Oxidative Stress Drivers and Modulators in Obesity and Cardiovascular Disease: From Biomarkers to Therapeutic Approach

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Abstract: This review article is intended to describe how oxidative stress regulates cardiovascular disease development and progression. Epigenetic mechanisms related to oxidative stress, as well as more reliable biomarkers of oxidative stress, are emerging over the last years as potentially useful tools to design therapeutic approaches aimed at modulating enhanced oxidative stress "*in vivo*", thereby mitigating the consequent atherosclerotic burden. As a paradigm, we describe the case of obesity, in which the intertwining among oxidative stress, due to caloric overload, chronic low-grade inflammation induced by adipose tissue

dysfunction, and platelet activation represents a vicious cycle favoring the progression of atherothrombosis. Oxidative stress is a major player in the pathobiology of cardiovascular disease (CVD). Reactive oxygen species (ROS)- dependent signaling pathways prompt transcriptional and epigenetic dysregulation, inducing chronic low-grade inflammation, platelet activation and endothelial dysfunction. In addition, several oxidative biomarkers have been proposed with the potential to improve current understanding of the mechanisms underlying CVD. These include ROS-generating and/or quenching molecules, and ROS-modified compounds, such as F2-isoprostanes. There is also increasing evidence that noncoding micro-RNA (mi-RNA) are critically involved in post- transcriptional regulation of cell functions, including ROS generation, inflammation, regulation of cell proliferation, adipocyte differentiation, angiogenesis and apoptosis. These molecules have promising translational potential as both markers of disease and site of targeted interventions. Finally, oxidative stress is a critical target of several cardioprotective drugs and nutraceuticals, including antidiabetic agents, statins, renin-angiotensin system blockers, polyphenols and other antioxidants. Further understanding of ROS-generating mechanisms, their biological role as well as potential therapeutic implications would translate into consistent benefits for effective CV prevention.

Keywords: Biomarker, inflammation, obesity, oxidative stress, platelet activation.

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INTRODUCTION

This review article is intended to describe how oxidative stress regulates cardiovascular disease development and progression, indicating its drivers and modulators. Epigenetic mechanisms related to oxidative stress, as well as more reliable biomarkers of oxidative stress are emerging over the last years as potentially useful tools to design therapeutic approaches aimed at modulating enhanced oxidative stress "*in vivo*", thereby mitigating the consequent atherosclerotic burden. As a paradigm, we describe the case of obesity, in which the intertwining among oxidative stress due to caloric overload, chronic low-grade inflammation induced by adipose tissue dysfunction, and platelet activation represents a vicious cycle favoring the progression of atherothrombosis.

A vast array of data from human studies suggests the involvement of oxidative stress in the onset of cardiovascular disease (CVD). Moreover, upregulation of oxidative stress markers may predict CVD [1-6]. Compelling evidence supports a pivotal role of oxidative stress in atherosclerosis ,

particularly through oxidative modification of LDL [7]. Within the atheroma, oxidized LDLs activate the innate immune system by ligating Toll-like receptors, and scavenger receptors, thus inducing an intracellular signaling cascade leading to increased expression of a range of proinflammatory molecules, including cytokines, chemokines, eicosanoids, proteases, and reactive oxygen species (ROS) [7].

ROS are a family of molecules, including oxygen and its derivatives, that are produced in aerobic cells. ROS are formed in all aerobic organisms from incomplete oxygen reduction during physiological respiration, and include both free radicals such as superoxide and hydroxyl radical, containing one or more unpaired electrons, and non radicals such as hydrogen peroxide.

The term "redox signaling" indicates a process in which the activity of several intracellular proteins and signaling pathways is modulated by physiological levels of ROS. This redox signaling is spatially and temporally fine-tuned, to generate specific effects [8], by several ROS-mediated mechanisms including post-translational covalent modification of cysteine thiols within the active and allosteric sites of proteins, oxidation of iron–sulfur cluster-containing proteins, S- glutathionylation and S-nitrosylation/S-nitrosation [8].

Endothelial cells engulf oxidized LDL via the lectin-like oxidized LDL receptor 1 (LOX-1), which renders the cells functionally deficient [9]. Intracellular accumulation of oxLDL within endothelial cells results in reduced •NO production, increased expression of leukocyte adhesion mole-cules, promotion of a prothrombotic surface, and synthesis of smooth muscle cell mitogenic factors. This proinflammatory, prooxidant atmosphere disrupts vascular function, primarily by decreasing nitric oxide (NO) bioavailability thus perpetu-ating conditions of oxidative stress through excess genera-tion of ROS and reactive nitrogen species [7]. The estab-lished cardiovascular risk factors, including smoking, dyslip-idemia, hypertension, and diabetes, may contribute to endo-thelial dysfunction, in part by unsettling the oxi-dant/antioxidant balance [9-12].

Despite the undisputable role of oxidative stress in this setting, randomized clinical trials and meta-analyses failed to show any benefit from antioxidant vitamins on the develop-ment of CVD or mortality [13-16]. Lack of efficacy of anti-oxidants reflects the complexity of redox reactions in bio-logical systems such as vascular cells and unravel the limita-tions of our current strategies to modulate the redox signal-ing for CVD prevention.

EPIGENETIC MECHANISMS OF OXIDATIVE STRESS

Although there are limited reports on the epigenetic regu-lation of oxidative stress in CVD, in the next future these mechanisms will become increasingly relevant.

Epigenetics supports the assumption that, in humans, ac-quired properties can be transferred to the progeny, but these changes are reversible leaving the genetic code unaltered. Epigenetic mechanisms (DNA methylation and modifica-tions of histone), which regulate chromosomal organization, may concur to determine the activity of gene expression. Thus, we have to reconsider the definition of phenotype, being not only defined by the DNA sequence present in the genome [17]. Epigenetic mechanisms may underlie poorly understood environmental and dietary effects on atherogene-sis as well as the rapid changes in the incidence of coronary heart disease observed in several populations, because hu-man genetic material does not change rapidly through classi-cal mutations or single nucleotide polymorphisms (SNPs) [17]. However, whether epigenetic changes are causally re-lated to pathogenetic steps, such as clonal proliferation of lipid accumulation, smooth muscle cells lesions, and modu-lation of immune responses in the lesions, or whether they occur as a consequence of the ongoing pathological process, remains to be elucidated.Human atherosclerotic lesions are characterized by hy-pomethylation of genomic DNA and methylation changes have been reported at the promoter level of several genes involved in the pathophysiology of atherosclerosis, including hypomethylation of endothelial NO synthase (eNOS) pro-moter area in healthy human vascular endothelial cells, as well as reduction in the methylation status of extracellular superoxide dismutase (SOD) promoter, characteristic of atherosclerotic lesions [17].

Sirtuin family, particularly SIRT1, is highly expressed in the vasculature and plays a critical role in the regulation of vascular function [18, 19], protecting against vascular senescence and age-related vas-cular diseases. SIRT1 has been involved in the processes of aging, metabolism, and tolerance to oxidative stress through its ability to deacetylate several substrates, including his-tones, transcription factors and coregulators.

