**Oxysterols and 4-hydroxy-2-nonenal contribute to atherosclerotic plaque destabilization.**

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Abstract: A growing bulk of evidence suggests that cholesterol oxidation products, known as oxysterols, and 4-hydroxy-2-nonenal (HNE), the major proatherogenic components of oxidized low density lipoproteins (oxLDLs), significantly contribute to atherosclerotic plaque progression and destabilization, with eventual plaque rupture. These oxidized lipids are involved in various key steps of this complex process, mainly thanks to their ability to induce inflammation, oxidative stress, and apoptosis. This review summarizes the current knowledge of the effects induced by these compounds on vascular cells, after their accumulation in the arterial wall and in the atherosclerotic plaque.

Prof. Giuseppe Poli
Prof. Neven Zarkovic
Guest Editors
FRBM Special Issue '4-Hydroxynonenal and related lipid oxidation products'

Turin, December 22, 2016

Dear Professors,
we are re-submitting a revised version of our manuscript No: FRBM-D-16-01213 "Oxysterols and 4-hydroxy-2-nonenal contribute to atherosclerotic plaque destabilization".

Looking forward to hearing from you
With best regards,
Gabriella Leonarduzzi

Turin, Decemberr 22, 2016

Dear Prof. Giuseppe Poli
Associate Editor
Free Radical Biology & Medicine

we are re-submitting a revised version of our manuscript No: FRBM-D-16-01213 “Oxysterols and 4-hydroxy-2-nonenal contribute to atherosclerotic plaque destabilization”.

We thank the Reviewers for the thoughtful review and helpful comments, which have guided the revision of the manuscript. We have answered to all queries raised by them.
Hereafter, please find our point by point response to the comments.

Reviewer #2:
- *A careful proofreading to correct typographical errors.*
It has been done

- *there is no mention about the role of cytosolic calcium deregulation which is involved in apoptosis evoked by oxysterols and HNE.*
This new part with the related references has been included in both section of oxysterols and HNE.

- *page 17: the authors write that "High plasma levels of HNE may damage the endothelial barrier function"...Are the authors sure about the plasma ? There is no reference associated with this sentence. If yes, it could be fine to give more precisions on the origin, level, and form of circulating plasma HNE (adducts on serum albumin? other proteins ?bioreactivity, toxicity etc...)*
Honestly, we did not find articles concerning the high plasma levels of HNE which could damage the endothelial barrier function. This has only been observed on various cell culture types incubated with HNE. The sentence has been modified and a reference has been added.

Reviewer #3:

- *Page 11, line 3: "An important feature of oxysterol-induced-apoptosis is the effect oxysterols have on cell cycle regulation:...." This sentence should be modified as "An important feature of oxysterol-induced-apoptosis is the effect of oxysterols on cell cycle regulation"*

- *Page 12, line 15: "absorbtion" please spell as "absorption"*

- *Throughout the manuscript, some sentences are too long and should be rewritten for improved clarity.*
*Example: Page 2, "The lesions primarily develop and progress at the arterial wall, mediated by interactions among cytokines, growth factors and vasoregulatory molecules, which regulate the function of cells intrinsic to the arterial wall and extracellular matrix (ECM) as well as blood cells and plasma constituents."*

- *Reference 148: "O. Rådmark, B. Samuelsson, 5-lipoxygenase: regulation and possible involvement in atherosclerosis, Prostaglandins Other Lipid Mediat. 83 (3) (2007) 162-74." The page numbers should be modified as "162-174" instead of "162-74".*

All the suggested changes have been done.

## Highlights

Atherosclerosis is characterized by oxidative stress and chronic inflammation

Oxysterols and HNE are proatherogenic and contribute to plaque progression

These oxidized lipids induce endothelial dysfunction and ECM degradation

# Oxysterols and 4-hydroxy-2-nonenal contribute to atherosclerotic plaque destabilization

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## Abstract

A growing bulk of evidence suggests that cholesterol oxidation products, known as oxysterols, and 4-hydroxy-2-nonenal (HNE), the major proatherogenic components of oxidized low density lipoproteins (oxLDLs), significantly contribute to atherosclerotic plaque progression and destabilization, with eventual plaque rupture. These oxidized lipids are involved in various key steps of this complex process, mainly thanks to their ability to induce inflammation, oxidative stress, and apoptosis. This review summarizes the current knowledge of the effects induced by these compounds on vascular cells, after their accumulation in the arterial wall and in the atherosclerotic plaque.

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*Keywords:* atherosclerosis, oxysterols, HNE,

*Abbreviations:* α-EPOX, 5α,6α-epoxide; β-EPOX, 5β,6β-epoxide; 22-OH, 22-hydroxycholesterol; 24S-OH, 24S-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; 27-OH, 27-hydroxycholesterol; 7α-OH, 7α-hydroxycholesterol; 7β-OH, 7β-hydroxycholesterol; 7-K, 7-ketocholesterol; AP-1,

activator protein-1; COX-2, cyclooxygenase-2; DPI, disulfide isomerase; ECs, endothelial cells; ECM, extracellular matrix; EDR, endothelium-dependent relaxation; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; HNE, 4-hydroxy-2-nonenal; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; LC3-II, light chain 3-II; 5-LO, 5-lipoxygenase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein; MIP-1β, macrophage inflammatory protein-1β; MMPs, matrix metalloproteinases; NAC, N-acetylcysteine; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; NOX, NADPH oxidase; Nrf2, nuclear factor E2-related factor 2; oxLDLs, oxidized low density lipoproteins; PDGFR, platelet-derived growth factor receptor; $PGE_2$, prostaglandin $E_2$; PI3K, phosphoinositide 3-kinase; PK, protein kinase; PPAR-γ, peroxisome proliferator-activated receptor-γ; ROS, reactive oxygen species; SMCs, smooth muscle cells; TGF-β1, transforming growth factor-β1; TIMPs, tissue inhibitors of MMPs; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α; Triol, cholesterol-3β,5α,6β-triol; TRX, thioredoxin; UPR, unfolded protein response; VCAM-1, vascular cell adhesion molecule.

## 1. Introduction

Atherosclerosis is a multifactorial and degenerative disease affecting large- and medium-sized arteries, which is characterized by chronic inflammation, oxidative stress, and by blood flow that is altered in certain areas of the vascular wall [1,2]. The lesions primarily develop and progress at the arterial wall, mediated by interactions among cytokines, growth factors and vasoregulatory molecules, which regulate the function of cells intrinsic to the arterial wall and extracellular matrix (ECM) as well as blood cells and plasma constituents. Development of atherosclerotic lesions is preceded by impaired vascular endothelium function, mainly caused by oxidative stress,

inflammation [1,3], and endoplasmic reticulum (ER) stress [4], all conditions that are induced by the factors promoting and accelerating atherosclerosis. Vascular endothelium dysfunction is also linked to cellular senescence [5]. The intercellular cross-talk that occurs among smooth muscle cells (SMCs), macrophages, endothelial cells (ECs) and leukocytes leads to a fibroproliferative response. During this response, the ECM plays a key role in plaque formation and progression, providing the structural integrity of the plaque itself, as well as contributing to cell migration and proliferation, and finally thrombosis. A stable atherosclerotic plaque is characterized by a thick and solid fibrous cap, where large amounts of ECM are deposited, and a small central lipid core. The strength of the fibrous cap depends on a dynamic balance between collagen synthesis and degradation. Conversely, an atherosclerotic plaque at high risk of rupture contains a large lipid core where, in addition to extracellular lipid deposition and debris, ECM degradation is enhanced, leading to increased plaque fragility; moreover, the lipid core is covered with a thin fibrous cap affected by ongoing inflammation and neovascularity [6,7].

Among the intrinsic and extrinsic factors that can trigger plaque progression, with subsequent vulnerability and rupture, key roles are played by lipid components, inflammation, cell death, and fibrous cap weakening, as well as hemodynamic stress and circumferential shear stresses [8].

One of the major risk factors of atherosclerosis is hypercholesterolemia, which promotes the accumulation of oxidatively modified low-density lipoproteins (oxLDLs) in the arterial wall, promoting endothelial cell dysfunction and, in turn, inflammatory response, with leukocyte invasion of the wall [9]. The development of vascular inflammation entails continuous recruitment of inflammatory and immune cells from the blood into the sub-intimal space, as a consequence of the inflammation-dependent increase in endothelial permeability; cell recruitment is favored by up-regulation of endothelial adhesion molecules, chemokines, cytokines, and growth factors. Once in the sub-intimal space, monocytes differentiate into macrophages and take up oxLDLs through the scavenger receptors CD36 and SR-A. Unlike LDL receptors, scavenger receptors are not regulated

by a negative feedback loop, so that macrophages avidly accumulate oxidized lipids, becoming foam cells, and meanwhile release a large variety of proinflammatory cytokines. Perpetuation of this process promotes a chronic inflammatory state leading to the progression and instability of the atherosclerotic plaque [10,11]. Accumulated foam cells and extracellular lipids form the lipid core that, depending on its individual characteristics and lipid components, may or may not contribute to plaque instability. It appears that large amounts of cholesteryl ester may soften the lipid core, whereas cholesterol crystals increase plaque rigidity [12]. In addition, it has been shown that a lipid fraction of human atherosclerotic plaques induces oxidative stress in mouse macrophages, and decreases high density lipoproteins' ability to trigger cholesterol efflux from macrophages [13]. Moreover, a lipid extract from human carotid plaques has been shown to increase expression of proinflammatory mediators in human THP-1 cells and macrophage-like cells. Of note, the fraction rich in cholesterol oxidation products was a major contributor to this effect [14]. These data support the hypothesis that the plaque itself is atherogenic, as plaque lipids may enhance plaque formation. Further, in the plaque, the death of foam cells, activated by inflammatory mediators, results in necrotic core formation: cell death leads to the spillage of lipids and, hence, enlargement of the soft lipid core [15,16].

Migration into the fibrous cap of numerous SMCs, which synthesize collagen, and their proliferation are crucial in determining whether the cap can maintain its thickness and structural integrity. Apoptosis of SMCs, which leads to depletion of their numbers, reduction in collagen production, and thinning of the fibrous cap, is another potential cause of plaque vulnerability: an increased number of apoptotic vascular SMCs has been found in advanced symptomatic plaques, compared with stable lesions [17-19]. Inhibition of SMC apoptosis stabilizes atherosclerotic plaques *in vivo* [20].

In addition, ongoing inflammation also plays an important role in weakening the fibrous cap [10,11]. Marked infiltration of inflammatory cells, predominantly macrophages and T-lymphocytes, has been observed at the site of plaque rupture [21]. Following activation of these inflammatory

cells, various cytokines and extracellular matrix-degrading metalloproteinases (MMPs) are released. Inflammatory molecules, together with oxidative stress compounds, can regulate the expression of genes involved in collagen synthesis, as well as in the expression and activity of MMPs [10,11]. Fibrous cap disruption is markedly initiated by an excess of MMPs over their inhibitors (TIMPs: tissue inhibitors of MMPs), which are produced by macrophages and macrophage-derived foam cells, while T-lymphocytes release interferon-γ, which inhibits collagen synthesis and SMC proliferation at the fibrous cap. Together, they also activate macrophages to release MMPs. In particular, MMP-9, MMP-8 and MMP-2 are markedly upregulated in macrophages and macrophage-derived foam cells. MMPs thus weaken the fibrous cap and promote plaque rupture thrombus formation, by destroying the ECM. They promote monocyte/macrophage invasion, thereby amplifying plaque inflammation, and apoptosis of ECs, SMCs, and macrophage-derived foam cells. Moreover, MMPs promote angiogenesis, favoring the development of vasa vasorum, which leaks erythrocytes into the plaque; this contributes to the formation of a large lipid core, which is an adverse feature in plaque stability [22-24].

## 2. Oxidized lipoproteins and atherosclerotic plaque progression

A causative role for oxLDLs in atherosclerosis has now been established [25,26]. After crossing the endothelial barrier and accumulating in the sub-endothelium, oxLDLs are significantly involved in the initiation, formation, progression, and destabilization of the atherosclerotic plaque, as they induce inflammation and oxidative stress. They also engender endothelial activation, monocyte recruitment, macrophage differentiation, and SMC migration and proliferation at moderate concentrations, whereas at higher concentrations they are proapoptotic [27].

Concerning endothelial function, oxLDLs can induce expression of angiotensin converting enzyme and receptor $AT_1R$ in primary human vascular ECs [28]. Of note, both the endothelium-

dependent relaxation (EDR) alteration in murine aortic vascular rings [29] and the foam cell formation in human macrophages [30] induced by oxLDLs were abolished on blocking $AT_1R$.

