Erythrocytes, leukocytes and platelets as a source of oxidative stress in chronic vascular diseases: Detoxifying mechanisms and potential therapeutic options

Jose Luis Martin-Ventura; Julio Madrigal-Matute; Roxana Martinez-Pinna; Priscila Ramos-Mozo; Luis Miguel Blanco-Colio; Juan Antonio Moreno; Carlos Tarín; Elena Burillo; Carlos Ernesto Fernandez-García; Jesús Egido; Olivier Meilhac; Jean-Baptiste Michel

Vascular Research Lab, IIS-Fundación Jiménez-Díaz Autonoma University, Madrid, Spain; 2Inserm, U698, Université Paris Diderot, Sorbonne Paris Cité, AP-HP, Hôpital Bichat, Paris, France

Summary
Oxidative stress is involved in the chronic pathological vascular remodelling of both abdominal aortic aneurysm and occlusive atherosclerosis. Red blood cells (RBCs), leukocytes and platelets present in both, aneurysmal intraluminal thrombus and intraplaque haemorrhages, could be involved in the redox imbalance inside diseased arterial tissues. RBC haemolysis may release the pro-oxidant haemoglobin (Hb), which transfers heme to tissue and low-density lipoproteins. Heme-iron potentiates molecular, cell and tissue toxicity mediated by leukocytes and other sources of reactive oxygen species (ROS). Polymorphonuclear neutrophils release myeloperoxidase and, along with activated platelets, produce superoxide mediated by NADPH oxidase, causing oxidative damage. In response to this pro-oxidant milieu, several antioxidant molecules of plasma or cell origin can prevent ROS production. Free Hb binds to haptoglobin (Hp) and once Hp-Hb complex is endocytosed by CD163, liberated heme is converted into less toxic compounds by heme oxygenase-1. Iron homeostasis is mainly regulated by transferrin, which transports ferric ions to other cells. Transferrin-bound iron is internalised via endocytosis mediated by transferrin receptor. Once inside the cell, iron is mainly stored by ferritin. Other non hemo-iron related antioxidant enzymes (e.g. superoxide dismutase, catalase, thioredoxin and peroxiredoxin) are also involved in redox modulation in vascular remodelling. Oxidative stress is a main determinant of chronic pathological remodelling of the arterial wall, partially linked to the presence of RBCs, leukocytes, platelets and oxidised fibrin within tissue and to the imbalance between pro-/anti-oxidant molecules. Understanding the complex mechanisms underlying redox imbalance could help to define novel potential targets to decrease atherothrombotic risk.

Keywords
Oxidative stress, atherosclerosis, vascular remodelling, antioxidants

Introduction
Chronic pathological vascular remodelling takes place in both, occlusive atherosclerosis and abdominal aortic aneurysm (AAA). Both manifestations of vascular disease show some differences (e.g. occlusive vs. dilating or intimal cap rupture vs. medial wall rupture) but also similarities, sharing common mechanisms (e.g. proteolysis, oxidative stress, neovascularisation or immune-inflammatory responses), as well as the presence of cell and plasma components of haemorrhages or thrombi (1). Whatever intraplaque or intraluminal localisation, haemorrhages and thrombi involve mainly fibrin, platelets, red blood cells (RBCs) and leukocytes. In this context, RBC-derived, iron-rich heme group as catalyser of enzymatic oxidant reaction of leukocyte origin, is probably the main source of oxidative stress, able to modify lipids and proteins, leading to progression of chronic pathological vascular remodelling (2). Recent data from samples obtained in acute events (myocardial infarction/acute coronary syndromes) support that oxidative stress and RBC composition of thrombus could be linked to the clinical consequences/therapeutic options of patients suffering an acute event (3–5). However, the focus of the present review will be the role of RBCs, leukocytes and platelets in chronic pathological vascular redox imbalance, highlighting the functional consequences of oxidative stress mainly in lipids, due to their key role in vascular diseases. Due to space limitations, we will not review the potential consequences of oxidative stress in DNA or proteins, although an exception will be done for fibrin(ogen) due to the importance of this protein as a main component of the thrombus.

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Red blood cells and oxidative stress in chronic pathological vascular remodelling

Lysis of RBCs within the intraluminal thrombus of AAA (1) or inside the plaque associated to haemorrhage due to microvessel rupture (6) or to RBC extravasation in initial plaques, may lead to subsequent release of the pro-oxidant haemoglobin, that when oxidised in methaemoglobin, liberates heme and iron within tissue and transfers them to lipoproteins (7).