Interestingly, statins inhibit oxidative-induced endothelial senescence by increasing eNOS activity and expression, leading in turn to SIRT1 upregulation and establishment of a positive feedback loop between SIRT and eNOS. Upregula-tion of SIRT1 then promotes mitochondria biogenesis and expression of catalase through induction of eNOS, SIRT1 and catalase expression [20]. The mammalian Shc locus en-codes the p66Shc isoform, that modulates intracellular redox balance by increasing concentration and functions of ROS as a critical mediator of intracellular oxidative signals transduc-tion, or by directly stimulating generation of mitochondrial ROS by an oxidoreductase activity [21]. In this context, the p66Shc-/- mouse is characterized by increased resistance to oxidative stress, thus representing a unique genetic model of prolonged lifespan in mammals [22]. p66Shc can also be posttranslationally upregulated by phosphorylation and ubiq-uitination following oxidative stress-inducing stimuli [23]. p66Shc expression is negatively regulated by SIRT1, at the transcriptional level, through epigenetic chromatin modifica-tion. 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 [23].

Interference by gene expression by modulating acetylation and methylation of histone/DNA complexes has been described in several clinical settings [24]. For instance, in diabetes mellitus, epigenetic changes involved in the modu-lation of transcription of ROS-generating and proinflamma-tory genes, may be involved in the pathways leading to the hyperglycemic memory [25], defined as persistence of hy-perglycemic stress, able to induce endothelial dysfunction, despite glucose control normalization [26]. Transient hyper-glycemia has been shown to induce long-lasting activation in the promoter of the nuclear factor KB (NF-KB) subunit p65 in aortic endothelial cells both *in vitro* and in nondiabetic mice, leading to increased p65 gene expression. Both the epige-netic changes and the gene expression changes persist for at least 6 days of subsequent normoglycemia, and are prevented by reducing mitochondrial superoxide production or super-oxide-induced a-oxoaldehydes [27]. This concept strength-ens the importance of early glycemic control and may ex-plain why diabetic macrovascular complications occur de-spite achievement of optimal glycemic control. In hypergly-cemic memory, persistent upregulation of the adaptor p66Shc and its mitochondrial translocation are associated with mitochondrial ROS production, reduced NO bioavail-ability, and apoptosis, whereas its downregulation clearly reverses the pathological features of hyperglycemic memory in vascular tissues. p66Shc gene overexpression is epigeneti-cally regulated by demethylation of promoter CpG and ace-tylation of histone 3 operated by acetyl-transferase general control nonderepressible 5 (GCN5) [28]. Thus, p66Shc-derived ROS production contributes to a vicious cycle despite restoration of normoglycemia.

Genetic deletion of p66Shc prevents age-related endothe-lial dysfunction, and p66Shc knockout mice show a 30% lifespan extension, suggesting that p66Shc plays a role in aging and age-associated disorders. Conversely, activation of SIRT1 inhibits oxidative stress-induced endothelial senes-cence [23] (Fig. **1**). Therefore, the cross-talk between two longevity genes may contribute to preventing vascular dis-eases through activation of anti-oxidative stress responses, thus inhibiting endothelial senescence.

In the next future, strategies targeting epigenetic regula-tion of specific genes in vascular cells may provide new pos-sibilities for the treatment of CVD, trying to regulate either healthspan and lifespan.

BIOMARKERS OF OXIDATIVE STRESS

Since it is difficult to detect ROS in biological samples because of their local production and evanescent half-life, indirect biomarkers of oxidative stress have been proposed and developed. These include (a) molecules involved in ROS generation or (b) quenching, as well as (c) ROS-modified lipoproteins, lipids, DNA and proteins. Many of these markers have also been directly implicated in disease pathways.

a. Sources of ROS

In vascular cells, circulating inflammatory cells and platelets, O2•- is synthesized by the enzymes NAD(P)H oxi- dase, xanthine oxidase, lipoxygenase, uncoupled eNOS, iNOS and mitochondrial electron transport [9]. A certain amount of O2•- within the cell, is required to maintain cellu-lar homeostasis; however, when these O2•--generating sources remain activated after a physiological stimulus has waned, the continued production of O2•- alters cellular re-dox homeostasis resulting in increased oxidant stress. Car-diovascular risk factors may therefore trigger ROS genera-tion thus playing a role in mediating oxidative stress in CVD [29].

Myeloperoxidase(MPO) is a leukocyte-derived enzyme catalyzing the formation of several ROS. Circulating MPO levels have been positively correlated to abdominal subcuta-neous and visceral adipose mass both in adults [30] and in prepubertal obese children [31], with significant association with CV risk markers such as CRP, MMP-9, insulin resis-tance, and endothelial dysfunction [31, 32]. A number of studies indicate that plasma MPO is associated with im-proved CVD risk stratification obtained with markers used in routine clinical practice [33]. In fact, elevated MPO levels predict angiographically defined CAD and have been linked to an increased risk of adverse outcomes in patients with acute coronary syndromes [33]. In a similar manner, serum NOX2 activity, the catalytic core of NADPH oxidase, is in-creased in children with obesity and/or hypercholesterolemia [34]. In adults with metabolic syndrome, successful weight loss significantly decreases serum NOX2, in parallel with improved endothelial dysfunction [35] .

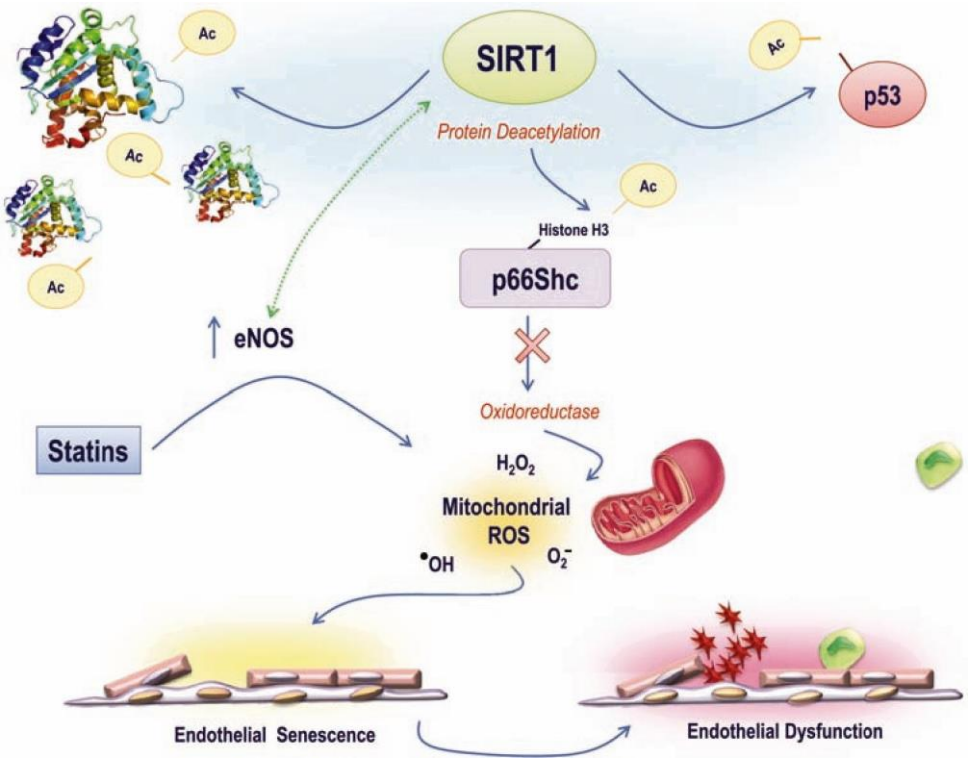

Fig. (1). SIRT1, belonging to the sirtuin family, is highly expressed in the vasculature and is an important regulator of vascular function, protecting against endothelial senescence and age- related vascular diseases. SIRT1 targets a wide range of transcriptional regulators, such as p53. SIRT1 is recruited to the p66Shc promoter region, whereby negatively regulates at the transcriptional level, through epigenetic chroma-tin modification, the p66Shc isoform. p66Shc modulates intracellular redox balance by increasing ROS concentration, eventually affecting endothelial function and aging. SIRT1, sirtuin 1; ROS, reactive oxygen species; Ac, acetylation.