It has also been demonstrated that oxLDLs modify and inhibit cellular proteins, contributing to the conformational change (misfolding) and impaired function of modified proteins. As a consequence of this effect, the regulation of pathways involved in cell homeostasis (e.g. the ubiquitin/proteasome system) [31], or of cell signaling pathways, (e.g. the platelet-derived growth factor receptor (PDGFR) pathway) [32] is disturbed or inhibited. Moreover, apoptosis of vascular cells induced by oxLDLs is associated to increases in ER stress and unfolded protein response (UPR) [33]. ER stress occurs when stress signals, such as oxidative stress, cause the accumulation of misfolded or unfolded proteins in the organelle. Under normal conditions, the UPR is then activated to restore ER homeostasis; if this response is not sufficient to contrast the stress stimuli, ER triggers apoptosis [34]. Toxic concentrations of oxLDLs may also modify protein disulfide isomerase (DPI), an ER-resident chaperone and oxidoreductase, which catalyzes the formation and rearrangement of disulfide bonds; it thus participates in protein folding. DPI modification during atherosclerotic lesion formation suggests that its activity could be inhibited, with consequent alteration to the folding of nascent proteins in the ER, and potentiation of both ER stress and apoptosis induced by oxLDLs. This leads it to contribute to plaque progression, destabilization, and rupture [35].

Several reactive molecules derive from oxidation of the LDL lipid fraction, including peroxides, hydroxides, aldehydes, oxidized phospholipids, and cholesterol oxidation products. Considerable information concerning the roles of these various components is now available [36,37]. In recent decades, cholesterol oxidation products and aldehydes have attracted the attention of various research groups, because of their close involvement in the pathogenesis of atherosclerosis.

Cholesterol oxidation products, known as oxysterols, are 27-atom carbon compounds that originate from the oxidation of cholesterol by either enzymatic or non-enzymatic mechanisms; they

present one or more carbonyl, keto, hydroxyl, or epoxide groups in the sterol ring and/or in the side chain (Fig. 1). They are present in both free and esterified forms [38-40].

With regard to atherosclerosis, there is no longer any doubt that oxysterols play a pivotal role in all the various steps of atheroma formation, from endothelial dysfunction to plaque fibrosis and rupture, through vascular cell infiltration/migration, proliferation and differentiation [17,19]. Of the oxysterols, 27-hydroxycholesterol (27-OH), 7-ketocholesterol (7-K), 5α,6α-epoxide (α-EPOX), 5β,6β-epoxide (β-EPOX) and cholesterol-3β,5α,6β-triol (Triol) are the most abundant oxysterols in plasma and atherosclerotic lesions [38,41,42]. It has been observed that the oxysterols commonly found in plasma from hypercholesterolemic patients are also found in atherosclerotic plaques, and a strong, direct correlation between total oxysterols and total cholesterol have been observed in plaques [43].

Among aldehydes that are end-products of n-6-polyunsaturated fatty acid peroxidation, 4-hydroxy-2-nonenal (HNE) may contribute to progression of the atherosclerotic plaque (Fig. 2). Its potential involvement in the pathogenesis of atherosclerosis is supported by its consistent detection in both oxLDLs [44] and atherosclerotic plaques in humans [45,46]. However, HNE accumulates mainly in the advanced fibrotic plaque and in the necrotic core, and not in very early stage plaque. Moreover, increased levels of HNE have been detected in the plasma of patients with acute myocardial infarction [47].

The hypothesis that these oxidized lipids contribute to atherosclerosis progression and destabilization is based on their marked proinflammatory, proapoptotic, and profibrogenic effects (Fig. 3).

## 3. Oxysterols and atherosclerotic plaque progression

A growing bulk of evidence suggests that oxysterols contribute significantly to atherosclerosis. They are involved in various key steps of this complex process: endothelial

dysfunction, adhesion of circulating blood cells, foam cell formation, migration and proliferation of vascular SMCs, fibrous cap formation, and modulation of ECM, leading to plaque progression and instability.

It is known that endothelial dysfunction is a key event in the development of atherosclerosis. It has been hypothesized that oxysterols alter the endothelium by inserting themselves into the cell membrane, where they modify the function of membrane-bound enzymes: disruption of ion transport by Na/K-ATPase and perturbation of membrane structure have been found in ECs treated with 7-K. This effect may contribute to endothelial rigidity [48]. Insertion of oxysterols into the lipid bilayer, and decreased lipid packing of the ordered domains rich in cholesterol, also alter the endothelium, as experimentally observed for 7-K [49,50]. Conversely, increasing the levels of membrane cholesterol may be protective [51].

One hallmark of endothelial dysfunction is the progressively impaired vasodilatory response to blood flow and vasodilating compounds, namely altered EDR. The incubation of aortic rings with 7-K, 7α-hydroxycholesterol (7α-OH), and 7β-hydroxycholesterol (7β-OH) confirmed the ability of these oxysterols to affect EDR, and its alteration was marked when oxysterols were used in a mixture, suggesting possible synergistic reactions *in vivo*. This effect on rabbit aortic rings was due to reduced NO bioavailability following direct interaction of oxysterols with nitric oxide (NO), and not to inhibition of endothelial nitric oxide synthase (NOS) [52]. Moreover, since oxysterols are well-known activators of NADPH oxidase (NOX) and inducers of reactive oxygen species (ROS), these compounds may increase the production of $O_2^-$, which rapidly reacts with NO, decreasing its bioavailability [53]. A recently characterized oxysterol, 5,6-secocholesterol, stemming from a non-free-radical-mediated autoxidation process, was also found to affect endothelial integrity, by inducing p53-dependent apoptosis and strongly inhibiting EDR, in *in vivo* and *in vitro* models [54]. Further, the ability of 7-oxysterols, including 7β-OH and 7-K, to induce endothelial dysfunction is suggested by research showing that these oxysterols induce EC apoptosis: they accumulate in

lysosomes at an early stage, then induce lysosomal activation, oxidative stress, and stimulate the mitochondrial pathway of programmed death [55].

The ability of some oxysterols to induce apoptosis appears consistently to be correlated with increased intracellular ROS generation [40,56]. Oxysterols may stimulate vascular ROS generation by up-regulating the activity of NOX isoenzymes, which are present in vascular cells. Inhibition of 7-K-dependent ROS production by selective inhibitors of NOX provided significant protection against macrophage apoptotic death induced by 7-K [57]. Macrophage apoptosis and defective clearance of these cells contribute to formation and enlargement of the lipid-rich necrotic core, favoring progression and instability of atherosclerotic plaques. Of note, it has been demonstrated that all events along the mitochondrial apoptotic pathway triggered by 7-K were markedly quenched when murine J774.A1 macrophages were coincubated with an identical concentration of 7β-OH [58], possibly because the two oxysterols compete to act as substrate for NOX [59]. In this connection, it has been shown that the NOX4 content is increased in atherosclerotic plaques, and it has been demonstrated that 7-K promotes ER stress and apoptosis by up-regulating NOX4 in human aortic SMCs [60]. Further, in U937 promonocytic cells, an oxysterol mixture mimicking that present in atherosclerotic plaques induced early generation of ROS, the effect being at least partly dependent on activation of NOX2 [61]. More recently, in the same cell model, 27-OH was shown to markedly up-regulate NOX2, but also induced derangement of the mitochondrial membrane potential, thus amplifying ROS production [62]. In human promonocytic U937 cells, 7-K has been shown to trigger cell death via oxidative stress and caspase activation. The key role exerted by ROS in oxysterol-mediated apoptosis is indirectly validated by its inhibition upon cell pre-treatment with antioxidants, such as glutathione or N-acetylcysteine (NAC): the number of apoptotic cells decreased drastically [63,64]. Moreover, supplementation of U937 cells with α-tocopherol counteracted 7-K-provoked lysosomal degradation, caspase activation, and phospholipidosis [65]. Thus, imbalance between prooxidants and antioxidants contributes to the inflammation and cell death seen in the atherosclerotic lesions.

Some findings support the involvement of death receptors in oxysterol-induced apoptosis. 27-OH and 22-hydroxycholesterol (22-OH) induce tumor necrosis factor α (TNF-α) in macrophagic cells [66-68] and up-regulation of TNF receptors has been observed in human aortic SMCs after incubation with 7-K [69].

With regard to the proapoptotic and antiapoptotic pathways, a large number of reports stress their activation by oxysterols [43,70]. To give some examples, 25-hydroxycholesterol (25-OH) and 7-K have been shown to induce phosphorylation and translocation of Bax to the mitochondria in vascular SMCs, also triggering ROS release, by activating soluble adenylyl cyclase/protein kinase (PK) A [71]. In the same cells, Triol was found to markedly impair mitochondrial membrane potential [72]; 7β-OH activated caspase-3 and caspase-9 in human promonocytic U937 cells [73] while 25-OH caused cytochrome c release and activation of caspase-3 and -9 in CHO-K1 [74]. In addition, in U937 cells, 7β-OH and 7-K have been shown to exert synergistic toxic effects through caspase activation, ROS production, cellular thiol depletion, and permeabilization of lysosomal and mitochondrial membranes. Of note, the apoptotic effect of these two oxysterols was quenched by simultaneous addition of 25-OH and 27-OH; however the combination of all four oxysterols as a mixture was proapoptotic, indicating that the major oxysterols accumulating in human atheroma are proapoptotic, and significantly contribute to plaque progression and instability [75]. In the regions of advanced plaques prone to fibrous cap rupture, the proapoptotic effects exerted by oxysterols on SMCs favor cap weakness and destruction [76]. It has been shown that 7-K contributes to SMC apoptosis, increasing plaque vulnerability and rupture, through activation of the caspase-3/7-dependent apoptotic pathway. The same study reportes that serotonin, released from activated platelets, appears to inhibit vascular SMC apoptosis induced by 7-K, helping to prevent the plaque vulnerability induced by the oxysterol [77]. The toxic response of oxysterols on SMCs occurred chiefly when the cells were incubated with high concentrations of 7-K [78]. The death of SMCs

induced by high concentrations of oxysterols is likely to destabilize plaques and increase the risk of thrombosis [79].

An important feature of oxysterol-induced-apoptosis is the effect of oxysterols on cell cycle regulation: they can lead to activation of p53, a key protein involved in DNA surveillance, promotion of cell cycle arrest, repair, and eventually apoptosis. Incubation of human vascular ECs with either 7β-OH or 7-K has been observed to promote p53 phosphorylation, probably by inducing ROS and translocating them into the nucleus. This would trigger a series of cell reactions, namely lysosomal membrane permeabilization, cathepsin release, and eventually mitochondrial membrane damage [55]. Besides up-regulation of p53 function, another common mechanism that interferes with cell cycle completion is the inhibition of certain cyclin-dependent kinases, with consequent accumulation of cells in the G1, S, and G2/M phases of the cycle. In this connection, treatment of THP-1 monocyte-like cells with 7β-OH or with 25-OH was found to block them in the G2/M phase, through oxysterol-dependent inhibition of cyclin B1 and its dependent kinase cdc2. The cells then underwent apoptosis via reduction of the antiapoptotic protein Bcl-2 and activation of caspase-9 and -3 [80].

Autophagy dysfunction has been associated with increased lipid accumulation, apoptosis, and inflammation, all events that promote advanced plaque formation and destabilization. In this connection, it has been demonstrated that dysfunctional autophagy is a characteristic of advanced human atherosclerotic plaques; this stage is associated with accumulation of oxidized lipids (such as oxysterols) that contribute to necrotic core formation [81]. Some oxysterols, for example 7-K, 7β-OH, and 24S-hydroxycholesterol (24S-OH), are reported to trigger apoptosis and, at the same time, autophagy; activation of the autophagic process was demonstrated by the presence of cytoplasmic phagosomes, and by increased levels of the specific marker of autophagy, the microtubule-associated protein light chain 3 (LC3)-II derived from LC3-I cleavage [82]. Oxysterols thus appear

able to induce a complex type of cellular death mechanism, which includes oxidative stress, apoptosis, and autophagy; this has very recently been termed “oxiapoptophagy”.

