

## Enzymatic Targets in Atherosclerosis

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### Abstract

Atherosclerosis, a prime cause of mortality across the developed societies, was targeted by diverse therapeutic strategies. These evolved in response to the complex etiology and evolution of the disease. Many enzymes are associated with atherosclerosis, either in the main stream of lipid biosynthesis and transport or in the collateral and intertwined pathways of oxidative stress, inflammation, vascular remodeling or chromatin stability and are therefore revised herein. Enzyme exploration led to important developments. At the beginning, there were the statins, derived as inhibitors of hydroxy-methyl-glutaryl CoA (HMG-CoA) reductase, currently used widely to decrease lipid levels. At the other end, the inhibitors of the recently discovered proprotein convertase subtilisin/kexin type 9 (PCSK9) are awaiting the validation in clinical trials with great hopes for the future. In between, one can find some palliatives, as aspirin, an inhibitor of cyclooxygenase (COX), but also many invalidated candidates. Classical pharmacological data and newer approaches, like genetic knockouts in murine atherosclerosis models, are reviewed in order to appreciate the involvement of a particular enzyme in atherogenesis. However, the pursuit of an efficacious drug has been long and, in many cases, disappointing. Conclusions can be drawn from the overview of both successes and failures, in a quest for the best.

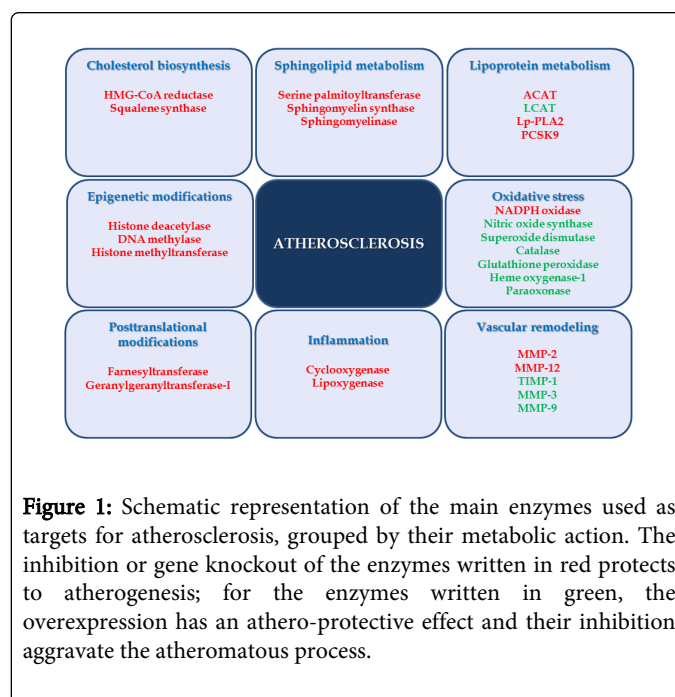
**Keywords:** Atherosclerosis; apoE; Therapeutic target

### Abbreviations

ACAT: Acyl-Coenzyme A-Cholesterol Acyltransferase; apoB: Apolipoprotein B; apoCI: Apolipoprotein CI; apoE: Apolipoprotein E; CAT: Catalase; COX: Cyclooxygenase; DNMT: DNA Methyltransferases; FTase: Farnesyltransferase; GGTaseI: Geranylgeranyltransferase I; GPx: Glutathion Peroxidase; HDAC: Histone Deacetylases; HDL: High-Density Lipoprotein; HMGCR: 3-Hydroxy-3-Methyl-Glutaryl-CoA Reductase; HO: Heme Oxygenase; IFN $\gamma$ :  $\gamma$  Interferon; LCAT: Lecithin-Cholesterol Acyltransferase; LDL: Low-Density Lipoprotein; LDLR: Low-Density Lipoprotein Receptor; LO: Lipoxygenase; Lp-PLA2: Lipoprotein-Associated Phospholipases A2; LPS: Lipopolysaccharide; miRNA: microRNA; MMP: Matrix Metalloproteinase; NF $\kappa$ B: Nuclear Factor KB; NO: Nitric Oxide; NOS: Nitric Oxide Synthase; oxLDL: Oxidized LDL; PCSK9: Proprotein Convertase Subtilisin/kexin type 9; PON: Paraoxonase; ROS: Reactive Oxygen Species; SMase: Sphingomyelin Phosphodiesterase; SMCs: Smooth Muscle Cells; SMS: Sphingomyelin Synthase; SOD: Superoxide Dismutase; SPT: Serine Palmitoyltransferase; SQS: Squalene Synthase; TNF $\alpha$ : Tumor Necrosis Factor  $\alpha$ ; VLDL: Very Low-Density Lipoprotein

