNF-α, may cause impairment of vascular responses, leukocyte adhesion to endothelium, and facilitation of pro-coagulant activity. Prospective studies have shown that a combined elevation of IL-1beta and IL-6 is independently associated with increased risk of T2DM, suggesting that subclinical inflammatory process has a role in the pathogenesis of T2DM (129).

In addition, modulation of gene expression by epigenetic modifications and the action of microRNAs are being recognized as critical processes affecting T2D risk (130).

Obesity, diabetes and metabolic syndrome have been recognized as programmable diseases,

characterized by epigenetic modifications of vital genes when exposed to oxidative stress (131).

In this regard, great attention has been given to the potential of future epigenome-wide studies, carried out across tissues and populations with correlations to pre-diabetes and T2D risk factors, to build up a library of epigenetic markers of risk and early progression of T2D (132).

The DNA methylation levels and plasticity of CpG sites in the promoter region of the metabolic regulator *PPARGC1A,* correlated with insulin sensitivity, have been extensively studied in relation to T2D. *PPARGC1A* encodes PGC1α, which is a transcriptional co-activator that regulates expression of numerous genes with a key role in mitochondrial function (133).

Adipose tissue specific CpG sites in numerous genes associated with T2D (*PPARG, IRS1,* and *TCF7L2*) were shown to exhibit differential DNA methylation in individuals with T2D compared to healthy controls (134).

Dysregulation of micro-ribonucleic acids (microRNA or miR) such as miR-15a, miR-126, miR-320, miR-223, miR-28-3p enabled the identification of 52% of normoglycemic subjects developing T2DM in a 10-year period. Also, in patients with newly diagnosed T2DM, miR-9, miR-29a, miR-30d, miR-34a, miR-146, miR-124, and miR-375 were significantly higher compared with subjects with normal glucose tolerance (135).

Strongly related to the miRs are the microparticles (MPs) that represent a heterogeneous population of vesicles with a diameter of 100 to 1000 nm that are released by budding of the plasma membrane and express antigens specific of their parental cells. MPs are found in the circulation of healthy subjects, carrying miRNA from cells to target cells, and their number is increased in CV disease and conditions predisposing to vascular disease (136).

MP characteristics or phenotype is associated with the type of vascular complication and might serve as a biomarker for the pro-coagulant state and vascular pathology in patients with T2DM (137).

Moreover, plasma MPs have been associated with the presence of hypertension and arterial stiffness in patients with T2DM, and another study has suggested that EMPs could be used as a surrogate marker of unstable plaques and might help to improve the CV prediction in T2DM patients at intermediate risk (138,139).

3. Vascular oxidative stress: therapeutic perspectives

Proposing a therapy for oxidative stress means to translate all the fascinating world of oxidant and antioxidant molecules into everyday practice, trying to create new treatment perspectives through the prevention of oxidative stress. Making a clear but not trivial simplification, there are two main possibilities to treat oxidative stress: blocking the excess of ROS generation, or using antioxidants to contrast the cell oxidation status.

Ascorbic acid (vitamin C) and α-tocopherol (vitamin E) are the two most studied antioxidants. Vitamin C improves endothelial function and reduces oxidative stress whereas vitamin E seems to increase functionality of glutathione S-transferase (GST) enzyme (140,141), although the effects on the intracellular antioxidant enzyme production are not consistent among studies (142).

Data about antioxidants are discordant (143). In fact, observational, prospective cohort studies suggest that higher dietary intake or supplementation of antioxidants is associated with a lower risk of cardiovascular disease and mortality (144,145).

On the contrary, unlike the short-term and relatively small-sized randomised controlled trials show the benefits of antioxidants in reducing cardiovascular risk factors (146,147), long-term and large-sized randomised controlled trials have consistently failed to demonstrate a protective effect of any single antioxidant or combination of antioxidants (vitamins C, E, and β-carotene) in the primary or

secondary prevention of cardiovascular events (33,148-150). Moreover, in some clinical trials the supplementation of vitamins C, E, and/or β-carotene has been associated with an increased risk of all-cause mortality (151,152).