b. Endogenous antioxidants

Endogenous antioxidants include the enzymatic and nonenzymatic antioxidant system. The latter consists of nonenzymatic low-molecular weight (MW) compounds, that can scavenge the oxidizing radical by donating electron(s) directly, such as GSH, histidine dipeptides, uric acid, vitamin C (ascorbic acid), and vitamin E (a-tocopherol). A number of studies reported an inverse relationship between plasma antioxidant, or total antioxidant capacity, and CVD [36]. The growing interest in the possible beneficial roles of antioxidant supplements in the treatment of CVD has contributed to a debate about their value in providing complementary therapies aimed at improving standard therapy [37] In contrast, the strength of the association between dietary antioxidant consumption and the prevention of coronary events is strongest in observational studies, which are confounded by self-selection of patients and co- consumption of other nutrients in whole foods [37], but appears controversial in clinical trials [36].

The major endogenous antioxidant enzymes include SOD, glutathione peroxidase (GPx), paraoxonase-1 (PON1), heme oxygenase (HO), and catalase. Serum extracellular *SOD* activity is negatively correlated with body mass index (BMI) and other components of metabolic syndrome in the absence of established vascular disease [38]. Similarly, low serum *GPx* activity has been associated with increased CV mortality in low HDL subjects [39]. In contrast, serum SOD is increased in the presence of carotid artery disease, possibly as a response to plaque-associated oxidative burden [38]. *PON-1* is a calcium-dependent esterase with peroxidase-like activity located on HDL, and is considered a major factor in their antioxidant and vasoprotective properties [40]. Consistent with this hypothesis, serum PON-1 activity predicts future development of major adverse CV events in both primary and secondary prevention populations, and reclassifies risk categories incrementally to traditional clinical variables [40]. Thiol-containing molecules are central in many redox biochemical and pharmacological reactions. High serum homocysteine as well as low glutathione (GSH) and cysteine are independently associated with CV risk scores at population level [41]. The ratio of reduced GSH to oxidized GSH (GSSG) is an indicator of cellular health, with reduced GSH constituting up to 98% of cellular GSH under normal conditions [42], and has been shown to vary with age in a manner compatible with the onset of vascular disease [43, 44] Similarly, clinical studies have shown that erythrocyte GPx-1 activity is an independent predictor of adverse CV events in individuals without traditional risk factors for atherosclerosis, as well as with suspected or documented CAD [45, 46].

This body of evidence suggests that measuring the endogenous redox potential could reflect oxidative uncoupling at cellular level, and may translate into clinically useful information for CV risk stratification

c. ROS-modified Compounds

Since ROS-generating enzymes and antioxidant molecules frequently exert additional redox-independent actions at vascular level, their association with CV endpoints may reflect only partially ongoing oxidative stress. By contrast, oxidatively modified biomolecules are immediate endproducts and, in some cases, effectors of oxidative stress.

A huge variety of lipid peroxidation end-products have been proposed and studied as biomarkers of oxidative stress in biological samples, including oxidized LDL (oxLDL) or phospholipids (oxPL), lipid hydroperoxides (LOOH), malondialdehyde (MDA), and F2-isoprostanes [47].

Urinary biomarkers of lipid peroxidation provide integrated, noninvasive estimates of systemic oxidative stress over a longer period of time as compared to blood levels, which make them more sensitive for the prediction of chronic conditions and more reliable within large-scale human studies [47, 48]. In addition, urine, as compared to plasma, minimizes the *ex vivo* artifactual formation of unmetabolized compounds resulting from auto-oxidation of lipids [11, 48-50].

MDA is a highly reactive dialdehyde formed as a byproduct of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism. Although relatively unstable, MDA has been frequently measured as an oxidative stress marker in plasma, urine, or tissue. The thiobarbituric acidreactive (TBAR) assay, although extensively criticized as being non-specific for MDA, is widely used to assess MDA [48].

F2-isoprostanes are prostaglandin-like products of the free radical-catalyzed peroxidation of arachidonic acid, and nowadays, F2-isoprostanes are considered the most sensitive and reliable biomarkers of lipid peroxidation [11, 49-51]. The 8-iso-prostaglandin (PG) F_2* is the most frequent F2- isoprostane measured in human biological samples, such as plasma or urine [11, 49-53]. Both stable-isotope dilution assays using gas chromatography/mass spectrometry and immunoassays for 8-iso-PGF_{2a} have been developed [11, 49- 53]. Besides serving as biomarkers of oxidative stress, F2- isoprostanes, including 8-iso-PGF_{2a}, exert biological actions such as vasoconstriction and platelet TP activation [11, 49- 53].

Increased urinary excretion of 8-iso-PGF_{2a}, often intertwined with markers of insulin resistance, endothelial dysfunction, low-grade inflammation and platelet activation, has been reported in association with several CVD risk factors (cigarette smoking, diabetes mellitus, hypercholesterolemia, obesity, arterial hypertension, hyperhomocysteinemia, Helicobacter pylori infection), [11, 49-55]. Moreover, an association has been reported between established vascular disease and isoprostane levels [56, 57]. Urinary 8-iso-PGF_{2a} is also able to monitor the antioxidant effect of interventions for CV risk reduction, including improved glycemic control, statins, weight loss, and antioxidant supplementation [11, 49-53, 58]. Finally, there is suggestive evidence that F2-isoprostane measurement may help to refine current CV risk prediction models both in apparently healthy subjects [59] and patients with acute coronary syndrome [60].

Oxidative damage to DNA can translate into a variety of mutagenic lesions, among which the predominant compounds produced are 8-oxo-7,8-dihydro-guanine (8-oxoGua) and 8- hydroxy-2*-deoxyguanosine (8-OHdG) [52]. These molecules can be measured in serum/plasma and urine by chromatography-based assays or ELISA [61]. Circulating and/or urinary levels of these oxidative DNA adducts are increased in association with obesity, hypercholesterolemia, diabetes, hypertension and premature CAD [62-64]. The question of whether or not intra-individual variation over time of these biomarkers are the main source of total varia-tion remains open [48]. Moreover, the influence of inter-individual variability in DNA repair capacity to the variation of these compounds in human populations is still uncertain [48]. A recent systematic review analyzing available human studies comprising either plasma or urine 8-OHdG concen-trations, reported significant positive associations between 8- OHdG and CVD in case-control studies and a significant association of 8-OHdG with either heart failure or stroke in prospective studies [65]. These findings await validation from larger prospective studies to investigate 8-OHdG as a predictor for CVD.