### Introduction

Atherosclerosis continues to remain the leading medical, social and economic challenge in the developed societies. Atherosclerosis occurs primarily as a result of the accumulation of cholesterol in the arterial wall, upon which subsequent events of calcification and inflammation lead to the formation of a characteristic plaque which reduces the blood vessel lumen. In the advanced stages, the plaque can be destabilized and obstruct blood flow, with lethal consequences.



**Figure 1:** Schematic representation of the main enzymes used as targets for atherosclerosis, grouped by their metabolic action. The inhibition or gene knockout of the enzymes written in red protects to atherogenesis; for the enzymes written in green, the overexpression has an athero-protective effect and their inhibition aggravate the atheromatous process.

Despite great research developments, the contemporary life style and genetic heritage afflict millions of individuals and their number raises. Due to the multifactorial, multistage manifestation of the disease, atherosclerosis was approached from different points of view, from which very diverse therapeutic strategies emerged. Most of them aimed lipid lowering as a primary therapeutic target, while others targeted later events to delay the progression of the disease, alleviating its phenotype. The current status of the most promising approaches,

either in use or in advanced clinical trials, have been recently reviewed by others [1,2]. The focus of the current paper was to summarize contemporary studies addressing the roles of various enzymes implicated in atherosclerosis, the rationale for targeting them, the main experimental models utilized, the challenges encountered, the puzzling/ controversial/ unexpected results. A diverse assortment of enzymes is presented (Figure 1), without any pretense of being exhaustive. This outline reflects the current status of both experimental and theoretical progresses, with an emphasis on the atherosclerotic murine knockout models, supplemented by pharmacological data.

From the multitude of the data overviewed below, unfortunately very few had come to a positive therapeutic end, of which the most well-known are statins. Others, like the novel PCSK9 (proprotein convertase subtilisin/kexin type 9) inhibitors, are expected with enhanced interest. In the same time, numerous inhibitors have proved to be at least ineffective, if not deleterious. More reflection and the development of new targeting and delivering strategies may help.

## Cholesterol Biosynthesis Related Enzymes

### HMG-CoA reductase

HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase, HMGCR) catalyzes the conversion of HMG-CoA to mevalonic acid, dependent on NADH (EC 1.1.1.88) or NADPH (EC 1.1.1.34) and controls cholesterol biosynthesis [3], as illustrated in Figure 2. Therefore, this enzyme is the target of the widely available cholesterol-lowering drugs known as statins, originally developed to competitively inhibit the binding of HMG-CoA to HMG-CoA reductase and to reduce mevalonate synthesis [4]. Statins lower LDL-cholesterol levels more dramatically than other lipid-lowering agents, and they also exert pleiotropic effects, including anti-inflammatory, anti-thrombotic and antioxidant effects [5,6]. The former statins, such as lovastatin, pravastatin, simvastatin, are derivatives of fungal products, while newer statins, including atorvastatin, cerivastatin, fluvastatin, rosuvastatin are synthetic. In various clinical trials involving patients with hypercholesterolemia, rosuvastatin and atorvastatin induced greater decreases in LDL-cholesterol than the other statins tested [7]. Despite that statins have been shown to be efficient in clinical trials and they are now commonly used for hyperlipidemia, they cannot completely eradicate the risk for cardiac heart disease [8,9]. Apart from the beneficial effects of statin therapy, the development of myopathies [10], side effects on hepatic and renal function [11], diabetes mellitus [12], as well as cognitive dysfunctions [13] were reported, with higher occurrence for lipophilic statins (atorvastatin, lovastatin, simvastatin), and lower prevalence for hydrophilic statins (pravastatin, rosuvastatin). However, the overall benefit for cardiovascular disease through statin therapy still exceeds the risk of side effects. Excepting cerivastatin, which was withdrawn because of severe myopathy and rhabdomyolysis, the tolerability of statins was similar. An earlier comprehensive review summarized the clinical benefits of statin therapy in wide-ranging cardiovascular disorders, such as acute coronary syndrome, cardiac arrhythmias, heart failure, peripheral arterial disease, cerebrovascular disease, as well as hypertension [14]. A plethora of studies [15] demonstrated that regression of atherosclerotic plaque does occur with statin therapy, and it was estimated that an average of two years of aggressive statin therapy was required for this regression to take place.