Potential reasons for these disappointing results may be attributed to the specific antioxidant, or the employed doses or dose regimens, or to the phenotype of patients included in the trials. Another plausible explanation is that these antioxidants do not reach the target tissue in sufficient concentrations (153).

Recently, vitamin E proved effective over placebo for the treatment of nonalcoholic steatohepatitis, a disease closely associated with insulin resistance, in non diabetic adults (154). Studies in healthy subjects (155,156) helped identifying the basal rate of lipid peroxidation as a major determinant of the response to vitamin E supplementation. The evidence that the same dose of vitamin E may have variable antioxidant effects in different patient populations characterized by variable rates of lipid peroxidation, is consistent with this concept. A linear correlation has been found between the basal rate of 8-iso-$PGF_{2\alpha}$ excretion and the slope of changes in this index of lipid peroxidation as a function of changes in plasma vitamin E associated with short-term dosing with 600 mg/day in different clinical settings. The issues of dose and duration of treatment may also affect the efficacy of vitamin E supplementation (157,158).

Many other drug molecules, such as angiotensin-converting enzyme inhibitors (ACEIs), angiotensin receptor blockers (ARBs), ß-blockers, statins, metformin, pentaerythritol tetranitrate (PETN) and polyphenols, can play an important role in reducing oxidative stress and in particular vascular oxidative stress (figure 5).

Polyphenols are a family of molecules, such as resveratrol and isoflavones, present in fruit, vegetables and red wine. Different classes of polyphenols can have different effects. Cardiovascular

risk reduction seems to be largely linked to the effect of non-alcoholic components of wine, mainly resveratrol and other polyphenols, on the vascular wall and blood cells.

Although heavy alcohol consumption has deleterious effects on heart health, moderate drinking is thought to have cardioprotective effects, reducing the risk of coronary artery disease and improving prognosis after a myocardial infarction (159). Red wine components, especially alcohol, resveratrol, and other polyphenolic compounds, may decrease oxidative stress, enhance cholesterol efflux from vessel walls (mainly by increasing levels of high-density lipoprotein cholesterol), and inhibit lipoproteins oxidation, macrophage cholesterol accumulation, and foam-cell formation (160).

Polyphenols facilitate redox enzyme as NADPH oxidase and favor BH_4 biosynthesis (161).

Polyphenols in olive oil or red wine reduce cellular ROS levels *in vitro* (162). Red wine extract decreases oxidative-stress-induced endothelial senescence (163). Acute red wine intake increases plasma total antioxidant capacity, suppressing NF-κB activation induced by a meal, but chronic red wine consumption compared with de-alcoholized red wine intake may increase 8-iso-$PGF_{2\alpha}$ levels (164).

PETN also induces antioxidative pathways at genomic level, increasing expression of HO-1 and ferritin, thus unraveling highly protective properties (165).

The ß -blocker nebivol inhibits the activity of NADPH oxidase and stimulates eNOS functionality (166). In hypertensive patients, nebivolol significantly reduces blood pressure and plasma LDL hydroperoxides, 8-isoprostanes, and ox-LDL. Similarly, nebivolol reduces ROS and $O2^{\circ -}$concentration in endothelial cells exposed to oxidative stress (167).

Statins suppress NADPH oxidase activity and enhances eNOS activity. Statins may also have antioxidant properties by reducing platelet ROS formation with a mechanism involving NADPH oxidase down-regulation (168).

Metformin reduces oxidative stress *in vitro*, in animal models and in human cellular models, directly scavenging ROS or modulating intracellular superoxide anion production in human leukocytes (169). Metformin treatment *in vivo* has been shown to mitigate oxidative stress, preserve the antioxidant function and decrease platelet activation (170). Metformin is believed to suppress gluconeogenesis by inhibiting a mitochondrion-specific isoform of glycerophosphate dehydrogenase, thus influencing the redox state with reduction in cytosolic dihydroxyacetone phosphate and increase in cytosolic NADH-NAD ratio (171). The recently observed improvement of mitochondrial integrity and platelet reactivity by metformin may contribute to the beneficial effects of this multifaceted drug on CVD (172).