Inflammation also participates in atherosclerotic plaque progression and instability. Oxysterols consistently contribute to the proinflammatory effect of oxLDLs by inducing the expression of various key inflammatory molecules [17,19,37]. In particular, they stimulate leukocytes’ adhesion to the arterial endothelium, but also their transmigration and cell differentiation. In this connection, monocyte chemoattractant protein 1 (MCP-1) and β1-integrin were found to be up-regulated in U937 cells by an oxysterol mixture mimicking that detectable in the plasma of hypercholesterolemic individuals, through the extracellular signaling-regulated kinase (ERK) and nuclear factor-κB (NF-κB) pathways. Of note, cells incubated with non-oxidized cholesterol did not show any variation compared to untreated cells, underlining that only the oxysterols have proatherogenic effects [83,84]. It has also been observed, in $ApoE^{-/-}$ mice, that dietary oxysterols accelerate plaque destabilization and rupture, by increasing monocyte infiltration and differentiation, inducing MCP-1 expression, increasing lipid core size in the plaque, and enhancing MMP-2 and MMP-9 activity. Inhibition of the absorption of oxysterols by ezetimide, an inhibitor of the cholesterol transporter Niemann-Pick C1 Like 1, prevented the acceleration of plaque destabilization [85]. The fate of an atherosclerotic plaque in fact depends on the balance between recruitment and activation of monocyte-derived macrophages and on macrophage polarization state [86]. The proinflammatory macrophage M1 may accelerate atherosclerosis progression, by producing a wide range of inflammatory molecules and ROS, while macrophage M2 shows an antiinflammatory phenotype and may be critical for decreasing inflammation [87]. It has recently been demonstrated that 7-K can affect human macrophage polarization by switching antiinflammatory M2 macrophages to proinflammatory M1 macrophages. In both macrophage phenotypes, this oxysterol selectively activated the production of many key proatherogenic mediators involved in inflammatory, invasive, and angiogenic mechanisms within the

atherosclerotic lesion. The proatherogenic effect of 7-K was counteracted by the antiinflammatory and antioxidant resveratrol, a polyphenolic compound [88,89].

Various oxysterols are also involved in the overexpression of interleukin (IL)-8 in human monocytes/macrophages [90,91]. IL-8 appears to be up-regulated by 7β-OH and 25-OH in THP-1 cells via the ERK/activator protein 1 (AP-1) pathway [91]. In addition, in human monocytic/macrophagic cells, 7β-OH, 25-OH, and also 7-K display potent induction of MCP-1, TNF-α, IL-8, IL-6, IL-1β, and macrophage inflammatory protein-1β (MIP-1β). These effects are associated with increased ROS generation, through overexpression of NOX, and with mitogen-activated protein kinase (MAPK)/NF-κB activation [92,93. Increased levels of IL-6 have also been found in human aortic SMCs incubated with 7-K through MAPK activation [94]. Of note, IL-6 regulates the expression of other inflammatory cytokines, including IL-1 and TNF-α [95]. Another effect of 7-K and 25-OH, which may contribute to inflammatory response, is inhibition of the secretion of IL-10, an important antiinflammatory cytokine, in SMCs [96]. Moreover, 27-OH which accumulates markedly in the atherosclerotic plaque, was found to up-regulate IL-1β, IL-8, TNF-α, and MMP-9 in U937 cells, through the Toll-like receptor (TLR)4/NF-κB pathway [68]. Moreover, 7β-OH and 7-K induce IL-1β, E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule (VCAM-1) expression in human vascular ECs [97,98]. Again, 7-K has been shown to enhance ROS-dependent secretion of VCAM-1 in human aortic ECs [99], and 25-OH has been found to increase leukocyte adhesion to ECs by up-regulating the same adhesion molecule [100]. Oxysterols may also be able to stimulate arachidonic acid release, after activation of cytosolic phospholipase $A_2$ and subsequent eicosanoid production. In human umbilical vein ECs, the oxysterol Triol up-regulates cyclooxygenase-2 (COX-2), leading to prostaglandin $E_2$ ($PGE_2$) release, involving phosphoinositide 3-kinase (PI3K)/Akt and endothelial NOS, as well as the activation of NF-κB and p38 MAPK [101]. Again, 27-OH contributes to inflammation by stimulating $PGE_2$ production, through COX-2 and microsomal prostaglandin E synthase 1

activation, and by increasing inducible NOS levels and subsequent NO release in U937 cells (Gargiulo et al, unpublished data).

Oxysterols thus contribute to sustaining inflammation by inducing the differentiation of monocytes into macrophages. An oxysterol mixture was found to induce foam cell formation through up-regulation of the scavenger receptor CD36, involving the PKC-δ, ERK, and peroxisome proliferator-activated receptor γ (PPAR-γ) pathways [102,103]. Among the oxysterols present in oxLDLs, 7β-OH and 7-K appear to be the major compounds responsible for monocyte differentiation into macrophages, as observed in oxLDL-treated THP-1 cells [104].

Vascular SMCs are crucial to plaque formation, progression, and stability. At the early phase of atherosclerotic lesion, these cells start to proliferate and migrate to the sub-endothelial space, also undergoing a phenotypic change: inflammation stimulates the shift of SMCs from the contractile phenotype to the fibroblast-like phenotype. The latter phenotype starts to synthesize ECM components, causing formation of the fibrous cap and contributing to arterial rigidity. With regard to the effects of oxysterols on these cell types, it has been shown that low doses of 7-K or of α-EPOX induce proliferation and migration of rat aortic SMCs, by activating the epidermal growth factor receptor (EGFR)/PI3K/Akt pathway; they also increase MMP-2 and MMP-9 activities. Cell treatment with the same concentration of cholesterol had no significant effect on SMC proliferation [105]. Further, $PGE_2$ has been shown to increase endothelial permeability and vascular tone, as well as vascular SMC proliferation and migration [106].

The strength of the fibrous cap depends on a dynamic balance between collagen synthesis and degradation. During fibrous cap formation, a key role in the deposition of ECM components is played by the inflammatory cytokine TGF-β1, which is strongly profibrogenic. In this connection, a biologically compatible mixture of oxysterols has been shown to induce expression and synthesis of TGF-β1 in the J774.A1 murine macrophagic cell line [107] and in Raw264.7 murine macrophages [108]. Conversely, irregular and uncontrolled ECM degradation, due to an imbalance in the

MMPs/TIMPs activity ratio, clearly contributes to plaque destabilization and rupture. Indeed, MMPs, principally MMP-9 and MMP-2, are the chief enzymes responsible for excessive proteolytic ECM degradation, as well as for vascular remodeling, SMC migration, and macrophage infiltration, with subsequent increase of plaque inflammation. The principal source of MMPs is activated macrophages, but also vascular SMCs and ECs, and expression of these cells may be modulated by ongoing inflammation [24,109].

In this connection, arteries from oxysterol-fed ApoE$^{-/-}$ mice showed enhanced macrophage activity of both MMP-2 and MMP-9, and infiltration of monocyte-mediated inflammation through MCP-1 release. These effects markedly accelerated plaque destabilization and rupture, pointing to a role of accumulated oxysterols in plaque vulnerability. In the same animal model, all these effects were prevented when oxysterol intestinal absorption was inhibited by administering ezetimibe [85]. In human promonocytic U937 cells, an oxysterol mixture of composition similar to that found in advanced atherosclerotic plaques was showed to up-regulate MMP-9, without affecting its inhibitors TIMP-1 and TIMP-2. Moreover, it has been demonstrated that oxysterols induce MMP-9 expression and synthesis, by enhancing ROS generation, up-regulating the MAPK signaling pathways via PKC, and also by up-regulating AP-1 and NF-κB DNA-binding, which are both redox transcriptional factors [61]. In addition, one the oxysterols that is most abundant in human blood, i.e. 27-OH, has been shown to markedly up-regulate MMP-9 expression and synthesis in human promonocytic U937 cells, through the TLR4/NF-κB pathway. The oxysterol also enhanced the release of IL-8, IL-1β and TNF-α, thereby augmenting the inflammatory response. Given that an inflammatory state can contribute to MMP activity, it is significant that these inflammatory cytokines have also been found to act on MMP-9 production, thus sustaining the release of this matrix-degrading enzyme and contributing to plaque fragility [68]. Interestingly, 7-K can affect macrophage polarization by skewing the M1/M2 balance towards a proinflammatory profile, by inducing many proinflammatory mediators, including MMP-9 and MMP-2 [88,89]. MMP-2 and

MMP-9 have also been found to be elevated, in vascular SMCs, by 7-K and α-EPOX, through activation of ERK (Liao *et al.* toxicol lett 197, 88-96, 2010).

**4. HNE and atherosclerotic plaque progression**

The biochemical effects of HNE are due to its lipophilicity and high reactivity towards thiol and amino groups, forming covalent adducts. HNE reacts mainly with the amino acids cysteine, histidine, and lysine, which, either free or protein-bound, readily undergo Michael addition reactions to the C-C double bond. Besides this type of reaction, other reactions may also occur, involving the carbonyl and hydroxyl groups. Alternatively, amino acids may react with the carbonyl group to form Schiff bases. The cytotoxicity of HNE is attributed to its covalent interaction with nucleophilic moieties in proteins, DNA, and phospholipids, with consequent modification of their structure. Thus the progressive accumulation of these adducts may alter normal cell function, leading to cell death [44,110-112]. Increased levels of HNE adducts have been found in atherosclerotic lesions, indicating an association of HNE with atherosclerosis, and it thus seems clear that HNE is responsible for the proatherogenic effects of oxLDLs, being one of their major reactive components [45,46].

HNE is transported by oxLDLs into the lipid core of the plaque, where it accumulates and chemically attracts monocytes/macrophages and SMCs. It has also been demonstrated that HNE covalently modifies oxLDLs by binding to lysine and histidine residues, leading to adduct formation: this favors oxLDL uptake by macrophages through the scavenger receptors, thus resulting in macrophage activation and foam cell formation [113]. It was demonstrated that HNE could stimulate the inflammatory processes by showing that it induces expression and synthesis of MCP-1 in murine macrophages (J774.A1), by activating PKC-βI and PKC-βII [114]. Marked

production of the inflammatory and fibrogenic cytokine TGF-β1 in macrophagic cells incubated with HNE has also been shown [115-117].

High plasma levels of HNE may also damage endothelial barrier function, due to impaired cell-cell communication and inhibition of membrane associated enzymes. It is known that NO plays an important role in mediating blood pressure and vascular tone, as well as in decreasing endothelium permeability, thus contrasting LDL infiltration. Reduction of the bioavailability of NO seems to be a key factor in cardiovascular disease [118]. HNE may modulate production of NO at the arterial wall affected by atherosclerosis, and it has been shown to reduce the production of inducible NOS and nitrites from NO, by inhibiting the NF-κB activation pathway in vascular SMCs, monocytes, and macrophages [119,120]. In addition, HNE may induce ER stress and impair vascular endothelium and coagulation functions. HNE can also directly impair endothelial barrier function and increase endothelial permeability [121]. In this connection, HNE may induce both actin stress fibers and intercellular gap formation, suggesting a link between F-actin cytoskeletal rearrangement and permeability changes [122,123]. Further, it may induce changes in cellular thiol redox status, thus disordering cell signaling pathways, leading to vascular endothelium dysfunction. After induction of ROS, HNE may act on endothelial permeability, modulating cell-cell adhesion by suppressing focal adhesion kinase (FAK) phosphorylation, which in turn affects focal adhesion, adherence and tight junction proteins, as well as integrin, all of which are natural FAK receptors. Moreover, HNE modifies cytoskeletal proteins, such as actin and microtubules, by regulating cell-cell contacts and endothelial barrier function. Notably, all these changes can be inhibited by pretreatment of cells with the thiol protectant NAC [124,125].

Further, HNE may stimulate the adhesion of monocytes, as well as lymphocytes, to ECs: it has been shown to react with the thioredoxin (TRX) system by binding to Cys73, with subsequent inhibition of its activity, followed by an increase in ROS generation, and the stimulation of adhesion of monocytes and immune cells to the vascular endothelium. HNE modification of TRX could thus potentiate adhesion of monocytes to ECs, an early event in atherosclerosis [126]. TRX is

an antioxidant system, which is ubiquitously expressed in ECs, where it regulates oxidative stress by scavenging ROS, and prevents apoptosis [127]; for this reason it is critical to maintaining normal endothelial function and protecting against atherosclerosis.

Regarding NF-κB, HNE can activate or inactivate this redox transcriptional factor, respectively by mediating or inhibiting its proinflammatory and oxidative effects [37]. It has been shown that inhibition of NF-κB in THP-1 cells and in human aortic ECs resulted in inhibition of the adhesion molecules and cytokines induced by inflammatory stimuli [128,129]. Conversely, HNE stimulates NF-κB in vascular SMCs [130]. The dual effect of HNE on NF-κB signaling pathway thus appears to depend on the cell type used, and it may have a significant impact on atherosclerosis development. Down-regulation of NF-κB dependent gene expression, as a consequence of inhibition of the transcriptional factor by HNE, may interfere with the immune response and thus amplify and sustain the inflammatory response. Conversely, marked activation of another redox transcriptional factor, AP-1, by HNE has been demonstrated in murine and human macrophages and aortic SMCs [130,131]. HNE may also potentiate inflammation and immune responses, by inducing COX-2 and in consequence prostaglandin production [132].