Advanced glycation end products (AGEs) are formed by glycoxidation or lipoxidation of proteins and amino acids [66, 67]. AGEs are prevalent in the diabetic vasculature and contribute to the development of atherosclerosis even in nondiabetic subjects [68, 69]. AGEs can also specifically interact with CD36 on platelets, accelerating platelet aggre-gation and thrombus formation [70].

There is a growing body of evidence demonstrating a link between increased circulating levels of AGEs and insulin resistance, endothelial dysfunction, coronary artery disease severity, and adverse CV outcomes irrespective of diabetes status [71, 72]. Elevated AGEs levels predict in-stent restenosis in diabetics on optimized glycemic control and have been proposed as a marker of adverse outcome after revascularization [73]. The accumulation of AGEs,

which can be measured using skin autofluorescence, is elevated in high-risk settings such as PAD [74] or end-stage renal dis-ease [75], and is predictive of major adverse CV events [76]. Taken together, these data support the notion that AGEs may represent useful markers of metabolic abnormalities and vas-cular risk. However, since their relationship with oxidative stress is complex, their use and interpretation as biomarkers of oxidative status should be very cautious.

THE CASE OF OBESITY

The prevalence of obesity and associated metabolic dis-orders is rising dramatically in the western countries. Obesity is accompanied by a state of chronic, low-grade, systemic inflammation that increases risk for CVD by exacerbating the vascular inflammatory response [7, 76, 77]. Excessive caloric intake, even before weight gain, is hypothesized to be a primary trigger of systemic inflammation and insulin resis-tance. A high metabolic load of carbohydrates and/or fats from as little as one meal can overload cells and causes ex-cessive mitochondrial oxidation, resulting in increased pro-duction of ROS. Oxidative stress and inflammation are key processes that drive the initiation, progression, and subse-quent rupture of the atherosclerotic lesion in the context of obesity and both mutually amplify each other within the vas-culature and in visceral fat. In visceral obesity, macrophages recruitment infiltrates adipose tissue, which results in the release of inflammatory adipokines. The further increase in oxidative stress, combined with the action of adipokines, exacerbates the vascular pro-oxidant and proinflammatory environment, inducing endothelial dysfunction and smooth muscle cell proliferation, thus accelerating the atheroscle-rotic process. Besides LDL oxidation, the oxidative imbal-ance promotes atherothrombosis through several mecha-nisms, including (a) HDL modification into a dysfunctional, proinflammatory, and pro-oxidant particle; (b) procoagulant action through decreased fibrinolysis and increased tissue factor expression; (c) alteration of local hemodynamic forces; and (d) enhanced platelet activation.

a. Oxidative stress and Inflammatory Mediators

It is well acknowledged that inflammation is one reflec-tion of oxidative stress, which in fact induces the pathways that generate mediators of inflammation, such as adhesion molecules and interleukins. Proinflammatory signaling may in turn increase ROS generation and oxidative stress, thus promoting a vicious cycle [78]. A direct correlation between markers of *in vivo* oxidative stress and several endothelial, platelet or myeloid-derived proinflammatory molecules have been reported in high CV risk settings such as diabetes melli-tus, obesity, and hypercholesterolemia [11].

Obesity occurs in mammalian species when caloric in-take exceeds energy expenditure. “Nutrient excess” induces cellular stress, with consequent ROS production exceeding that required for physiological responses. Thus, in obesity, enhanced oxidative stress may arise as a consequence of a high fat- and/or refined sugar-diet. Furthermore, the excess caloric intake-associated oxidative stress may unleash the impairment of the insulin-signaling cascade, leading to insu-lin resistance [77]. However, in obesity, environmental, life-style, genetic, and epigenetic interactions translate into com-plex pathological processes [79] in which the oxidative stress caused by ROS is crucial. Several processes are involved in obesity-associated oxidative stress, triggered by a nutrient overload in terms of high-fat and high-carbohydrate meals. High fat levels drive enhanced energy storage, mitochondrial oxidation of nutrients, and an imbalance between ROS gen-eration and elimination by the cellular defence systems [80]. Sustained hyperglycemia leads to the overproduction of AGEs and to the activation of the AGEs receptor (RAGE) which in turn leads to inflammation, impaired vascular reac-tivity, and insulin resistance [78]. Both mitochondrial and endoplasmic reticulum (ER) stress responses can regulate or induce adaptation to the ROS production initiated by nutrient excess [81]. Interestingly, free fatty acids (FFA), which are elevated in obesity, have the potential to induce ER stress in several cells, including adipocytes [82]. However, the mo-lecular mechanisms of obesity-induced ER stress in adipo-cytes are not fully elucidated. High-fat diet-induced obesity has been shown to induce ER stress and activate unfolded protein response (UPR) signaling in adipose tissue [82]. Conversely, alleviation of ER stress using chemical chaper-ones suppressed the inflammatory response in the adipose tissue of high-fat diet-fed mice and improved insulin signal-ing [82]. The adipokine vaspin, a visceral adipose tissue-derived

serine protease inhibitor, ameliorates ER stress in obesity through binding cell-surface glucose-regulated pro-tein (GRP78), a molecule recruited from ER to plasma membrane under ER stress[82].

Furthermore, the adaptor protein p66Shc, and some isoforms of PKC family are relevant participants in redox-sensitive signaling pathways in obesity [84] (Fig. **2**). In this setting, excess glucose activates biochemical pathways, such as autoxidation of glyceraldehydes, and oxidative phosphorylation, which cause an increase in intracellular ROS production that may promote PKC activation. Once activated, PKC leads to p66Shc phosphorylation, thus allowing p66Shc to be imported into mitochondria, where p66Shc acts as ROS producer and thus further increases intracellular ROS levels [84]. Using the p66Shc-null mice model, p66Shc-generated ROS has been shown to modulate the impact of insulin on the energetic metabolism, by promoting fat deposition and fat-related disorders [85]. Specifically, the redox enzyme activity of p66Shc is activated by insulin specifically in adipocytes, with consequent ROS generation. This in turn regulates insulin signaling through a number of mechanisms, including AKT phosphorylation, Foxo localization, and regulation of insulin target genes. In the context of insulin resistance, under nutrient and insulin overload, p66shc elicits the signal-inhibitory phosphorylation of the major insulin transducer IRS-1, thereby promoting obesityassociated insulin desensitization which underlies animal susceptibility to metabolic syndrome and type 2 diabetes [86].