ACEIs and ARBs reduce NADPH activity and vascular oxidative stress. Angiotensin II produces vasoconstriction, modifies vascular smooth muscle, induces inflammation and hypercoagulability and may generate vascular superoxide production by uncoupling eNOS. Thus ARBs and ACE inhibitors may induce endothelium-dependent vasorelaxation, through less superoxide production and improvement of NO bioavailability (1). In addition, ARBs improve intracellular antioxidant enzyme expression, both in experimental (173) and clinical studies (174).

3.1 New emerging molecules

Considering that the NADPH oxidase family of enzymes, particularly those that contain NOX1 or NOX2 catalytic subunits, are important sources of ROS production in the arterial wall, several

compounds are considered as inhibitors of NADPH oxidase and promise to be optimal anti-oxidant molecules. Apocynin and diphenyleneiodonium (DPI) are the most widely studied but their lack of selectivity for NADPH oxidases over other enzymes limits their clinical utility. Triazolopyrimidines, such as VAS2870 and VAS3947, have also emerged as promising inhibitors of NADPH oxidase activity. These compounds inhibit NADPH oxidase-derived ROS in several cell lines expressing NADPH oxidases and in primary endothelial and VSMC cultures, with no effect on ROS generated by xanthine oxidase or on eNOS activity (175,176).

Pyrazolopyridines, like GK-136901, are potent inhibitors of NOX1 oxidase- and NOX4 oxidase-dependent ROS generation from disrupted cell membrane preparations (177).

Moreover, 2-acetylphenothiazine is a specific NOX1 oxidase inhibitor at nanomolar concentrations, with only marginal activity on other cellular ROS-producing sources, including xanthine oxidase and the other NADPH oxidases (178). At present, all these molecules need to be better studied and tested.

Another emerging molecule is the aldehyde dehydrogenase 2 (ALDH2), a mitochondrial enzyme, and its activator Alda-1. It is known that ROS-dependent peroxidation of polyunsaturated fatty acids, associated with generation of toxic aldehydes, is related to dysfunction of mitochondria and plays a role in atherogenesis and steatosis of the liver. It has been observed that use of ALDA is associated with inhibition of atherogenesis and attenuation of hepatic steatosis in apoE−/− mice (179).

Moreover, recently, the beneficial influence of ALDH2 stimulation in acute ischemia-reperfusion injury of heart or brain has been attributed to many possible mechanisms, including attenuation of oxidative stress and clearance of reactive aldehydes (180).

3.2 Mitochondria-targeted antioxidants

Mitochondria-ROS (Mito-ROS), accumulated in mitochondria, cannot be modified through anti-oxidant drugs, but there are novel, targeted scavengers, like Mito-Q, that diminishes radical formation and oxidative mitochondrial damage but not respiratory activity. Mito-Q has a good *in vivo* tolerance and wide organ distribution with apparently no toxicity. Mito-Q reduces cardiac ischemia–reperfusion damage, chronic nitrate-induced endothelial damage and blood pressure (181). However, further clinical studies on Mito-Q are needed in CVD setting.

3.3 MicroRNAs

In vascular oxidative stress therapy, a role can be played by microRNAs (miR). As previously stated, endothelial dysfunction plays a key role in CVD initiation and progression. Endothelial dysfunction coincides with the occurrence of vascular oxidative stress with increment of ROS production and LDL oxidization. miR are important regulators of gene expression that modify cellular responses and function, at a post-transcriptional level.

A particular role is played by miRNA-126 (miR-126), that is a strongly expressed microRNA specific to endothelial cells, able to fine-tune their phenotype (182).

MiR-126 expression is also affected in the course of several physiological and pathological processes, such as angiogenesis, atherosclerosis, and the proinflammatory process (183,184).

Deletion of miR-126 causes loss of vascular integrity and produces defects in endothelial cell proliferation, migration, and angiogenesis (185).

A recent study focused on the effect of laminar shear stress (LSS) on human endothelial cells, with an emphasis on the role of miR-126, demonstrating that miR-126 is overexpressed by long-term LSS and it is involved in up- and downregulation of genes of atherosclerosic process (186).