HNE also promotes foam cell formation, by increasing expression and synthesis of the class A scavenger receptors at the transcriptional level, as well as of the CD36 scavenger receptor on murine macrophages [133]. It also enhances the CD36 scavenger receptor in vascular SMCs by inducing nuclear accumulation of the nuclear factor E2-related factor 2 (Nrf2) [134].

HNE is responsible for DNA-damage in SMCs: bulky DNA adducts have been found in abdominal and thoracic aorta samples from atherosclerotic patients [135]. High levels of miscoding etheno-DNA adducts have also been detected in human aorta SMCs from subjects affecting by atherosclerosis. This DNA damage leads to SMCs proliferation [136].

HNE may also exert a dual effect on cell proliferation, depending on the local concentration and the degree of oxidative stress. This dual effect may result from the modification and subsequent

dysfunction of tyrosine kinase receptors induced by HNE [137], such as PDGFR-β, which regulates SMCs migration and proliferation. Brief incubation of cells with low concentrations of HNE induces autophosphorylation of PDGFR-β and subsequent SMC proliferation [138]; longer incubation with high concentrations of the aldehyde increases formation of HNE-PDGFR-β adducts, with subsequent inhibition of PDGFR-β phosphorylation and SMC proliferation. This reduced SMC proliferation contributes to decreasing the stability of a vulnerable atherosclerotic plaque [139]. Activation of PDGFR-β and ERK1/2 by HNE in human coronary SMCs is also a key mechanism in the production of MMP-1 and MMP-2, which may stimulate SMC proliferation and migration into the intima [140]. Further, the accumulation of HNE adducts on EGFR may inhibit PI3K/Akt pathway activation, inhibiting cell proliferation and inducing apoptosis [138]. In addition, HNE was found to react directly with membrane proteins of human ECs, inducing formation of HNE-EGFR and HNE-PDGFR adducts [137]. Of note, HNE-adducts on PDGFR and EGFR have been detected in carotid atherosclerotic plaques [139].

HNE has also been shown to induce derivatization and ubiquitination of cell proteins, and to inhibit the proteasome pathway, all events involved in the mechanism of oxLDL-induced apoptosis. Modification of cell protein structure impairs function, and eventually alters cell viability, while inhibition of proteasome reduces cellular defenses [31]. In addition, HNE appears to modify and inhibit the enzymatic activity of DPI, a major chaperone in protein folding in the ER; this leads to alteration of the folding of nascent proteins in the ER, and is a factor in activating ER stress/URP response, and induces apoptosis in vascular cells if the ER stress is prolonged [35]. ER stress thus appears to have a negative effect on EC stability, which may lead to plaque fragility and rupture: cell apoptosis mediated by ER stress may promote loss of the endothelial layer covering the plaque.

HNE also induces expression of 5-lipoxygenase (5-LO) through the NF-κB/ERK and Sp1/p38 MAPK pathways via EGFR in murine macrophages [141]. The p38 MAPK and ERK pathways are also involved in HNE-induced activation of 5-LO in vascular SMCs, inducing their

migration and proliferation leading to plaque instability [142]. Of note, up-regulation of 5-LO by activated macrophages in symptomatic plaques has been shown to lead to enhanced production of MMPs, with subsequent plaque instability [143,144]. It has also been demonstrated that the association between HNE and 5-LO derivatives increases the secretion of MMP-9, by means of ERK and p38 MAPK activation [145]. A similar synergistic action between these products has also been demonstrated in vascular SMCs. In this case, a marked production of MMP-2 was evident, again after activation of the ERK and p38 pathways [142,146]. Both MMP-9 and MMP-2 play pathogenic roles in the progression of plaque rupture. Thus 5-LO, induced by HNE, plays a key role in the development and progression of atherosclerosis, and it has been suggested to be a modulator of plaque instability [143,147,148]. Moreover, HNE has also been shown to induce MMP-2 production in vascular SMCs, via mitochondrial ROS-mediated activation of the Akt/NF-κB signaling pathways [142]. HNE was also found to enhance cell release of inflammatory cytokines (IL-8, IL-1β, and TNF-α) and to up-regulate MMP-9 via the TLR4/NF-κB-dependent pathway. These events may sustain inflammatory response and matrix degradation. Using specific antibodies, these cytokines have been shown to increase MMP-9 up-regulation, thus enhancing the release of this matrix-degrading enzyme by macrophage cells, and contributing to plaque instability [68]. HNE may also contribute to ECM modification and degradation by antagonizing the elastogenic activity of TGF-β in fibroblasts [149].

## 5. Conclusions

Increasing experimental evidence points to a significant contribution of oxysterols and HNE in development of the atherosclerotic plaque. Of note, these proatherogenic compounds appear to have a dual effect: i) they first initiate impairment of vascular endothelium function, mainly caused by oxidative stress and inflammation; ii) subsequently, following excessive accumulation, they

might contribute to foam cell formation, migration and proliferation of vascular SMCs, fibrous cap formation, and modulation of ECM, leading to plaque progression and instability with eventual rupture (Fig. 4). However, further studies will be needed to identify all the molecular pathways that are modulated or altered by these oxidized lipids, in order to find targeted therapeutic strategies to prevent or treat this disabling disease.

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**Conflicts of interest statement**

The authors declare no conflict of interest.

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**Figure legends**

**Fig. 1 Chemical structures of oxysterols.** Oxysterols are 27-carbon-atom compounds deriving from cholesterol oxidation. Oxidation of the side chain is usually enzymatic, whereas oxidation of the sterol rings is non-enzymatic and depends on oxidative stress.

**Fig. 2 Chemical structure of 4-hydroxynonenal.** HNE is a highly reactive hydroxyalkenal deriving from the non-enzymatic breakdown of n-6-polyunsaturated fatty acids (PUFA), such as arachidonic, linoleic, and linolenic acids.

**Fig. 3 Accumulation of oxysterols and HNE contributes to plaque progression.** Continuous LDL oxidation and lipid release from apoptotic foam cells produce increasing levels of oxysterols and HNE, which contribute to all stages of atheroma formation. These oxidized lipids sustain the inflammatory process and endothelial activation, the formation of foam cells, the migration of SMCs from the tunica media toward the intima, and degradation of the fibrous cap, favoring plaque rupture and thrombus formation. LDL, low density lipoprotein; oxLDL, oxidized LDL; SMC, smooth muscle cells.

**Fig. 4 Scheme of key steps promoting plaque vulnerability induced by oxysterols and HNE.**

# Oxysterols and 4-hydroxy-2-nonenal contribute to atherosclerotic plaque destabilization

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## Abstract

A growing bulk of evidence suggests that cholesterol oxidation products, known as oxysterols, and 4-hydroxy-2-nonenal (HNE), the major proatherogenic components of oxidized low density lipoproteins (oxLDLs), significantly contribute to atherosclerotic plaque progression and destabilization, with eventual plaque rupture. These oxidized lipids are involved in various key steps of this complex process, mainly thanks to their ability to induce inflammation, oxidative stress, and apoptosis. This review summarizes the current knowledge of the effects induced by these compounds on vascular cells, after their accumulation in the arterial wall and in the atherosclerotic plaque.

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*Keywords:* atherosclerosis, oxysterols, HNE,

*Abbreviations:* α-EPOX, 5α,6α-epoxide; β-EPOX, 5β,6β-epoxide; 22-OH, 22-hydroxycholesterol; 24S-OH, 24S-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; 27-OH, 27-hydroxycholesterol; 7α-OH, 7α-hydroxycholesterol; 7β-OH, 7β-hydroxycholesterol; 7-K, 7-ketocholesterol; AP-1,

activator protein-1; COX-2, cyclooxygenase-2; DPI, disulfide isomerase; ECs, endothelial cells; ECM, extracellular matrix; EDR, endothelium-dependent relaxation; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; HNE, 4-hydroxy-2-nonenal; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; LC3-II, light chain 3-II; 5-LO, 5-lipoxygenase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein; MIP-1β, macrophage inflammatory protein-1β; MMPs, matrix metalloproteinases; NAC, N-acetylcysteine; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; NOX, NADPH oxidase; Nrf2, nuclear factor E2-related factor 2; oxLDLs, oxidized low density lipoproteins; PDGFR, platelet-derived growth factor receptor; $PGE_2$, prostaglandin $E_2$; PI3K, phosphoinositide 3-kinase; PK, protein kinase; PPAR-γ, peroxisome proliferator-activated receptor-γ; ROS, reactive oxygen species; SMCs, smooth muscle cells; TGF-β1, transforming growth factor-β1; TIMPs, tissue inhibitors of MMPs; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α; Triol, cholesterol-3β,5α,6β-triol; TRX, thioredoxin; UPR, unfolded protein response; VCAM-1, vascular cell adhesion molecule.

## 1. Introduction

Atherosclerosis is a multifactorial and degenerative disease affecting large- and medium-sized arteries, which is characterized by chronic inflammation, oxidative stress, and by blood flow that is altered in certain areas of the vascular wall [1,2]. The lesions primarily develop and progress at the arterial wall, mediated by interactions among cytokines, growth factors and vasoregulatory molecules; these mediators regulate the function of cells intrinsic to the arterial wall and extracellular matrix (ECM) as well as blood cells and plasma constituents. Development of atherosclerotic lesions is preceded by impaired vascular endothelium function, mainly caused by

oxidative stress, inflammation [1,3], and endoplasmic reticulum (ER) stress [4], all conditions that are induced by the factors promoting and accelerating atherosclerosis. Vascular endothelium dysfunction is also linked to cellular senescence [5]. The intercellular cross-talk that occurs among smooth muscle cells (SMCs), macrophages, endothelial cells (ECs) and leukocytes leads to a fibroproliferative response. During this response, the ECM plays a key role in plaque formation and progression, providing the structural integrity of the plaque itself, as well as contributing to cell migration and proliferation, and finally thrombosis. A stable atherosclerotic plaque is characterized by a thick and solid fibrous cap, where large amounts of ECM are deposited, and a small central lipid core. The strength of the fibrous cap depends on a dynamic balance between collagen synthesis and degradation. Conversely, an atherosclerotic plaque at high risk of rupture contains a large lipid core where, in addition to extracellular lipid deposition and debris, ECM degradation is enhanced, leading to increased plaque fragility; moreover, the lipid core is covered with a thin fibrous cap affected by ongoing inflammation and neovascularity [6,7].

Among the intrinsic and extrinsic factors that can trigger plaque progression, with subsequent vulnerability and rupture, key roles are played by lipid components, inflammation, cell death, and fibrous cap weakening, as well as hemodynamic stress and circumferential shear stresses [8].

One of the major risk factors of atherosclerosis is hypercholesterolemia, which promotes the accumulation of oxidatively modified low-density lipoproteins (oxLDLs) in the arterial wall, promoting endothelial cell dysfunction and, in turn, inflammatory response, with leukocyte invasion of the wall [9]. The development of vascular inflammation entails continuous recruitment of inflammatory and immune cells from the blood into the sub-intimal space, as a consequence of the inflammation-dependent increase in endothelial permeability; cell recruitment is favored by up-regulation of endothelial adhesion molecules, chemokines, cytokines, and growth factors. Once in the sub-intimal space, monocytes differentiate into macrophages and take up oxLDLs through the scavenger receptors CD36 and SR-A. Unlike LDL receptors, scavenger receptors are not regulated

by a negative feedback loop, so that macrophages avidly accumulate oxidized lipids, becoming foam cells, and meanwhile release a large variety of proinflammatory cytokines. Perpetuation of this process promotes a chronic inflammatory state leading to the progression and instability of the atherosclerotic plaque [10,11]. Accumulated foam cells and extracellular lipids form the lipid core that, depending on its individual characteristics and lipid components, may or may not contribute to plaque instability. It appears that large amounts of cholesteryl ester may soften the lipid core, whereas cholesterol crystals increase plaque rigidity [12]. In addition, it has been shown that a lipid fraction of human atherosclerotic plaques induces oxidative stress in mouse macrophages, and decreases high density lipoproteins' ability to trigger cholesterol efflux from macrophages [13]. Moreover, a lipid extract from human carotid plaques has been shown to increase expression of proinflammatory mediators in human THP-1 cells and macrophage-like cells. Of note, the fraction rich in cholesterol oxidation products was a major contributor to this effect [14]. These data support the hypothesis that the plaque itself is atherogenic, as plaque lipids may enhance plaque formation. Further, in the plaque, the death of foam cells, activated by inflammatory mediators, results in necrotic core formation: cell death leads to the spillage of lipids and, hence, enlargement of the soft lipid core [15,16].