The initial oxidative process in haemoglobin (Hb) involves the spontaneous oxidation of its ferrous iron (Fe²⁺), known as auto-oxidation of Hb, leading to non-functional methaemoglobin (metHb) and superoxide (O₂⁻). Moreover, hydrogen peroxide (H₂O₂), coming from O₂⁻ dismutation, could react with Hb, either Fe²⁺ or ferric (Fe³⁺), forming ferrylHb (Fe⁴⁺) or ferryl protein radicals, respectively. Finally, the formation of ferrylHb could also result in the formation of heme degradation products. Oxidised forms of Hb could act as a dangerous signal for the immune system, thus contributing to the innate immune response that takes place in vascular diseases (8). In a recent study, FerryHb, but not haemoglobin or metHb, induced the expression of proinflammatory genes in endothelial cells in vitro and the recruitment of polymorphonuclear cells (PMNs) in vivo (9). The presence of these oxidised forms of Hb in ruptured advanced atherosclerotic plaques was recently observed (7). Moreover, in this interesting study, the authors demonstrated that exposure of RBCs to lipids from atherosclerotic lesions causes haemolysis and oxidation of Hb, and conversely, Hb promoted further lipid oxidation. In contrast, Boyle et al. (10) reported that erythrocytes, Hb and heme, released by in-traplaque haemorrhage, stimulated cholesterol efflux and decrease oxidative stress in macrophages (11). These effects are mediated by activating transcription factor-1 (12). Similarly, Finn et al. reported that haemoglobin/haptoglobin stimulated macrophages expressed more ferroportin, had less intracellular iron and reactive oxygen species (ROS) production and were more resistant to cholesterol loading due to upregulation of the ATP-binding cassette transporter (13).

Regarding the relationship between lipids and Hb, a previous study identified by mass spectrometric analysis two peaks corresponding to alpha and beta chains of Hb associated to proinflammatory high-density lipoprotein (HDL). Biochemical analysis confirmed the differential association of Hb with HDL from hyperlipidaemic mice. Interestingly, HDL-associated Hb is predominantly in the oxygenated (oxyHb) form with distinct physical and chemical properties. Furthermore oxyHb-containing proinflammatory HDL potentially consumed nitric oxide (NO) and contracted arterial vessels ex vivo (14). Apart from the oxidative toxicity of Hb, Hb is a potent scavenger of NO, a critical regulator of vascular tone, endothelial function and thrombosis. Hb functions as an allosterically and redox-regulated nitrite reductase whose “enzyme activity” couples hypoxia to increased NO-dependent blood flow (15). Hb potently inactivates the NO radical forming nitrate and metHb, producing endothelial dysfunction under haemolytic conditions (16).

Besides, heme exerts important biological functions as a prothletic group in haemoproteins and as a regulator of protein expression. Heme also has toxic properties mainly derived from its hydrophobic nature and from the iron atom contained in the porphyrin ring (17). Heme can enter into RBCs membrane, which shortens erythrocyte life span, enhancing haemolysis (18). In addition, heme potentiates cell cytotoxicity mediated by leukocytes and other sources of ROS (19). Furthermore, heme induces neutrophil migration (20) through a mechanism involving leukotriene B4 (21). Free heme can threaten vascular endothelial cell integrity through oxidative modification of low-density lipoproteins (oxLDLs) (22). Initially, most of this oxLDL is contained within foam cell lysosomes primarily in the form of the peroxidised lipid-protein complex ceroid (23). Specifically, ceroids are insoluble sudanophilic polymers of oxidised cholesterol and proteins. Ceroids are hallmarks of atherothrombotic pathology (24). Development of ceroids could colocalise with iron deposits within cell and tissue (25), and presence of Hb and myeloperoxidase has been recently identified in ceroids by Raman and fluorescence spectroscopy (26). Similarly, ceroids can be found in intraluminal thrombus of AAA (Fig. 1). These data provide also evidences of the role played by heme-derived iron in the genesis of tissue oxidative process, modifying both lipids and proteins.