Adiponectin has been shown to exert a direct impact on redox state in human arteries and veins, through its com bined effect on BH4-mediated improvement of eNOS coupling and PI3/Akt-mediated phosphorylation of eNOS [87]. Thus, increased oxidative stress in the vessel wall leads to the release of peroxidation products (i.e. 4-hydroxynonenal, 4-HNE) that upregulate adiponectin gene expression in perivascular adipose tissue, via a PPAR-gamma dependent mechanism. These findings raise for the first time the hypothesis of a bidirectional crosstalk between vascular wall and perivascular adipose tissue, with potentially important implications in vascular biology. Moreover, 4-HNE displayed additional function such as activation of the canonical Wnt pathway both *in vitro* and in a rat model of diabetic retinopathy [88]. In mesenchymal osteoblast progenitor cells, acute increase in ROS exerts the so called phenomenon of “Wnt antagonism”, consisting in antagonizing the osteogenic and favoring adipogenic differentiation by diverting the pool of R-catenin away from prototypical Wnt-mediated (TCF/LEF) transcription factors [89]. Taken together, these data underscore the pivotal role of the “adipose oxidative stress” in regulating commitment and differentiation of precursor cells, partly through the modulation the Wnt signaling pathway [90]. Along these lines, lipid peroxidation byproducts may serve as reliable “molecular signatures” of adipose oxidative stress.

Individuals with obesity exhibit higher levels of biomarkers of oxidative damage, which appear directly related to BMI and the percentage of body fat [91]; in contrast, an inverse relationship between body fat, central adiposity, and antioxidant capacity has been shown [92]. The Framingham study reported a close association between BMI and sys-temic oxidative stress, as reflected by the urinary excretion of F2-isoprostanes [4]. Isoprostanes are chemically stable end-products of non-enzymatic lipid peroxidation derived from arachidonic acid [11]. In addition to their being a marker of *in vivo* oxidative stress, isoprostanes have biologi-cal activities relevant to CVD pathophysiology [11]. In fact, 8-iso-PGF2a is a potent vasoconstrictor, and may activate platelets via interaction with the thromboxane receptor (TP) [11]. We showed enhanced isoprostane generation in women with android obesity, with weight loss significantly reducing oxidative stress [93]. Thus, the highly significant correlation between any metabolic perturbation (such as BMI, blood glucose and cholesterol) and isoprostane levels suggests that lipid peroxidation may be related to the metabolic abnor-malities rather the attendant vascular disease. Moreover, dietary or pharmacologic interventions, inducing reductions in either BMI, plasma glucose or cholesterol and in iso-prostane levels, seem to confirm their linear relation under basal conditions [77].

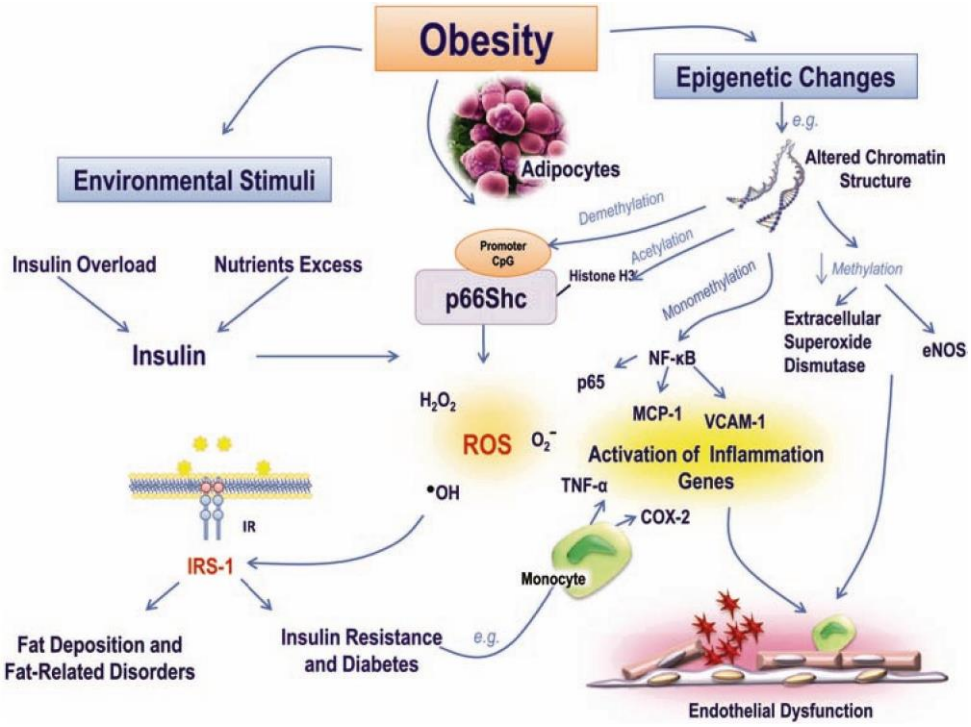

Fig. (2). Genetic, epigenetic, lifestyle, and environmental interactions reflect complex pathophysiological perturbations in which the oxidative stress caused by ROS plays a pivotal role. ROS participates through highly regulated redox-sensitive signaling pathways, to the modulation of the adaptor protein p66Shc, which in turn promotes endothelial dysfunction and activation of inflammatory genes relevant to the development of diabetes mellitus and atherothrombosis. The redox enzyme activity of p66Shc is specifically activated by insulin in adipocytes, and insulin signaling is regulated by p66Shc- generated ROS. Under nutrient and insulin overload, p66shc promotes the signal-inhibitory phosphorylation of the major insulin transducer IRS-1, thereby participating in obesity-induced insulin resistance and diabetes, fat deposition and fat-related d**i**sorders.

Finally, activation, by oxidized LDL, of the RAGE am-plifies the inflammatory response in the vascular wall, trig-gering further generation of ROS and thus inducing acceler-ated atherosclerosis [77]. Obesity may be implicated in RAGE hyperactivation; in fact, in healthy obese women, low endogenous soluble RAGE is associated with reduced adi-ponectin and increased thromboxane-dependent platelet acti-vation, in part dependent on enhanced lipid peroxidation [94].

b- miRNAs

miRNAs are single-stranded, noncoding RNAs that act post-transcriptionally on gene expression. Micro-RNAs (miRNAs) are associated with biological processes that may contribute to the formation of atherosclerosis, type 2 diabe-tes, obesity and CVD. For a comprehensive review please refer to reference [95]. Several miRNAs have been associ-ated with obesity, energy metabolism, pancreatic O-cells, insulin sensitivity and non-alcoholic fatty liver disease, as recently reviewed [96-98]. In obese adipose tissue, miRNAs are hypothesized to regulate adipocyte differentiation, lipid metabolism, oxidative stress, and macrophages infiltration and activation [99-101].

Some of them, such as the miR-21, miR-103, miR-17-92 cluster, miR143, and miR-371, are able to increase adipo-genesis, or accelerate adipocyte differentiation [95]. Between subjects with different amounts of fat mass, differences on miRNA expression can be observed. Thus, in lean and obese subjects, miR-210 and miR-130b are both downregulated during adipocyte differentiation, whereas miR-221, and miR-222, miR-100, and miR-125b, are down-regulated in lean subjects but up-regulated in obese ones [96]. In contrast, miR-107, miR-143, miR-103, and miR-185 are upregulated in normal adipocytes, but downregulated in the obese state [96]. Thus, adipose hypertrophy and hyperplasia are associ-ated with a dysfunctional phenotype with increased mito-chondrial oxidative stress and a pro-inflammatory signaling, which can be induced by changes in miRNA expression pro- files [95] (Fig. **3**). miRNAs play a role in obesity-associated insulin resistance by influencing insulin signaling and glu-cose homeostasis [102]. For instance, miR-21 and miR-223 inhibit the TGF-signaling pathway that inhibit adipogenesis, thus linking increased inflammation and impaired adipo-genesis in dysfunctional adipose tissue [95]. miR-223 regu-lates macrophage activation in adipose tissue, with conse-quent attenuation of diet-induced inflammation and insulin resistance [103].