Migration into the fibrous cap of numerous SMCs, which synthesize collagen, and their proliferation are crucial in determining whether the cap can maintain its thickness and structural integrity. Apoptosis of SMCs, which leads to depletion of their numbers, reduction in collagen production, and thinning of the fibrous cap, is another potential cause of plaque vulnerability: an increased number of apoptotic vascular SMCs has been found in advanced symptomatic plaques, compared with stable lesions [17-19]. Inhibition of SMC apoptosis stabilizes atherosclerotic plaques *in vivo* [20].

In addition, ongoing inflammation also plays an important role in weakening the fibrous cap [10,11]. Marked infiltration of inflammatory cells, predominantly macrophages and T-lymphocytes, has been observed at the site of plaque rupture [21]. Following activation of these inflammatory

cells, various cytokines and extracellular matrix-degrading metalloproteinases (MMPs) are released. Inflammatory molecules, together with oxidative stress compounds, can regulate the expression of genes involved in collagen synthesis, as well as in the expression and activity of MMPs [10,11]. Fibrous cap disruption is markedly initiated by an excess of MMPs over their inhibitors (TIMPs: tissue inhibitors of MMPs), which are produced by macrophages and macrophage-derived foam cells, while T-lymphocytes release interferon-γ, which inhibits collagen synthesis and SMC proliferation at the fibrous cap. Together, they also activate macrophages to release MMPs. In particular, MMP-9, MMP-8 and MMP-2 are markedly upregulated in macrophages and macrophage-derived foam cells. MMPs thus weaken the fibrous cap and promote plaque rupture thrombus formation, by destroying the ECM. They promote monocyte/macrophage invasion, thereby amplifying plaque inflammation, and apoptosis of ECs, SMCs, and macrophage-derived foam cells. Moreover, MMPs promote angiogenesis, favoring the development of vasa vasorum, which leaks erythrocytes into the plaque; this contributes to the formation of a large lipid core, which is an adverse feature in plaque stability [22-24].

## 2. Oxidized lipoproteins and atherosclerotic plaque progression

A causative role for oxLDLs in atherosclerosis has now been established [25,26]. After crossing the endothelial barrier and accumulating in the sub-endothelium, oxLDLs are significantly involved in the initiation, formation, progression, and destabilization of the atherosclerotic plaque, as they induce inflammation and oxidative stress. They also engender endothelial activation, monocyte recruitment, macrophage differentiation, and SMC migration and proliferation at moderate concentrations, whereas at higher concentrations they are proapoptotic [27].

Concerning endothelial function, oxLDLs can induce expression of angiotensin converting enzyme and receptor $AT_1R$ in primary human vascular ECs [28]. Of note, both the endothelium-

dependent relaxation (EDR) alteration in murine aortic vascular rings [29] and the foam cell formation in human macrophages [30] induced by oxLDLs were abolished on blocking $AT_1R$.

It has also been demonstrated that oxLDLs modify and inhibit cellular proteins, contributing to the conformational change (misfolding) and impaired function of modified proteins. As a consequence of this effect, the regulation of pathways involved in cell homeostasis (e.g. the ubiquitin/proteasome system) [31], or of cell signaling pathways, (e.g. the platelet-derived growth factor receptor (PDGFR) pathway) [32] is disturbed or inhibited. Moreover, apoptosis of vascular cells induced by oxLDLs is associated to increases in ER stress and unfolded protein response (UPR) [33]. ER stress occurs when stress signals, such as oxidative stress, cause the accumulation of misfolded or unfolded proteins in the organelle. Under normal conditions, the UPR is then activated to restore ER homeostasis; if this response is not sufficient to contrast the stress stimuli, ER triggers apoptosis [34]. Toxic concentrations of oxLDLs may also modify protein disulfide isomerase (DPI), an ER-resident chaperone and oxidoreductase, which catalyzes the formation and rearrangement of disulfide bonds; it thus participates in protein folding. DPI modification during atherosclerotic lesion formation suggests that its activity could be inhibited, with consequent alteration to the folding of nascent proteins in the ER, and potentiation of both ER stress and apoptosis induced by oxLDLs. This leads it to contribute to plaque progression, destabilization, and rupture [35].

Several reactive molecules derive from oxidation of the LDL lipid fraction, including peroxides, hydroxides, aldehydes, oxidized phospholipids, and cholesterol oxidation products. Considerable information concerning the roles of these various components is now available [36,37]. In recent decades, cholesterol oxidation products and aldehydes have attracted the attention of various research groups, because of their close involvement in the pathogenesis of atherosclerosis.

Cholesterol oxidation products, known as oxysterols, are 27-atom carbon compounds that originate from the oxidation of cholesterol by either enzymatic or non-enzymatic mechanisms; they

present one or more carbonyl, keto, hydroxyl, or epoxide groups in the sterol ring and/or in the side chain (Fig. 1). They are present in both free and esterified forms [38-40].

With regard to atherosclerosis, there is no longer any doubt that oxysterols play a pivotal role in all the various steps of atheroma formation, from endothelial dysfunction to plaque fibrosis and rupture, through vascular cell infiltration/migration, proliferation and differentiation [17,19]. Of the oxysterols, 27-hydroxycholesterol (27-OH), 7-ketocholesterol (7-K), 5α,6α-epoxide (α-EPOX), 5β,6β-epoxide (β-EPOX) and cholesterol-3β,5α,6β-triol (Triol) are the most abundant oxysterols in plasma and atherosclerotic lesions [38,41,42]. It has been observed that the oxysterols commonly found in plasma from hypercholesterolemic patients are also found in atherosclerotic plaques, and a strong, direct correlation between total oxysterols and total cholesterol have been observed in plaques [43].

Among aldehydes that are end-products of n-6-polyunsaturated fatty acid peroxidation, 4-hydroxy-2-nonenal (HNE) may contribute to progression of the atherosclerotic plaque (Fig. 2). Its potential involvement in the pathogenesis of atherosclerosis is supported by its consistent detection in both oxLDLs [44] and atherosclerotic plaques in humans [45,46]. However, HNE accumulates mainly in the advanced fibrotic plaque and in the necrotic core, and not in very early stage plaque. Moreover, increased levels of HNE have been detected in the plasma of patients with acute myocardial infarction [47].

The hypothesis that these oxidized lipids contribute to atherosclerosis progression and destabilization is based on their marked proinflammatory, proapoptotic, and profibrogenic effects (Fig. 3).

## 3. Oxysterols and atherosclerotic plaque progression

A growing bulk of evidence suggests that oxysterols contribute significantly to atherosclerosis. They are involved in various key steps of this complex process: endothelial

dysfunction, adhesion of circulating blood cells, foam cell formation, migration and proliferation of vascular SMCs, fibrous cap formation, and modulation of ECM, leading to plaque progression and instability.

It is known that endothelial dysfunction is a key event in the development of atherosclerosis. It has been hypothesized that oxysterols alter the endothelium by inserting themselves into the cell membrane, where they modify the function of membrane-bound enzymes: disruption of ion transport by Na/K-ATPase and perturbation of membrane structure have been found in ECs treated with 7-K. This effect may contribute to endothelial rigidity [48]. Insertion of oxysterols into the lipid bilayer, and decreased lipid packing of the ordered domains rich in cholesterol, also alter the endothelium, as experimentally observed for 7-K [49,50]. Conversely, increasing the levels of membrane cholesterol may be protective [51].

One hallmark of endothelial dysfunction is the progressively impaired vasodilatory response to blood flow and vasodilating compounds, namely altered EDR. The incubation of aortic rings with 7-K, 7α-hydroxycholesterol (7α-OH), and 7β-hydroxycholesterol (7β-OH) confirmed the ability of these oxysterols to affect EDR, and its alteration was marked when oxysterols were used in a mixture, suggesting possible synergistic reactions *in vivo*. This effect on rabbit aortic rings was due to reduced NO bioavailability following direct interaction of oxysterols with nitric oxide (NO), and not to inhibition of endothelial nitric oxide synthase (NOS) [52]. Moreover, since oxysterols are well-known activators of NADPH oxidase (NOX) and inducers of reactive oxygen species (ROS), these compounds may increase the production of $O_2^-$, which rapidly reacts with NO, decreasing its bioavailability [53]. A recently characterized oxysterol, 5,6-secocholesterol, stemming from a non-free-radical-mediated autoxidation process, was also found to affect endothelial integrity, by inducing p53-dependent apoptosis and strongly inhibiting EDR, in *in vivo* and *in vitro* models [54]. Further, the ability of 7-oxysterols, including 7β-OH and 7-K, to induce endothelial dysfunction is suggested by research showing that these oxysterols induce EC apoptosis: they accumulate in

lysosomes at an early stage, then induce lysosomal activation, oxidative stress, and stimulate the mitochondrial pathway of programmed death [55].

The ability of some oxysterols to induce apoptosis appears consistently to be correlated with increased intracellular ROS generation [40,56]. Oxysterols may stimulate vascular ROS generation by up-regulating the activity of NOX isoenzymes, which are present in vascular cells. Inhibition of 7-K-dependent ROS production by selective inhibitors of NOX provided significant protection against macrophage apoptotic death induced by 7-K [57]. Macrophage apoptosis and defective clearance of these cells contribute to formation and enlargement of the lipid-rich necrotic core, favoring progression and instability of atherosclerotic plaques. Of note, it has been demonstrated that all events along the mitochondrial apoptotic pathway triggered by 7-K were markedly quenched when murine J774.A1 macrophages were coincubated with an identical concentration of 7β-OH [58], possibly because the two oxysterols compete to act as substrate for NOX [59]. In this connection, it has been shown that the NOX4 content is increased in atherosclerotic plaques, and it has been demonstrated that 7-K promotes ER stress and apoptosis by up-regulating NOX4 in human aortic SMCs [60]. Further, in U937 promonocytic cells, an oxysterol mixture mimicking that present in atherosclerotic plaques induced early generation of ROS, the effect being at least partly dependent on activation of NOX2 [61]. More recently, in the same cell model, 27-OH was shown to markedly up-regulate NOX2, but also induced derangement of the mitochondrial membrane potential, thus amplifying ROS production [62]. In human promonocytic U937 cells, 7-K has been shown to trigger cell death via oxidative stress and caspase activation. The key role exerted by ROS in oxysterol-mediated apoptosis is indirectly validated by its inhibition upon cell pre-treatment with antioxidants, such as glutathione or N-acetylcysteine (NAC): the number of apoptotic cells decreased drastically [63,64]. Moreover, supplementation of U937 cells with α-tocopherol counteracted 7-K-provoked lysosomal degradation, caspase activation, and phospholipidosis [65]. Thus, imbalance between prooxidants and antioxidants contributes to the inflammation and cell death seen in the atherosclerotic lesions.

Some findings support the involvement of death receptors in oxysterol-induced apoptosis. 27-OH and 22-hydroxycholesterol (22-OH) induce tumor necrosis factor α (TNF-α) in macrophagic cells [66-68] and up-regulation of TNF receptors has been observed in human aortic SMCs after incubation with 7-K [69].

With regard to the proapoptotic and antiapoptotic pathways, a large number of reports stress their activation by oxysterols [43,70]. To give some examples, 25-hydroxycholesterol (25-OH) and 7-K have been shown to induce phosphorylation and translocation of Bax to the mitochondria in vascular SMCs, also triggering ROS release, by activating soluble adenylyl cyclase/protein kinase (PK) A [71]. In the same cells, Triol was found to markedly impair mitochondrial membrane potential [72]; 7β-OH activated caspase-3 and caspase-9 in human promonocytic U937 cells [73] while 25-OH caused cytochrome c release and activation of caspase-3 and -9 in CHO-K1 [74]. In addition, in U937 cells, 7β-OH and 7-K have been shown to exert synergistic toxic effects through caspase activation, ROS production, cellular thiol depletion, and permeabilization of lysosomal and mitochondrial membranes. Of note, the apoptotic effect of these two oxysterols was quenched by simultaneous addition of 25-OH and 27-OH; however the combination of all four oxysterols as a mixture was proapoptotic, indicating that the major oxysterols accumulating in human atheroma are proapoptotic, and significantly contribute to plaque progression and instability [75]. In the regions of advanced plaques prone to fibrous cap rupture, the proapoptotic effects exerted by oxysterols on SMCs favor cap weakness and destruction [76]. It has been shown that 7-K contributes to SMC apoptosis, increasing plaque vulnerability and rupture, through activation of the caspase-3/7-dependent apoptotic pathway. The same study reportes that serotonin, released from activated platelets, appears to inhibit vascular SMC apoptosis induced by 7-K, helping to prevent the plaque vulnerability induced by the oxysterol [77]. The toxic response of oxysterols on SMCs occurred chiefly when the cells were incubated with high concentrations of 7-K [78]. The death of SMCs

induced by high concentrations of oxysterols is likely to destabilize plaques and increase the risk of thrombosis [79].