Iron is an essential element that plays crucial roles in cell proliferation and metabolism by serving as a functional constituent of various enzymes, including ribonucleotide reductase and cytochrome P450. However, when present in excess, free iron generates ROS via the Fenton reaction. In this respect, another iron species named non-transferrin bound iron seem to play an important role in the ROS-induced toxicity both at the cell membrane but also intracellularly (27). On the opposite, ROS themselves can modulate iron homeostasis as a defence mechanism against iron-induced oxidative stress, since the expression of many genes involved in iron transport and storage is regulated by ROS (28).
Human carotid atherosclerotic lesions contain 3- to 17-fold more iron than healthy control arteries (29). Iron can mediate both lipid and protein oxidation; however, it has been later suggested that elevated levels of iron contribute to the extent of protein, but not lipid, oxidation in advanced human atherosclerotic lesions (30). Similarly, a previous study suggested that higher oxidative stress in AAA tissue could be the result of higher iron levels (31). Moreover, peroxidase activity potentially associated to iron in intraluminal thrombus of AAA can be detected (**Fig. 2**), further suggesting the role of iron as a main mediator or oxidative stress in AAA thrombus. The source of redox-active iron in vascular tissue could involve both, RBC lysis and erythrophagocytosis. Most of the iron within vascular lesions is associated with phagocytes. In this respect, we have recently shown that magnetic resonance imaging allows in vivo demonstration of super-paramagnetic iron oxide uptake at the luminal interface of the AAA thrombus, potentially reflecting increased iron phagocytosis by leukocytes (32). The storage and processing of iron from erythrophagocytosis or other sources within vascular lesions could have an important role in disease progression.

**Leukocytes and oxidative stress in chronic pathological vascular remodelling**

Leukocytes produce ROS (O$_2^-$ and H$_2$O$_2$) during respiratory burst. Immune cells use NADPH oxidase to reduce O$_2$ to oxygen free radical and then to H$_2$O$_2$. Moreover, neutrophils and monocytes utilize myeloperoxidase (MPO) to further combine H$_2$O$_2$ with Cl$^-$ to produce hypochlorite, causing oxidative damage.

Superoxide radical (O$_2^-$) is unstable in aqueous solution and is rapidly dismutated to H$_2$O$_2$, favoured by the action of superoxide dismutase (SOD). It is a poorly membrane-permeable molecule, being generally restricted to the cell compartment where it is produced. The main O$_2^-$ functions are serving as a precursor for other ROS (e.g. H$_2$O$_2$) in redox signalling, causing rapid inactivation of NO and further oxidative damage indirectly through more reactive radicals such as hydroxyl radical (33). Different studies have demonstrated increased O$_2^-$ levels in atherosclerotic or AAA tissue compared to normal healthy aortas (34, 35).

Among ROS, H$_2$O$_2$ is a non-radical, uncharged oxidant that is chemically more stable than other ROS and that can permeate through the vascular wall. In addition, H$_2$O$_2$ can accumulate extracellularly in the tissue and survive long enough to induce numerous paracrine functions. In this respect, we have recently observed increased H$_2$O$_2$ levels in ILT of AAA (36). H$_2$O$_2$ itself is not very reactive. However, the danger of H$_2$O$_2$ comes from its rapid conversion to hydroxyl radical by interaction with a range of transition metal ions, probably being iron the most important in vivo. Thus, thrombus is a privilege site for ROS formation because they can be formed, among other mechanisms, either by MPO-catalyzed or by Fe$^{2+}$-catalysed conversion of H$_2$O$_2$ (37).

Phagocytic NADPH oxidase O$_2^-$ production is normally dormant in resting neutrophils and activation involves the translocation of cytosolic subunits to the membrane, generating O$_2^-$ mainly in the extracellular compartment. Several works have demonstrated a key role of vascular and phagocytic NADPH oxidase (Nox) isoforms in the development of human vascular diseases (38). It has been previously demonstrated that NADPH oxidase activity positively correlated with carotid intima-media thickness (IMT) in asymptomatic subjects (39). We should also take into account the fact that other enzymes have been proposed as sources of ROS in atherothrombosis such as lipoygenase, xanthine oxidase, and NO synthase.

**MPO** is a haemoprotein mainly released by activated leukocytes. MPO catalyses the reaction of H$_2$O$_2$ with halide and pseudohalide ions (Cl$^-$, Br$^-$, I$^-$, and SCN$^-$) to form hypohalous acids (hypochlorous acid, HClO, from Cl$^-$; hypobromous acid, HOBr, from Br$^-$; and hypothiocyanous acid, HOSCN, from SCN$^-$), which are potent oxidants mainly involved in killing bacteria and other pathogens (40, 41). HOCl converts free and protein-bound tyrosine residues to 3-chlorotyrosine (42). HOSCN reacts with great specificity with thiols [both glutathione (GSH) and protein cysteine (Cys) residues] and selenium-containing species (43, 44). In addition, MPO along with H$_2$O$_2$ converts nitrite into nitrogen dioxide.