Characterization of the miRNA signature in healthy and diseased individuals may allow the design of novel, highly specific strategies, tailored to the miRNA signature of an individual, to restore the healthy expression of an entire rep-ertoire of dysregulated miRNAs in the disease state. Based on the potential of a single miRNA to post-transcriptionally regulate the expression of several target genes, altering the function of a single miRNA may modulate the behavior of treated cells. This ability makes miRNAs a target for direct therapeutic intervention. Up to date, very limited experience exists to experimentally alter the activity of miRNAs to yield a particular biological effect, and researchers are trying to determine the clinical potential of therapies utilizing miRNA manipulations. In fact, pharmacologically returning miRNA expression to normal levels may improve both clinical out-comes and current therapies. miRNA levels can be experi-mentally reduced *in vivo* using antisense oligonucleotide miRNAs (antimiRs). As an alternative, miRNA mimics or mimetics, can be used to pharmacologically increase the levels of a particular miRNA. In the context of insulin resis-tance, combining traditional therapeutics with miRNA ma-nipulation technologies to improve clinical outcomes may represent a class of novel therapies. In fact, manipulation of few specific miRNAs could potentially improve the efficacy of drugs traditionally used to improve glucose tolerance and insulin sensitivity.

Pathogenic mechanisms that occur in obese adipose tis-sue and in the vasculature may be linked by miRNAs, that display similar functions in both tissue types, including in-flammation, regulation of cell proliferation, angiogenesis, and apoptosis. Moreover, adipocytes secrete microvesicles containing miRNAs; thus, intercellular communication

be-tween adipose tissue and vasculature may occur through this way. Thus, miRNAs common to both vascular and adipose tissue may be relevant targets for treatment of obesity-associated vascular diseases [95].

b. Platelet Activation and Lipid Peroxidation

Platelet activation may play a relevant role in the patho-genesis of atherothrombosis in patients with visceral obesity [104]. Mean platelet volume (MPV) is a parameter mirroring *in vivo* platelet activation and an independent predictor of vascular events. MPV is increased in obese patients, with a significant correlation between BMI and MPV and concur-rent reversal after weight loss [76]. This may reflect an in-crease in young, mRNA rich platelets (circulating reticulated cells) that endow increased proaggregating potential. In fact, obese subjects present higher levels of reticulated platelets, together with increased P-selectin expression [76]. Persistent platelet activation is accompanied by enhanced expression of activation-dependent adhesion molecules, and with augmented levels of circulating P-selectin in obese insulinresistant subjects [76]. Obese patients have also increased levels of CD40 ligand, an inflammatory protein largely derived from platelets [105].

The major enzymatic metabolite of thromboxane, 11- dehydro-TXB_2, has been validated as a reliable and integrated index of *in vivo* platelet activation [106]. This biochemical index is significantly augmented in healthy obese women, in comparison with the non-obese controls [93].

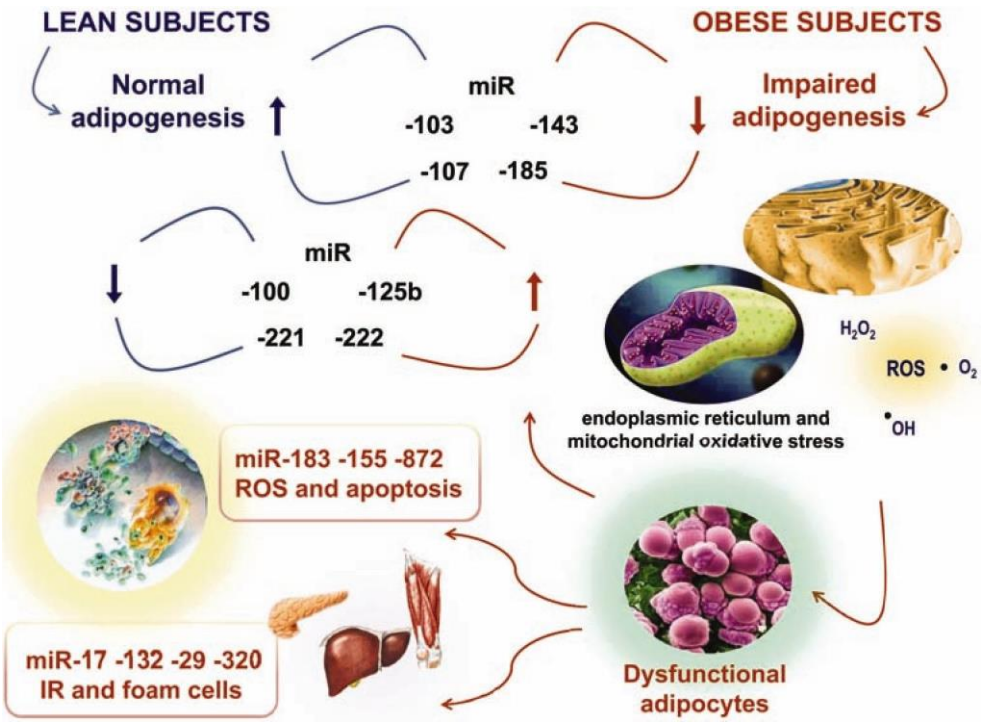

Fig. (3). Adipose hypertrophy and hyperplasia are associated with changing in miRNA expression profiles. For instance, miR-130b and miR210 are both 30 downregulated during adipocyte differentiation in lean and obese subjects, whereas miR-100, miR-125b, miR-221, and miR222 are down- or up-regulated according to body weight (lean vs. obese subjects, respectively). In contrast, miR-103, miR-107, miR-143, and miR-185 are upregulated in normal adipocytes, but downregulated in the obese state. These changes result in dysfunctional adipocytes with increased endoplasmic reticulum and mitochondrial oxidative stress, which further induces an inflammatory phenotype, by regulation cell proliferation, angiogenesis, and apoptosis, as well as insulin resistance.

As stated before, increased oxidative stress in obesity leads to enhanced generation of F2-isoprostanes, such as 8-iso-PGF_{2a} [93]. Besides being an *in vivo* index oxidative stress, 8-iso-PGF_{2a} induces platelet activation by low concentrations of other agonists, via interaction with the TP receptor [11]. Thus, in the setting of visceral obesity, we characterized 8-iso-PGF_{2a} as a potential biochemical link between platelet activation and obesity. We found a significant relationship between 8-iso-PGF_{2a} and 11-dehydro-TXB_2 levels, with down-regulation of both metabolites after weight loss in obesity [93]. These changes leading to platelet activation were driven by inflammatory triggers such as C-reactive protein, again down-regulated by a successful weight-loss program [93]. Thus, in abdominal obesity, low-grade inflammation may trigger platelet activation mediated by increased lipid peroxidation [11, 93, 107] (Fig. **4**).