Furthermore, it has been reported that oxysterols can induce mitochondrial apoptotic pathway through modulation of cytosolic $Ca^{2+}$ levels. These oxidized lipids can trigger the increase of intracellular calcium by induction of ROS release which can damage ER membrane integrity. In human macrophagic cells, increase of intracellular $Ca^{2+}$ may act as initial trigger of apoptosis induced by 7β-OH and 7-K [80,81]. The cell death provoked by this event can depend on Bad dephosphorylation due to activation of the calcium-dependent phosphatase calcineurin, as observed for 7-K [80]; it can also depend on activation of the calcium-dependent calpains involved in Bid cleavage and cytochrome c release [82].

Of note, beside the proapoptotic pathways, 7-K is able to activate a calcium-dependent survival pathway [83], underlying the complexity of the programmed death process induced by oxysterols. In addition, it has been demonstrated that the rise of $Ca^{2+}$ and the activation of extracellular signaling-regulated kinase (ERK) are early features of ultrastructural changes related to apoptosis evoked by 7β-OH in human aortic SMCs [84]. In the same cell type, apoptosis induced by 7-K and 25-OH was inhibited by cell treatment with $Ca^{2+}$ channel blockers [85,86]. Finally, the oxysterol Triol causes an elevation of intracellular $Ca^{2+}$ levels leading to apoptosis of vascular ECs [87]. In contrast to these data, it has been shown that alteration of calcium homeostasis is not involved in β-EPOX-induced cell death in U937 cells [81].

An important feature of oxysterol-induced-apoptosis is the effect of oxysterols on cell cycle regulation: they can lead to activation of p53, a key protein involved in DNA surveillance, promotion of cell cycle arrest, repair, and eventually apoptosis. Incubation of human vascular ECs with either 7β-OH or 7-K has been observed to promote p53 phosphorylation, probably by inducing ROS and translocating them into the nucleus. This would trigger a series of cell reactions, namely lysosomal membrane permeabilization, cathepsin release, and eventually mitochondrial membrane

damage [55]. Besides up-regulation of p53 function, another common mechanism that interferes with cell cycle completion is the inhibition of certain cyclin-dependent kinases, with consequent accumulation of cells in the G1, S, and G2/M phases of the cycle. In this connection, treatment of THP-1 monocyte-like cells with 7β-OH or with 25-OH was found to block them in the G2/M phase, through oxysterol-dependent inhibition of cyclin B1 and its dependent kinase cdc2. The cells then underwent apoptosis via reduction of the antiapoptotic protein Bcl-2 and activation of caspase-9 and -3 [88].

Autophagy dysfunction has been associated with increased lipid accumulation, apoptosis, and inflammation, all events that promote advanced plaque formation and destabilization. In this connection, it has been demonstrated that dysfunctional autophagy is a characteristic of advanced human atherosclerotic plaques; this stage is associated with accumulation of oxidized lipids (such as oxysterols) that contribute to necrotic core formation [89]. Some oxysterols, for example 7-K, 7β-OH, and 24S-hydroxycholesterol (24S-OH), are reported to trigger apoptosis and, at the same time, autophagy; activation of the autophagic process was demonstrated by the presence of cytoplasmic phagosomes, and by increased levels of the specific marker of autophagy, the microtubule-associated protein light chain 3 (LC3)-II derived from LC3-I cleavage [90]. Oxysterols thus appear able to induce a complex type of cellular death mechanism, which includes oxidative stress, apoptosis, and autophagy; this has very recently been termed "oxiapoptophagy".

Inflammation also participates in atherosclerotic plaque progression and instability. Oxysterols consistently contribute to the proinflammatory effect of oxLDLs by inducing the expression of various key inflammatory molecules [17,19,37]. In particular, they stimulate leukocytes' adhesion to the arterial endothelium, but also their transmigration and cell differentiation. In this connection, monocyte chemoattractant protein 1 (MCP-1) and β1-integrin were found to be up-regulated in U937 cells by an oxysterol mixture mimicking that detectable in the plasma of hypercholesterolemic individuals, through ERK and nuclear factor-κB (NF-κB)

pathways. Of note, cells incubated with non-oxidized cholesterol did not show any variation compared to untreated cells, underlining that only the oxysterols have proatherogenic effects [91,92]. It has also been observed, in ApoE$^{-/-}$ mice, that dietary oxysterols accelerate plaque destabilization and rupture, by increasing monocyte infiltration and differentiation, inducing MCP-1 expression, increasing lipid core size in the plaque, and enhancing MMP-2 and MMP-9 activity. Inhibition of the absorption of oxysterols by ezetimide, an inhibitor of the cholesterol transporter Niemann-Pick C1 Like 1, prevented the acceleration of plaque destabilization [93]. The fate of an atherosclerotic plaque in fact depends on the balance between recruitment and activation of monocyte-derived macrophages and on macrophage polarization state [94]. The proinflammatory macrophage M1 may accelerate atherosclerosis progression, by producing a wide range of inflammatory molecules and ROS, while macrophage M2 shows an antiinflammatory phenotype and may be critical for decreasing inflammation [95]. It has recently been demonstrated that 7-K can affect human macrophage polarization by switching antiinflammatory M2 macrophages to proinflammatory M1 macrophages. In both macrophage phenotypes, this oxysterol selectively activated the production of many key proatherogenic mediators involved in inflammatory, invasive, and angiogenic mechanisms within the atherosclerotic lesion. The proatherogenic effect of 7-K was counteracted by the antiinflammatory and antioxidant resveratrol, a polyphenolic compound [96,97].

Various oxysterols are also involved in the overexpression of interleukin (IL)-8 in human monocytes/macrophages [98,99]. IL-8 appears to be up-regulated by 7β-OH and 25-OH in THP-1 cells via the ERK/activator protein 1 (AP-1) pathway [99]. In addition, in human monocytic/macrophagic cells, 7β-OH, 25-OH, and also 7-K display potent induction of MCP-1, TNF-α, IL-8, IL-6, IL-1β, and macrophage inflammatory protein-1β (MIP-1β). These effects are associated with increased ROS generation, through overexpression of NOX, and with mitogen-activated protein kinase (MAPK)/NF-κB activation [100,101]. Increased levels of IL-6 have also

been found in human aortic SMCs incubated with 7-K through MAPK activation [102]. Of note, IL-6 regulates the expression of other inflammatory cytokines, including IL-1 and TNF-α [103]. Another effect of 7-K and 25-OH, which may contribute to inflammatory response, is inhibition of the secretion of IL-10, an important antiinflammatory cytokine, in SMCs [104]. Moreover, 27-OH which accumulates markedly in the atherosclerotic plaque, was found to up-regulate IL-1β, IL-8, TNF-α, and MMP-9 in U937 cells, through the Toll-like receptor (TLR)4/NF-κB pathway [68]. Moreover, 7β-OH and 7-K induce IL-1β, E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule (VCAM-1) expression in human vascular ECs [105,106]. Again, 7-K has been shown to enhance ROS-dependent secretion of VCAM-1 in human aortic ECs [107], and 25-OH has been found to increase leukocyte adhesion to ECs by up-regulating the same adhesion molecule [108]. Oxysterols may also be able to stimulate arachidonic acid release, after activation of cytosolic phospholipase $A_2$ and subsequent eicosanoid production. In human umbilical vein ECs, the oxysterol Triol up-regulates cyclooxygenase-2 (COX-2), leading to prostaglandin $E_2$ ($PGE_2$) release, involving phosphoinositide 3-kinase (PI3K)/Akt and endothelial NOS, as well as the activation of NF-κB and p38 MAPK [109]. Again, 27-OH contributes to inflammation by stimulating $PGE_2$ production, through COX-2 and microsomal prostaglandin E synthase 1 activation, and by increasing inducible NOS levels and subsequent NO release in U937 cells (Gargiulo et al, unpublished data).

Oxysterols thus contribute to sustaining inflammation by inducing the differentiation of monocytes into macrophages. An oxysterol mixture was found to induce foam cell formation through up-regulation of the scavenger receptor CD36, involving the PKC-δ, ERK, and peroxisome proliferator-activated receptor γ (PPAR-γ) pathways [110,111]. Among the oxysterols present in oxLDLs, 7β-OH and 7-K appear to be the major compounds responsible for monocyte differentiation into macrophages, as observed in oxLDL-treated THP-1 cells [112].

Vascular SMCs are crucial to plaque formation, progression, and stability. At the early phase of atherosclerotic lesion, these cells start to proliferate and migrate to the sub-endothelial space, also undergoing a phenotypic change: inflammation stimulates the shift of SMCs from the contractile phenotype to the fibroblast-like phenotype. The latter phenotype starts to synthesize ECM components, causing formation of the fibrous cap and contributing to arterial rigidity. With regard to the effects of oxysterols on these cell types, it has been shown that low doses of 7-K or of α-EPOX induce proliferation and migration of rat aortic SMCs, by activating the epidermal growth factor receptor (EGFR)/PI3K/Akt pathway; they also increase MMP-2 and MMP-9 activities. Cell treatment with the same concentration of cholesterol had no significant effect on SMC proliferation [113]. Further, $PGE_2$ has been shown to increase endothelial permeability and vascular tone, as well as vascular SMC proliferation and migration [114].

The strength of the fibrous cap depends on a dynamic balance between collagen synthesis and degradation. During fibrous cap formation, a key role in the deposition of ECM components is played by the inflammatory cytokine TGF-β1, which is strongly profibrogenic. In this connection, a biologically compatible mixture of oxysterols has been shown to induce expression and synthesis of TGF-β1 in the J774.A1 murine macrophagic cell line [115] and in Raw264.7 murine macrophages [116]. Conversely, irregular and uncontrolled ECM degradation, due to an imbalance in the MMPs/TIMPs activity ratio, clearly contributes to plaque destabilization and rupture. Indeed, MMPs, principally MMP-9 and MMP-2, are the chief enzymes responsible for excessive proteolytic ECM degradation, as well as for vascular remodeling, SMC migration, and macrophage infiltration, with subsequent increase of plaque inflammation. The principal source of MMPs is activated macrophages, but also vascular SMCs and ECs, and expression of these cells may be modulated by ongoing inflammation [24,117].

In this connection, arteries from oxysterol-fed $ApoE^{-/-}$ mice showed enhanced macrophage activity of both MMP-2 and MMP-9, and infiltration of monocyte-mediated inflammation through MCP-1 release. These effects markedly accelerated plaque destabilization and rupture, pointing to a

role of accumulated oxysterols in plaque vulnerability. In the same animal model, all these effects were prevented when oxysterol intestinal absorption was inhibited by administering ezetimibe [93]. In human promonocytic U937 cells, an oxysterol mixture of composition similar to that found in advanced atherosclerotic plaques was showed to up-regulate MMP-9, without affecting its inhibitors TIMP-1 and TIMP-2. Moreover, it has been demonstrated that oxysterols induce MMP-9 expression and synthesis, by enhancing ROS generation, up-regulating the MAPK signaling pathways via PKC, and also by up-regulating AP-1 and NF-κB DNA-binding, which are both redox transcriptional factors [61]. In addition, one the oxysterols that is most abundant in human blood, i.e. 27-OH, has been shown to markedly up-regulate MMP-9 expression and synthesis in human promonocytic U937 cells, through the TLR4/NF-κB pathway. The oxysterol also enhanced the release of IL-8, IL-1β and TNF-α, thereby augmenting the inflammatory response. Given that an inflammatory state can contribute to MMP activity, it is significant that these inflammatory cytokines have also been found to act on MMP-9 production, thus sustaining the release of this matrix-degrading enzyme and contributing to plaque fragility [68]. Interestingly, 7-K can affect macrophage polarization by skewing the M1/M2 balance towards a proinflammatory profile, by inducing many proinflammatory mediators, including MMP-9 and MMP-2 [96,97]. MMP-2 and MMP-9 have also been found to be elevated, in vascular SMCs, by 7-K and α-EPOX, through activation of ERK [113].