**Figure 2**: DiAmino Benzidin (DAB) staining of ILT showing large areas of DAB positivity (X 4) corresponding to degenerated RBC (b, formol fixed section X 20).
radical (45, 46). Recently, it has been shown that under oxidative stress, MPO may serve as a source of free iron through a mechanism that involves heme depletion (47). MPO has been implicated in lipoprotein oxidation in vivo (48). Previous studies have shown that catalytically active MPO and its oxidative species are present in human atherothrombotic lesions (49). Moreover, plasma MPO levels are increased in atherothrombosis (50, 51).

**Platelets and oxidative stress in chronic pathological vascular remodelling**

Platelets, similarly to RBCs, are present in intraluminal thrombus of AAA (1) and can infiltrate the atherosclerotic plaque via rupture of newly formed microvessels and/or intraplaque haemorrhage (6). Platelet activation is a common feature of atherothrombosis, accompanied by increased platelet aggregation, platelet-leukocyte interaction and platelet-induced ROS production. Activated platelets produce intracellular O$_2^*$ by NADPH oxidase (52), which conversely increases platelet recruitment favouring thrombus formation (53). It has been recognised that lipoproteins can influence platelet function and conversely, platelets are able to bind, transport and oxidised lipoproteins (54, 55). Lipid oxidation and oxidative stress appear to trigger prothrombotic effects mainly mediated by CD36 (56–58).

**Consequences of oxidative stress on fibrin(ogen)**

Fibrinogen is proteolysed to fibrin by thrombin, releasing fibrinopeptide A and B, which are finally cleaved to induce polymerisation into protofibrils of overlapping fibrin units. Components of the fibrin(ogen) system are present in human atherosclerotic plaques (59) and in AAA (1). Moreover, the modification of fibrin structure and/or function has been related to different chronic vascular-associated diseases such as coronary or peripheral artery disease (60). Among mechanisms modifying fibrin properties are genetic factors, thrombin concentrations, platelet activation and oxidative stress. Interestingly, fibrinogen is very susceptible to oxidation, potentially preventing other proteins from oxidation. Exposure of fibrinogen to Fe$^{3+}$-ascorbate promotes fibrin formation and enhances platelet aggregation (61). Previous studies have shown that elevated concentrations of fibrinogen are associated to increase risk of atherosclerosis and AAA (62, 63). More recently, oxidative modification of fibrinogen has been suggested to be a prothrombotic risk factor (64, 65) and also the key molecule responsible for advanced oxidation protein products in plasma (66). On the whole, this data suggest that oxidative stress could modify fibrin(ogen) and finally, clot structure.

**Oxidative stress detoxifying mechanisms in chronic vascular remodelling**

However, oxidative stress is the result of imbalance between pro-oxidant and anti-oxidant molecules. In response to the high oxidant milieu inside vascular wall associated to the presence of RBCs, leukocytes and platelets, several antioxidant mechanisms can be modulated to prevent harmful ROS production.

**Scavengers**

Free Hb binds to **haptoglobin**, which are cleared by the scavenger receptor CD163. When released into plasma, Hb forms a high affinity complex with haptoglobin, preventing thereby peroxidative modifications of Hb (67). Haptoglobin is a circulating powerful acute phase protein whose main roles are to prevent renal injury (caused by Hb accumulation) and Hb/iron loss by urinary secretion, as well as to stabilise Hb/iron within Hb and protect globin from oxidative modification (68). Interestingly, not only Hb can bind HDL (as stated above) but also Hb-Hp complex has been associated to HDL in modifying its functional properties (69). Remarkably, the haptoglobin genotype is a determinant of oxidative activity of free Hb, and of iron content in human plaques (70). Hb-Hp complex formation facilitates the delivery of Hb to CD163 expressing-monocytes/macrophages. Interestingly, Hp/Hb binding to CD163 triggers signal transduction pathways (61).