A miR-network has been identified among the molecular mechanisms that control cellular homeostasis, vascular inflammation and metabolism, with a direct link between altered miR expression profiles and pathophysiology of a disease, thus identifying putative miR targets for novel therapeutic strategies (187). Since miR can have profound effects on biological pathways, cell function and homeostasis in the vessel wall, modulation of specific miR, using anti-miR or miR mimics, might contribute to reduce or increase a specific miR involved in oxidative stress mechanisms (188). For a miR-based therapeutic approach, we need to understand pharmacodynamics of miR inhibitors *in vivo*, in order to identify miR-specific targets and to develop new technologies to facilitate tissue-specific delivery (189).

4. Conclusions

ROS play an important role in the pathogenesis of vascular disease and they are intimately linked to atherosclerosis, diabetes and hypertension. **However, the conflicting results and sometimes opposite outcomes obtained with antioxidant supplementation forced the research to reconsider the entire oxidative stress story, recognizing that ROS have both deleterious and beneficial effects. It has been even hypothesized that diabetes, dementias, cardiovascular disease and some cancers are accelerated by failure of the endoplasmic reticulum to generate sufficient oxidative redox potential for disulphide bonds to be formed (190). Tumorigenesis is characterized by O_2 consumption and ROS accumulation (191) which result in a change in the redox balance (192). Changes in the cellular redox balance affect proliferation, migration, and survival of cancer cells contributing to disease progression (193). In cancer cells, ROS act as secondary messengers of oncogenic signaling pathways and can also induce cellular senescence and apoptosis. When mice carrying mutations that increase their risk of lung cancer were treated with antioxidants, their early precancerous lesions progressed more quickly, and the mice developed more tumors**

and at more advanced stages. The antioxidants did reduce oxidative stress and DNA damage as expected, but at the same time, they also reduced the expression of p53, a key tumor suppressor protein. Thus, a procarcinogenic role of antioxidants in people who are already at a higher risk of cancer, such as smokers, may be speculated by these data in animal models. This would explain the neutral or detrimental effect of antioxidants in a large part of trials, due to the enrollment of subjects with early or not-yet diagnosed cancers, for whom antioxidant supplementation may prove harmful (194).

Interestingly, physical activity may help prevent both vascular and neoplastic diseases by generating ROS. The therapeutic effect of physical activity in cancer might rely on the restoration of the low ROS levels caused by cancerous metabolic rewiring. Thus, further studies are required to better understand the twofold role of ROS (195), giving new perspectives to the treatment of several diseases related to oxidative stress.

Meanwhile, several oxidative biomarkers have been proposed to detect oxidative stress levels and to improve current understanding of the mechanisms underlying vascular disease. There is considerable pathophysiologic and clinical interest in the development of novel biomarkers for oxidative stress that may help in the detection of individuals at high risk for future vascular events. It is important to underline that there is an interindividual variability in the degree of oxidative stress, but the extent to which such diversity of metabolic phenotype translates into different vascular outcomes is still a matter of debate, and prospective and adequately sized studies are needed. Therefore, we are still lacking reliable and cost-effective markers able to identify vascular disease at an early stage and use of drugs aimed to lower oxidative stress is far from clinical practice. Among all the oxidative stress markers, urinary F_2-isoprostane detection, and in particular 8-iso-$PGF_{2\alpha}$, has been one of the most reliable and best characterized. Indeed, it has proven able to monitor the antioxidant effect of interventions for CV risk reduction, such as improved glycemic

control, statins, weight loss, antioxidant supplementation, as well as to refine current CV risk prediction models (54-76): for these reasons it appears to be a potential candidate biomarker in predicting specific patient groups more or less likely to benefit from targeted interventions, or for dose-finding studies of antioxidant interventions.

Even if oxidative stress has a fundamental role in this setting, randomized clinical trials have failed to show significant benefit from antioxidant vitamins on the development of CVD or mortality. This aspect reflects the complexity of redox reactions in biological systems such as vascular cells and unravel the limitations of our current strategies to modulate the redox signaling for CVD prevention. The inclusion of subjects without enhanced oxidative stress is likely to dilute the benefit of antioxidant supplementation and might explain the negative results of these trials. Validation of biomarkers to be employed in clinical trials will be instrumental to answer unsolved controversial issues around the efficacy of antioxidant compounds.