## 4. HNE and atherosclerotic plaque progression

The biochemical effects of HNE are due to its lipophilicity and high reactivity towards thiol and amino groups, forming covalent adducts. HNE reacts mainly with the amino acids cysteine, histidine, and lysine, which, either free or protein-bound, readily undergo Michael addition reactions to the C-C double bond. Besides this type of reaction, other reactions may also occur,

involving the carbonyl and hydroxyl groups. Alternatively, amino acids may react with the carbonyl group to form Schiff bases. The cytotoxicity of HNE is attributed to its covalent interaction with nucleophilic moieties in proteins, DNA, and phospholipids, with consequent modification of their structure. Thus the progressive accumulation of these adducts may alter normal cell function, leading to cell death [44,118-120]. Increased levels of HNE adducts have been found in atherosclerotic lesions, indicating an association of HNE with atherosclerosis, and it thus seems clear that HNE is responsible for the proatherogenic effects of oxLDLs, being one of their major reactive components [45,46].

HNE is transported by oxLDLs into the lipid core of the plaque, where it accumulates and chemically attracts monocytes/macrophages and SMCs. It has also been demonstrated that HNE covalently modifies oxLDLs by binding to lysine and histidine residues, leading to adduct formation: this favors oxLDL uptake by macrophages through the scavenger receptors, thus resulting in macrophage activation and foam cell formation [121]. It was demonstrated that HNE could stimulate the inflammatory processes by showing that it induces expression and synthesis of MCP-1 in murine macrophages (J774.A1), by activating PKC-βI and PKC-βII [122]. Marked production of the inflammatory and fibrogenic cytokine TGF-β1 in macrophagic cells incubated with HNE has also been shown [123-125].

HNE may also damage endothelial barrier function, due to impaired cell-cell communication and inhibition of membrane associated enzymes [126]. It is known that NO plays an important role in mediating blood pressure and vascular tone, as well as in decreasing endothelium permeability, thus contrasting LDL infiltration. Reduction of the bioavailability of NO seems to be a key factor in cardiovascular disease [127]. HNE may modulate production of NO at the arterial wall affected by atherosclerosis, and it has been shown to reduce the production of inducible NOS and nitrites from NO, by inhibiting the NF-κB activation pathway in vascular SMCs, monocytes, and macrophages [128,129]. In addition, HNE may induce ER stress and impair vascular endothelium and

coagulation functions. HNE can also directly impair endothelial barrier function and increase endothelial permeability [130]. In this connection, HNE may induce both actin stress fibers and intercellular gap formation, suggesting a link between F-actin cytoskeletal rearrangement and permeability changes [131,132]. Further, it may induce changes in cellular thiol redox status, thus disordering cell signaling pathways, leading to vascular endothelium dysfunction. After induction of ROS, HNE may act on endothelial permeability, modulating cell-cell adhesion by suppressing focal adhesion kinase (FAK) phosphorylation, which in turn affects focal adhesion, adherence and tight junction proteins, as well as integrin, all of which are natural FAK receptors. Moreover, HNE modifies cytoskeletal proteins, such as actin and microtubules, by regulating cell-cell contacts and endothelial barrier function. Notably, all these changes can be inhibited by pretreatment of cells with the thiol protectant NAC [126,133].

Further, HNE may stimulate the adhesion of monocytes, as well as lymphocytes, to ECs: it has been shown to react with the thioredoxin (TRX) system by binding to Cys73, with subsequent inhibition of its activity, followed by an increase in ROS generation, and the stimulation of adhesion of monocytes and immune cells to the vascular endothelium. HNE modification of TRX could thus potentiate adhesion of monocytes to ECs, an early event in atherosclerosis [134]. TRX is an antioxidant system, which is ubiquitously expressed in ECs, where it regulates oxidative stress by scavenging ROS, and prevents apoptosis [135]; for this reason it is critical to maintaining normal endothelial function and protecting against atherosclerosis.

Regarding NF-κB, HNE can activate or inactivate this redox transcriptional factor, respectively by mediating or inhibiting its proinflammatory and oxidative effects [37]. It has been shown that inhibition of NF-κB in THP-1 cells and in human aortic ECs resulted in inhibition of the adhesion molecules and cytokines induced by inflammatory stimuli [136,137]. Conversely, HNE stimulates NF-κB in vascular SMCs [138]. The dual effect of HNE on NF-κB signaling pathway thus appears to depend on the cell type used, and it may have a significant impact on atherosclerosis

development. Down-regulation of NF-κB dependent gene expression, as a consequence of inhibition of the transcriptional factor by HNE, may interfere with the immune response and thus amplify and sustain the inflammatory response. Conversely, marked activation of another redox transcriptional factor, AP-1, by HNE has been demonstrated in murine and human macrophages and aortic SMCs [138,139]. HNE may also potentiate inflammation and immune responses, by inducing COX-2 and in consequence prostaglandin production [140].

HNE also promotes foam cell formation, by increasing expression and synthesis of the class A scavenger receptors at the transcriptional level, as well as of the CD36 scavenger receptor on murine macrophages [141]. It also enhances the CD36 scavenger receptor in vascular SMCs by inducing nuclear accumulation of the nuclear factor E2-related factor 2 (Nrf2) [142].

HNE is responsible for DNA-damage in SMCs: bulky DNA adducts have been found in abdominal and thoracic aorta samples from atherosclerotic patients [143]. High levels of miscoding etheno-DNA adducts have also been detected in human aorta SMCs from subjects affecting by atherosclerosis. This DNA damage leads to SMCs proliferation [144].

HNE may also exert a dual effect on cell proliferation, depending on the local concentration and the degree of oxidative stress. This dual effect may result from the modification and subsequent dysfunction of tyrosine kinase receptors induced by HNE [145], such as PDGFR-β, which regulates SMCs migration and proliferation. Brief incubation of cells with low concentrations of HNE induces autophosphorylation of PDGFR-β and subsequent SMC proliferation [146]; longer incubation with high concentrations of the aldehyde increases formation of HNE-PDGFR-β adducts, with subsequent inhibition of PDGFR-β phosphorylation and SMC proliferation. This reduced SMC proliferation contributes to decreasing the stability of a vulnerable atherosclerotic plaque [147]. Activation of PDGFR-β and ERK1/2 by HNE in human coronary SMCs is also a key mechanism in the production of MMP-1 and MMP-2, which may stimulate SMC proliferation and migration into the intima [148]. Further, the accumulation of HNE adducts on EGFR may inhibit

PI3K/Akt pathway activation, inhibiting cell proliferation and inducing apoptosis [146]. In addition, HNE was found to react directly with membrane proteins of human ECs, inducing formation of HNE-EGFR and HNE-PDGFR adducts [145]. Of note, HNE-adducts on PDGFR and EGFR have been detected in carotid atherosclerotic plaques [147].

Some cytotoxic effects of oxLDLs can be attributed to HNE, but the mechanisms are not fully elucidated. Lipid peroxidation products, such as HNE, are inducers of oxidative stress and are able to trigger $Ca^{2+}$ mobilitation. In this connection, it has been reported that HNE induces α-fodrin cleavage and upregulates the heat shock protein 70 expression, both substrate of the calcium-dependent protease calpain. The activity of this enzyme leads to the release of cathepsins which cause cell death [149]. Moreover, it has been found that HNE induces cytotoxicity in cardiomyocytes by increasing intracellular $Ca^{2+}$ and ROS levels, and causing mitochondrial dysfunction [150]. The role of calcium in HNE-mediated apoptosis is also supported by studies carried out on neuronal cells [151,152].

HNE has also been shown to induce derivatization and ubiquitination of cell proteins, and to inhibit the proteasome pathway, all events involved in the mechanism of oxLDL-induced apoptosis. Modification of cell protein structure impairs function, and eventually alters cell viability, while inhibition of proteasome reduces cellular defenses [31]. In addition, HNE appears to modify and inhibit the enzymatic activity of DPI, a major chaperone in protein folding in the ER; this leads to alteration of the folding of nascent proteins in the ER, and is a factor in activating ER stress/URP response, and induces apoptosis in vascular cells if the ER stress is prolonged [35]. ER stress thus appears to have a negative effect on EC stability, which may lead to plaque fragility and rupture: cell apoptosis mediated by ER stress may promote loss of the endothelial layer covering the plaque.

HNE also induces expression of 5-lipoxygenase (5-LO) through the NF-κB/ERK and Sp1/p38 MAPK pathways via EGFR in murine macrophages [153]. The p38 MAPK and ERK pathways are also involved in HNE-induced activation of 5-LO in vascular SMCs, inducing their migration and proliferation leading to plaque instability [154]. Of note, up-regulation of 5-LO by

activated macrophages in symptomatic plaques has been shown to lead to enhanced production of MMPs, with subsequent plaque instability [155,156]. It has also been demonstrated that the association between HNE and 5-LO derivatives increases the secretion of MMP-9, by means of ERK and p38 MAPK activation [157]. A similar synergistic action between these products has also been demonstrated in vascular SMCs. In this case, a marked production of MMP-2 was evident, again after activation of the ERK and p38 pathways [154,158]. Both MMP-9 and MMP-2 play pathogenic roles in the progression of plaque rupture. Thus 5-LO, induced by HNE, plays a key role in the development and progression of atherosclerosis, and it has been suggested to be a modulator of plaque instability [155,159,160]. Moreover, HNE has also been shown to induce MMP-2 production in vascular SMCs, via mitochondrial ROS-mediated activation of the Akt/NF-κB signaling pathways [154]. HNE was also found to enhance cell release of inflammatory cytokines (IL-8, IL-1β, and TNF-α) and to up-regulate MMP-9 via the TLR4/NF-κB-dependent pathway. These events may sustain inflammatory response and matrix degradation. Using specific antibodies, these cytokines have been shown to increase MMP-9 up-regulation, thus enhancing the release of this matrix-degrading enzyme by macrophage cells, and contributing to plaque instability [68]. HNE may also contribute to ECM modification and degradation by antagonizing the elastogenic activity of TGF-β in fibroblasts [161].

## 5. Conclusions

Increasing experimental evidence points to a significant contribution of oxysterols and HNE in development of the atherosclerotic plaque. Of note, these proatherogenic compounds appear to have a dual effect: i) they first initiate impairment of vascular endothelium function, mainly caused by oxidative stress and inflammation; ii) subsequently, following excessive accumulation, they might contribute to foam cell formation, migration and proliferation of vascular SMCs, fibrous cap

formation, and modulation of ECM, leading to plaque progression and instability with eventual rupture (Fig. 4). However, further studies will be needed to identify all the molecular pathways that are modulated or altered by these oxidized lipids, in order to find targeted therapeutic strategies to prevent or treat this disabling disease.

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**Conflicts of interest statement**

The authors declare no conflict of interest.

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**Figure legends**

**Fig. 1 Chemical structures of oxysterols.** Oxysterols are 27-carbon-atom compounds deriving from cholesterol oxidation. Oxidation of the side chain is usually enzymatic, whereas oxidation of the sterol rings is non-enzymatic and depends on oxidative stress.

**Fig. 2 Chemical structure of 4-hydroxynonenal.** HNE is a highly reactive hydroxyalkenal deriving from the non-enzymatic breakdown of n-6-polyunsaturated fatty acids (PUFA), such as arachidonic, linoleic, and linolenic acids.

**Fig. 3 Accumulation of oxysterols and HNE contributes to plaque progression.** Continuous LDL oxidation and lipid release from apoptotic foam cells produce increasing levels of oxysterols and HNE, which contribute to all stages of atheroma formation. These oxidized lipids sustain the inflammatory process and endothelial activation, the formation of foam cells, the migration of SMCs from the tunica media toward the intima, and degradation of the fibrous cap, favoring plaque

rupture and thrombus formation. LDL, low density lipoprotein; oxLDL, oxidized LDL; SMC, smooth muscle cells.