**CD163** is a member of the scavenger receptor cysteine-rich domain containing proteins. In addition to different CD163 splice variants, a soluble version circulates in plasma coming from proteolytic shedding of the membrane bound version. Among possible proteases, we have recently demonstrated that neutrophil elastase promotes CD163 shedding, resulting in a decreased clearance of Hb by macrophages (71). As commented above, a recent study described the presence of a novel macrophage phenotype associated to intraplaque haemorrhage, characterised by high CD163 and heme oxygenase-1 (HO-1) levels (10). Interestingly, heme induces HO-1 and CD163 expression in macrophages (10).

Free heme binds to **hemopexin**, which is cleared by endocytosis mediated by low-density lipoprotein receptor-related protein/CD91. Hemopexin is an acute phase protein that transports heme in the body under a non-toxic form, decreasing the peroxidative and catalytic activity of heme and preventing heme-induced oxidative damage. In addition to the heme-detoxifying properties, hemopexin-heme complexes may activate different signalling pathways (such as JNK or NF-κB), modulate hemoxygenase, ferritin and transferrin receptor expression or regulate the immune response (17).

**Anti-oxidants**

After uptake of haptoglobin-haemoglobin complex by CD163, liberated heme is converted into less toxic compounds (such as fer-
rous ion, carbon monoxide (CO) and biliverdin-bilirubin] by heme oxygenase-1 in the cytosol. However, there is evidence that the functional properties of this enzyme are not limited to heme degradation, but includes anti-inflammatory, antiapoptotic and antioxidant actions (72). These beneficial effects have been mainly associated to its products, such as CO, but other mechanisms such as the expression of ferritin by HO-derived iron could also be involved (73). Expression of HO-1 is low in vascular tissues under physiological conditions, but highly upregulated in human and mouse vascular lesions, suggesting an antioxidant response to different stimuli such as oxidised LDLs in atherosclerosis (74) or blood flow in AAA (75). In one hand, decrease or absence of HO-1 expression in mice resulted in increased lipid uptake and foam cell formation associated to increased ROS generation and cytokine expression (76). In the other hand, HO-1 activation shows a protective role against atherosclerotic lesion formation, possibly by inhibiting lipid peroxidation and influencing the NO pathway (77).

As a response to oxidative stress, cells upregulate both HO-1 and ferritin. Ferritin modulates the potential toxic effects of the HO-1 product iron by its storage and/or through ferroxidase activity (19). However, although iron storage in ferritin is safe in normal conditions, under pathological conditions ferritin may have antioxidant actions (78). In addition to its well-known intracellular iron storage functions, extracellular ferritin has been involved in regulation of immune-inflammatory responses (e.g. inducing IL-10) or angiogenesis (e.g. by binding to kininogen and decreasing its proteolysis) (79), key mechanisms involved in atherothrombosis. Similarly to the protective effect of ferritin at the intracellular level, circulating iron homeostasis is regulated by transferrin, a glycoprotein containing an iron-binding site, which accepts ferric ions and transports them to other cells where transferrin receptor internalises transferrin-bound iron via endocytosis.

The presence of ferritin in experimental and human atherosclerotic lesions is well documented (80). Very recently, the expression of ferritin, along with transferrin receptor, in phagocytes was associated with severity of human carotid plaques (81). Moreover, transferrin has been inversely correlated to lesion volume in patients with stroke, suggesting that transferrin may play a protective role in the early phases of stroke progression (82).

In relation to other antioxidant enzymes not directly involved in hemo/iron induced-oxidative stress modulation, SOD, catalase, thioredoxin (TRX), peroxiredoxin (PRX) and glutathione peroxidases (GPX) have been involved in redox balance in chronic vascular diseases. As explained before, SOD (either Mn or Cu-Zn SOD) antioxidant function involves $O_2^-$ dismutation to $H_2O_2$, which is further processed by catalase or PRX to water; however, due to the production of $H_2O_2$, SOD could potentially be pro-oxidative in certain situations, in case other $H_2O_2$ antioxidant proteins do not respond similarly to SOD. In this respect, AAA formation is associated with early increases in SOD expression in an experimental model (83). In contrast, SOD and GPX levels are low in AAA tissue (84). We have recently observed a decrease of MnSOD, catalase and TRX reductase in PMNs of AAA patients compared to controls, which suggest a global decrease in antioxidant enzymes in PMNs under chronic pathological conditions (36).