In conclusion, an efficient therapeutic approach to vascular diseases cannot exclude evaluation and treatment of oxidative stress. Further studies are needed to better understand the relations between atherosclerosis, diabetes, hypertension and ROS and the role of the antioxidants and to discover new oxidative targets that would be precious for an effective treatment and prevention of vascular disease. In particular, starting from the point that oxidative stress is a wide world of molecules and biochemical reactions, it is fundamental to select molecules or reactions as ideal target to limit oxidative stress, thus identifying drugs that can act at a cellular level, such as endothelial cells. In this regard, new therapeutic horizons, such as miRNA, have the potential to represent a real hope as a targeted therapy able to address the real protagonists of oxidative stress.

Conflict of interest

The Authors have no conflict of interest to disclose.

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FIGURE LEGENDS

Figure 1.

NADPH oxidase, xanthine oxidase, uncoupled endothelial nitric oxide synthase (eNOS) and mitochondria generate superoxide anion (O_2−radical dot) in the vascular wall. O_2−radical dot is converted to hydrogen peroxide (H_2O_2) thanks to superoxide dismutase (SOD). H_2O_2 have the possibility to be converted in: hydroxyl radical (spontaneously); H_2O and O_2 via glutathione peroxidase (GPx), catalase, or thioredoxin (Trx) peroxidase. Myeloperoxidase (MPO) can use H_2O_2 to oxidize chloride to the strong oxidizing agent hypochlorous acid (HOCl). Paraoxonase (PON) can limit mitochondrial O_2−radical dot generation.

Figure 2.

ROS are generated by several enzymatic systems, such as NOS, NADPH oxidase, myeloperoxidase (MPO), as well as in mitochondria. Antioxidant enzymes contrast ROS, that can modify macromolecules and fundamental cellular components. Biomarkers of ROS and oxidative stress are NO breakdown product, advanced glycation end-products (AGEs), MDA, isoprostanes, lipid hydroperoxides (LOOHs). AGEs derive from glycoxidation and lipoxidation of proteins and amino acids. MDA is an aldehyde, which results from lipid peroxidation in vivo, as an arachidonate by-product. LOOHs measurement is utilized as a marker of peroxidative damage of membrane lipids and oxidative stress in vivo. F_2-isoprostanes are a series of prostaglandin-like products of the peroxidation of arachidonic acid catalized by free radicals. F_2-isoprostanes represent the most sensitive and reliable biomarkers of lipid peroxidation.

Figure 3.

Peroxidation of arachidonic acid. The chemical structures of intermediates and products are depicted.

Figure 4.

In endothelial cell, ROS promote production and activity of angiotensin II instead of NO. This represents one fundamental cellular mechanism, that can contribute to generate hypertension. In particular, superoxide anion is a critical determinant of NO biosynthesis and bioavailability and angiotensin II (ANG II) has a crucial role in NADPH generation of ROS and activation of reduction-oxidation signaling cascades. ANG II elicits its effects thanks to the receptors AT1 (that leads to vasoconstriction) and AT2 (that leads to vasodilatation), representing pharmacological targets to maintain a normal balance between the vasodilator agent NO and ROS.

Figure 5.

Angiotensin-converting enzyme inhibitors (ACEIs), angiotensin II receptor type 1 blockers (ARBs), 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins), the third generation β-blocker nebivolol, the plant-derived polyphenol resveratrol, and organic nitrate pentaerithrityl tetranitrate (PETN), all are able to limit ROS generation by inhibiting NADPH oxidase or by preventing eNOS uncoupling. ARBs also reduce mitochondrial O_2^- production. Peroxynitrite ($ONOO^-$), resulting from the reaction of O_2^- with nitric oxide (NO), oxidizes the endothelial nitric oxide synthase (eNOS) cofactor tetrahydrobiopterin (BH_4) to dihydrobiopterin (BH_2). BH_4 deficiency leads to O_2^- production by eNOS (eNOS 'uncoupling'), with further oxidative stress. Angiotensin II may reduce vascular BH_4

levels by stimulating NADPH oxidase, but also by downregulating the enzyme dihydrofolate reductase (DHFR), catalyzing the regeneration of BH_4 from BH_2 .