**Fig. 4 Scheme of key steps promoting plaque vulnerability induced by oxysterols and HNE.**

Figure 1

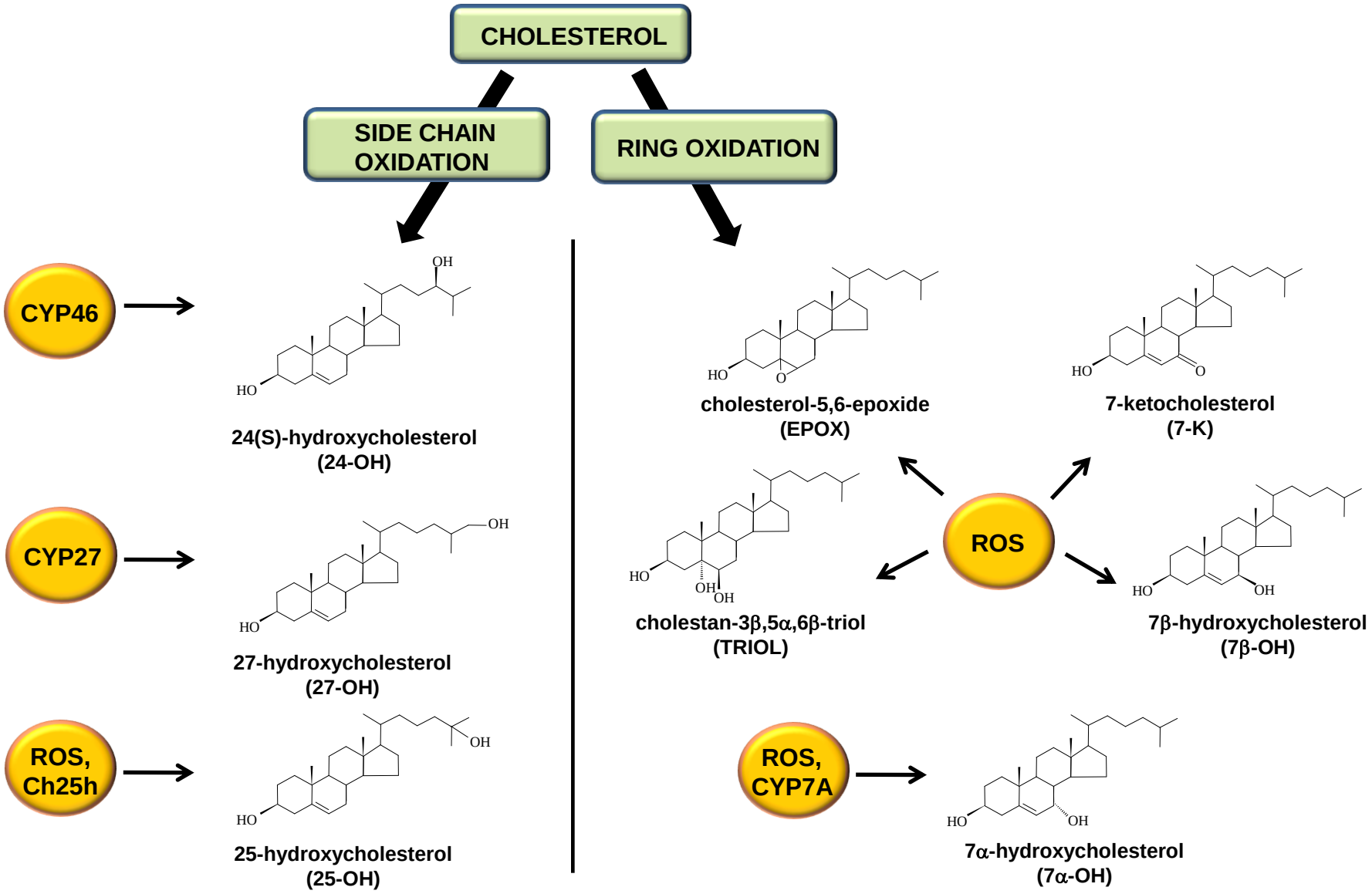
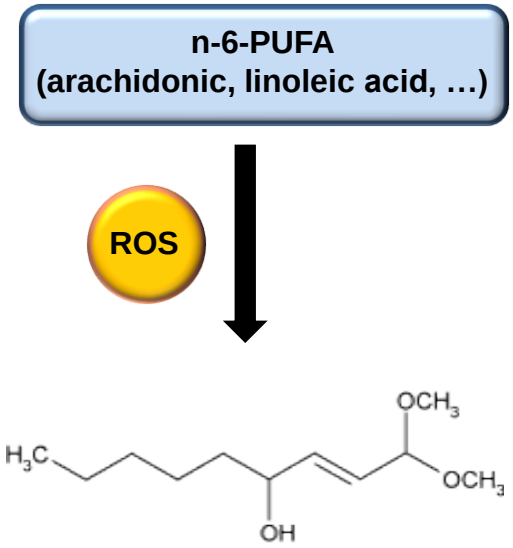

CHOLESTEROL
SIDE CHAIN OXIDATION
RING OXIDATION
CYP46
24(S)-hydroxycholesterol (24-OH)
CYP27
27-hydroxycholesterol (27-OH)
ROS, Ch25h
25-hydroxycholesterol (25-OH)
cholesterol-5,6-epoxide (EPOX)
7-ketocholesterol (7-K)
ROS
cholestan-3β,5α,6β-triol (TRIOL)
7β-hydroxycholesterol (7β-OH)
ROS, CYP7A
7α-hydroxycholesterol (7α-OH)

Figure 2




4-Hydroxynonenal (HNE)

Figure 3

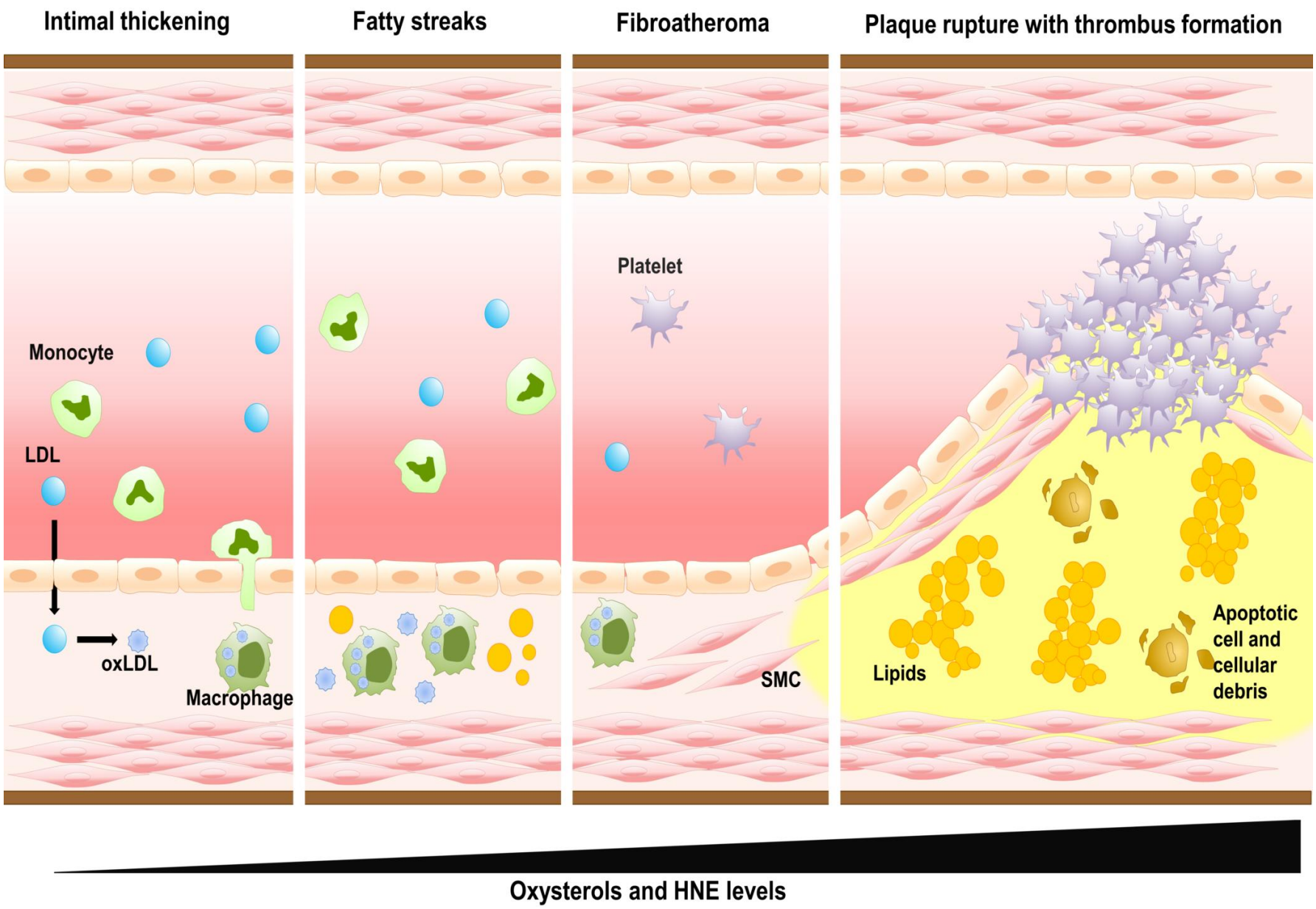
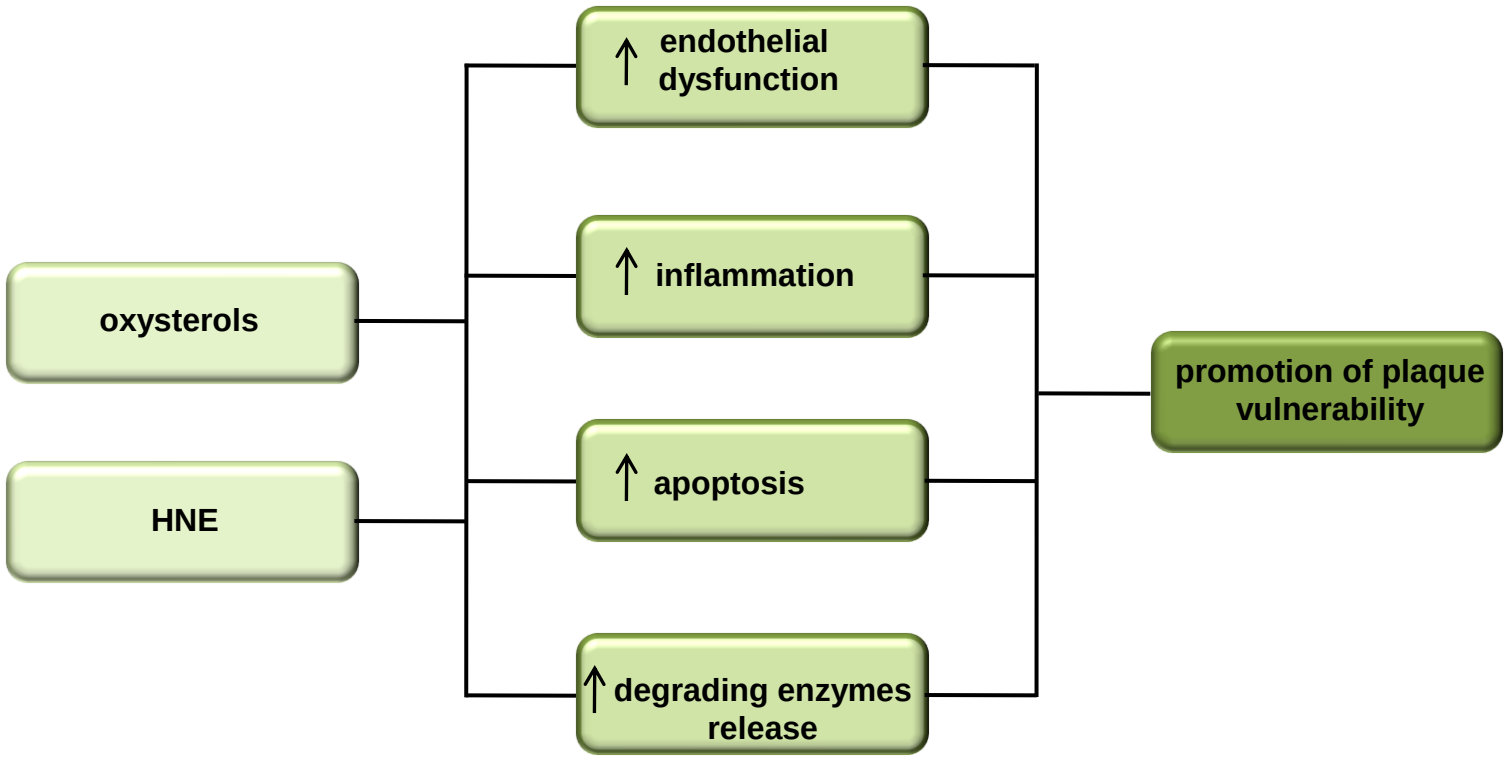

Intimal thickening
Fatty streaks
Fibroatheroma
Plaque rupture with thrombus formation
Monocyte
LDL
oxLDL
Macrophage
Platelet
SMC
Lipids
Apoptotic cell and cellular debris
Oxysterols and HNE levels

Figure 4


oxysterols
HNE
↑ endothelial dysfunction
↑ inflammation
↑ apoptosis
↑ degrading enzymes release
promotion of plaque vulnerability