Because NAD(P)H oxidase is present in platelets, activated platelets may themselves produce intracellular ROS [108]; in fact, platelet activation induces activation of a gp91phox- dependent enzyme [109]. This pathway further enhances platelet activation, promoting intraplatelet isoprostane generation, increasing glycoprotein IIb/IIIa surface expression as well as CD40L release. Consistently, platelets from gp91phox-deficient patients produce only few amount of ROS [110].

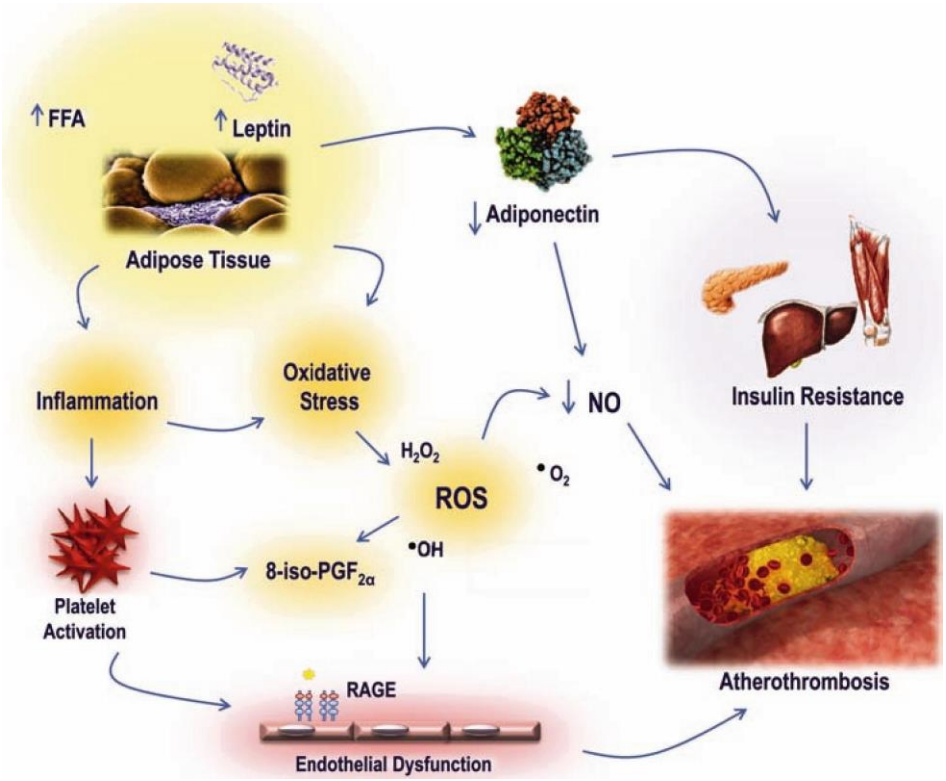

Fig. (4). The mechanisms through which dysfunctional adipose tissue exerts its detrimental cardiovascular effects may be related to adi-pokine release, promoting liver and muscle insulin resistance and impaired insulin secretion, formation of ROS, which, in turn, are involved in the development of endothelial dysfunction, low-grade inflammation, AGE/RAGE axis hyperactivation and lipid peroxidation, with gen-eration of biologically active F2-isoprostane (i.e., 8-iso-PGF2.). These mechanisms all contribute to platelet activation and consequent atherothrombosis.

THERAPEUTIC APPROACHES

Obesity treatment should be focused on weight loss and prevention of its metabolic and vascular complications. Behavioral interventions with diet and/or physical exercise are the cornerstone of treatment. Additional pharmacological interventions are mainly aimed at targeting concomitant risk factors such as arterial hypertension, dyslipidemia, and insulin resistance. Despite the critical role of oxidative stress in obesity and vascular disease, there is no antioxidant-based treatment approved for the management of obesity and the prevention of its complications.

Trials assessing scavenger antioxidants, such as vitamins E and C or [i-carotene, as therapeutic options in CVD prevention were mostly negative [111] or showed only minor responses in pathophysiological end-points [112, 113]. A recent systematic review of randomized controlled trials concludes that there is no evidence to support the prescription of vitamin and antioxidant supplements for the prevention of CVD [111].

Several molecules of different drug-classes frequently used in obese subjects, such as angiotensin-converting enzyme inhibitors (ACEIs), angiotensin receptor blockers (ARBs), beta-blockers, statins, and metformin, have showed pleiotropic antioxidant properties contributing to their beneficial effects (Fig. **5**).

Peroxynitrite is a product formed by the reaction of ROS with nitric oxide (NO) and oxidizes the essential eNOS co-factor tetrahydrobiopterin (BH4) to dihydrobiopterin (BH2) [114]. BH4 deficiency leads to O2- production by eNOS (eNOS 'uncoupling'), thereby worsening the preexisting oxidative stress. Angiotensin II promotes endothelial dysfunction by reducing vascular BH4 levels through enhanced peroxynitrite and ROS generation from NADPH oxidase, as well as through the downregulation of dihydrofolate reduc-tase (DHFR), the enzyme catalyzing the regeneration of BH4 from BH2 [114].

Upregulation of the AT1 receptor, potentiating the activa-tion of angiotensin II-induced NADPH oxidase, has been associated with hyperlipidemia and diabetes. Therefore, ACEIs reduce NADPH oxidase activity and vascular oxidative stress. The observed reduction of atherosclerotic lesion formation achieved by ARBs may be in part attributed to the antioxidant properties of these drugs, such as inhibition of NADPH oxidase expression and activity, with lower mito-chondrial O2- production, and prevention of eNOS uncou-pling through stimulation of BH4 de novo synthesis, im-provement of BH4 recycling from BH2 by upregulation of DHFR, and reduction of eNOS S-glutathionylation [114].

Also statins have antioxidant effects synergistic with ACEIs and ARBs, such as suppression of NADPH oxidase expression and activity, induction of antioxidant enzymes, prevention of eNOS uncoupling by increased BH4 biosynthesis, and enhancement of eNOS expression and activity [115].

The P-blocker nebivolol inhibits NADPH oxidase ex-pression and activity, enhances endothelial NO production by stimulating eNOS activity, and prevents eNOS uncou-pling [116].

The organic nitrate pentaerithrityl tetranitrate (PETN) in-duces several antioxidative enzymes. PETN (but not isosorbide-5-mononitrate) reduces NADPH oxidase activity and prevents O2- production from uncoupled eNOS by increasing BH4 levels [117].

Polyphenols such as resveratrol and isoflavones exert a number of biologic activities on the CV system that may explain the protective properties of fruit, vegetables and red wine. The effects of polyphenols against oxidative injury are mainly attributable to their impact on redox enzymes (NADPH oxidase, catalase) rather than to their moderate direct ROS-scavenging activity. Resveratrol enhances eNOS expression and activity and prevents eNOS uncoupling by upregulating BH4 biosynthesis [118, 119].

An optimal orally available, non-toxic, regenerating tar-geted scavenger should abolish detrimental ROS, while leaving normal redox signaling intact. However, compounds able to reduce oxidative stress by preventing ROS production and accelerating ROS inactivation are likely to be superior to ROS scavenging 'antioxidants'. In this

context, therapies utilizing miRNA manipulations using antimiRs or miRNA mimics, to pharmacologically reduce or increase the levels of a particular miRNA involved in key oxidative stress mechanisms, seem of relevant clinical interest, even if still speculative. Interesting results have also been obtained in mice models by targeting ER stress with chemical chaperones [82]. The verification of the therapeutic potential of new compounds with such properties remains a fascinating bet in the CV research field.