TRX reductase, together with TRX, PRX and NADPH constitute a ubiquitous system that regulates cellular redox status. TRX has a redox active disulfide/dithiol site within two conserved cysteine residues, and it functions as an antioxidant molecule by protecting cells against $H_2O_2$, regulating HO-1 expression or inducing MnSOD in the mitochondria. Moreover, it has a protective role against NO-induced stress, regulating NO synthases activity and through other NO regulating processes. However, the main antioxidant properties of TRX result from PRX action, which recycle $H_2O_2$ through reduction of several hydroperoxides into water and alcohol (85). PRXs are a recent discovery among the peroxidases lacking the heme group, which were initially described mainly as antioxidant proteins because of its ability to inactivate $H_2O_2$, ONOO$^-$, and other hydroperoxides. However, other cellular roles have been recently proposed for PRX, including the modulation of cytokine-induced $H_2O_2$ levels, which have been shown to mediate the signalling cascade that leads to cell proliferation, differentiation, apoptosis, and proinflammatory actions. TRX up-regulation in response to increased oxidative stress has been associated with intraplaque haemorrhage of coronary culprit lesions, suggesting its potential role as a marker of plaque instability (86). Moreover, we have observed increased TRX and PRX-1 levels in the intraluminal thrombus and plasma of AAA patients (87, 88). Finally, glutathione (GSH), along with the TRX system, maintain the intracellular milieu in a reduced state. GSH is used by the GPX to reduce peroxides, producing oxidised GSH (GSSG) while GSH reductase reduces this oxidised form to GSH. The relationship between GPX1 activity and cardiovascular disease is well described. GPX1 activity is inversely correlated with coronary artery disease (89), and decreased erythrocyte intracellular GPX1 activity is associated with an increase in the number of vascular beds with atherosclerotic manifestations (90). Glutathione depletion in platelets leads to attenuated GPX activity, decreased levels of α-tocopherol, and increased lipid peroxidation (91).

**Therapeutic options**

Due to the important role of oxidative stress in different pathological states, such as chronic vascular diseases, great efforts have been made to address the potential therapeutic effect of antioxidants. Regarding the molecules discussed in the present review, we will highlight some examples of different strategies that have been addressed in experimental models or in patients with different manifestations of chronic vascular diseases.

Regarding experimental models, it has been shown that treatment with the iron chelator desferrioxamine inhibits atherosclerotic lesion development and decreases lesion iron concentrations in the cholesterol-fed rabbit (92). In a recent study, a small molecule inhibitor of bone morphogenetic protein signalling (LDN) was used to suppress hepcidin expression, which in turns reduces macrophage intracellular iron levels. Interestingly, the authors demonstrated that injection of LDN to ApoE mice decreased lesion size, lowering intracellular iron and $H_2O_2$ production, and
raising cholesterol efflux to ApoA-1 (93). Although there is no data in experimental models of pathological vascular remodelling, new approaches to target free heme and free Hb complexes has been recently used (94). Targeting ROS production by administration of apocynin (NADPH oxidase inhibitor) has been recently found to attenuate AAA experimental formation (95). In the opposite hand, diminished experimental AAA formation by tamoxifen treatment has been associated to increased catalase expression, which was accompanied by decreased PMN infiltration (96). More specifically, Ebselen, a GPx1-mimetic, reduces atherosclerotic lesions in diabetic apoE−/− mice (97).

Regarding the data obtained in humans, most of the studies performed have been focused on the role of vitamins (mainly vitamin C and E), with the majority of trials showing negative results (98). Among potential explanations for the negative results obtained in these trials, it should be taking into account that oxidative stress varies from patient to patient, and patients included in those trials were not assessed for their "oxidative stress status". Other factors such as the type, dosage and duration of antioxidant used have also been questioned. Furthermore, Levy et al demonstrated that beneficial effects of Vitamin E are shown in specific haptoglobin genotype (99–101).

In any case, future studies should also take into account the physiological role of oxidative stress; they should take advantage on the novel results obtained with more specific therapies at the experimental level to be used in selected patients, which could benefit from this therapy in the way to the dreamed era of individualised medicine.

In conclusion, oxidative stress is a main determinant of chronic pathological remodelling of the vascular wall, depending, at least in part, on the presence of RBCs, leukocytes, platelets within tissue (Fig. 3). Understanding the complex cellular and molecular mechanisms underlying redox balance could help to define novel potential therapeutic targets to decrease atherothrombotic risk.
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Conflicts of interest

None declared.

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