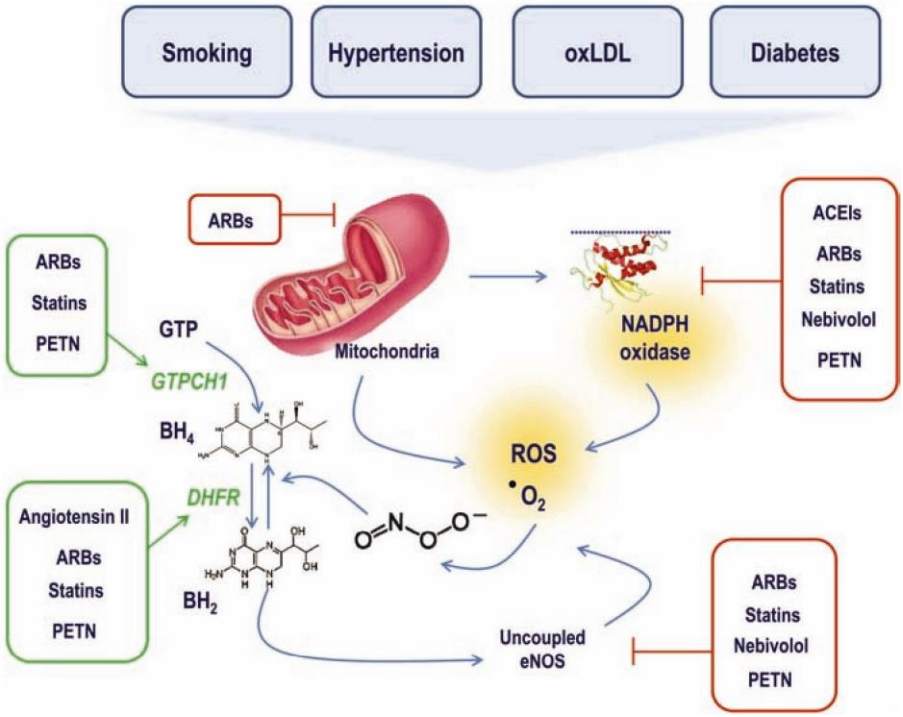

Fig. (5). Oxidative stress burden associated to cardiovascular risk factors and pharmacological prevention. Cardiovascular risk factors such as hypercholesterolemia, diabetes mellitus, hypertension, and smoking induce oxidative stress primarily by stimulating superoxide ($O2^-$) production by NADPH oxidase. Upregulation and activation of NADPH oxidase eliciting mitochondrial $O2^-$ generation have been associated with enhanced levels of oxidized low-density lipoprotein (oxLDL) in hypercholesterolemia, with compounds in cigarette smoke, with hyperglycemia in diabetes. Peroxynitrite ($ONOO^-$) resulting from the reaction of $O2^-$ with nitric oxide (NO), oxidizes the endothelial nitric oxide synthase (eNOS) cofactor tetrahydrobiopterin (BH4) to dihydrobiopterin (BH2). BH4 deficiency leads to $O2^-$ production by eNOS (eNOS 'uncoupling'), with further oxidative stress. Angiotensin II may reduce vascular BH4 levels by stimulating NADPH oxidase, but also by downregulating the enzyme dihydrofolate reductase (DHFR), catalyzing the regeneration of BH4 from BH2. Angiotensin-converting enzyme inhibitors (ACEIs), angiotensin II receptor type 1 blockers (ARBs), 3-hydroxy-3-methylglutaryl- coenzyme A reductase inhibitors (statins), the third generation (3-blocker nebivolol, the plant- derived polyphenol resveratrol, and organic nitrate pentaerithrityl tetranitrate (PETN), all are able to inhibit NADPH oxidase or to prevent eNOS uncoupling by stimulating GTP cyclohydrolase 1 (GTPCH1) (the rate-limiting enzyme in the de novo BH4 biosynthesis pathway from GTP) and/or DHFR. ARBs, resveratrol, and MitoQ also reduce mitochondrial $O2^-$ production.

CONCLUSIONS

A vast array of data from human studies suggests the involvement of oxidative stress in the onset of CVD [1-6]. Compelling evidence supports a pivotal role of oxidative modification of LDL leading to increased expression of a range of proinflammatory molecules, including cytokines, chemokines, eicosanoids, proteases, and ROS [7]. In obesity, enhanced oxidative stress may arise as a consequence of a high fat- and/or refined sugar-diet, triggering AGE/RAGE axis upregulation [78], mitochondrial and ER stress [81, 82], epigenetic changes involved in turn in the modulation of transcription of ROS-generating and proinflammatory genes [25-28]. Excess caloric intake-associated oxidative stress may also unleash the impairment of the insulin-signaling cascade, leading to insulin resistance [77]. In vascular cells, the main enzymes involved in the synthesis of ROS in vascular, circulating inflammatory cells and platelets, are the NAD(P)H oxidase, xanthine oxidase, lipoxygenase, uncoupled eNOS, iNOS and mitochondrial electron transport [9]. In addition to enzymatic pathways, non-enzymatic lipid peroxidation derived from arachidonic acid to generate chemically stable end-products called isoprostanes, has emerged as a potentially relevant mechanism, consistently described in association with several traditional and non traditional risk factors and in close relationship with inflammation and thromboxane-dependent platelet activation [51-60].

There is considerable pathophysiologic and clinical inter-est in the development of novel biomarkers for oxidative stress that may help in the detection of individuals at high risk for future vascular events. We are aware that not every patient carries the same degree of oxidative stress, but the extent to which such diversity of metabolic phenotype trans-lates into diverse vascular outcomes is still a matter of de-bate, and prospective and adequately sized studies are needed.

Therefore, we are still lacking reliable and cost-effective markers able to identify atherosclerotic vascular disease at an early stage and use of drugs aimed to lower oxidative stress is far from clinical practice.

Among proposed markers of oxidative stress, urinary F2 isoprostane detection, with particular reference to 8-iso-PGF2,,,, has been one of the most reliable and best character-ized. Its platelet activating properties, the ability to monitor the antioxidant effect of interventions for CV risk reduction, including improved glycemic control, statins, weight loss, and antioxidant supplementation, its capacity to refine cur-rent CV risk prediction models both in apparently healthy subjects and patients with acute coronary syndrome [51-60], make it 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 in-terventions.

Compounds that reduce oxidative stress by preventing ROS production and accelerating ROS inactivation seem to be superior to ROS scavenging antioxidants, for which trials assessing the efficacy on vascular endpoint are negative or inconclusive [111-113]. Inclusion of a variable proportion of subjects without enhanced oxidative stress is likely to dilute the benefit of antioxidant supplementation, and might ex-plain the largely negative results of these trials. Validation of biomarkers to be employed in clinical trials will be instru-mental to answer unsolved controversial issues around the efficacy of antioxidant compounds.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no con-flicts of interest.

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