### Squalene synthase

Squalene synthase (farnesyl-diphosphate farnesyl transferase; FDFT1; SQS; EC 2.5.1.21) catalyzes the two step NADPH-dependent reductive dimerization of two C15-farnesyl pyrophosphate moieties with the formation of the C30-cholesterol precursor squalene, as illustrated in Figure 2. Squalene synthase is localized exclusively to the membrane of the endoplasmic reticulum [16]. Ablation of SQS in mice is lethal, the enzyme being essential for the development of the central nervous system [17]. Increased expression of SQS has been shown to elevate cholesterol levels in mice [18]. The reaction catalyzed by SQS occurs beyond the branching point of isoprenoid biosynthesis, being the first committed step in cholesterol synthesis. Thus, it was expected that SQS pharmacological targeting would be an attractive approach for the treatment of atherosclerosis due to reduced side effects. To this aim, various SQS inhibitors were assessed for their capacity of lowering cholesterol level [19]. Among these, the benzoxazepine derivative lapaquistat acetate (TAK-475) seemed the most promising. In animal studies on WHHLMI rabbits, a model for coronary atherosclerosis and myocardial infarction, TAK-475 slowed down the progression of coronary atherosclerosis and changed the coronary atheromatous plaques from an unstable, lipid-rich to a stable, fibromuscular phenotype [20]. In humans, administration of TAK-475, alone or together with statins, significantly reduced low-density lipoprotein (LDL)-cholesterol in a dose-dependent manner. It also decreased other risk markers, such as C-reactive protein. However, its hepatotoxic effects such as raised levels of aminotransferase and bilirubin, led to the termination of advanced clinical trials [21]. For an extensive review of SQS inhibitors as antiatherosclerotic agents, the reader is directed to the recent comprehensive review of Kourounakis [19].

## Sphingolipid Metabolism Related Enzymes

Sphingolipids are essential constituents of the plasma membrane, key signaling molecules and modulators of plasma lipoprotein metabolism [22]. A preference for cholesterol association with sphingolipids in the plasma membrane lipid raft microdomains is believed to serve as a signal transduction centers for immune responses, which may be important for the inflammatory component of atherogenesis [23]. Plasma sphingomyelin (SM) emerged recently as a risk factor for cardiovascular diseases [24], being a key regulator of plasma lipoprotein metabolism and atherosclerosis [25]. Recently sphingolipids have been attributed a rather active role in cardiovascular pathology, since inhibition of their de novo synthesis raises anti-atherogenic lipoproteins and decreases atherosclerosis in murine models [26]. Thus, genetic and pharmacological interventions on the enzymes involved in sphingolipid biosynthetic pathways open a new avenue for therapeutic approaches in this field.

### Serine palmitoyltransferase

Serine palmitoyltransferase (palmitoyl-CoA:L-serine C-palmitoyltransferase (decarboxylating); SPT; EC 2.3.1.50) catalyzes the condensation between palmitoyl-CoA and L-serine, the first reaction in sphingolipid biosynthesis. In eukaryotes the enzyme is anchored to the endoplasmic reticulum [27] and it consists of a dimer of SPTLC1 and SPTLC2 or SPTLC3 [28] or it could aggregate in even higher oligomers [29]. SPTLC1 or SPTLC2 knockout in mice embryos is lethal, while heterozygous animals (Sptlc1(+/-), Sptlc2(+/-)) are viable, but present a modified plasma lipid profile. Thus, they exhibit decreased plasma sphingolipid levels, but no change in plasma

triglyceride, total cholesterol, phospholipids, plasma and liver sphingomyelin [30]. SPT is specifically inhibited by the fungal antibiotic myriocin [31]. Treatment with myriocin of apoE-deficient mice led to reduced levels of plasma sphingolipid, increased level of phosphatidylcholine, but similar cholesterol or triglyceride level, while an substantial decrease of the size of the atherosclerotic lesions was noticed [32,33]. The anti-atherosclerotic effect was due to the decreased level of sphingolipids, but not of glycosphingolipids, since EtDO-P4, a glucosylceramide synthesis inhibitor, had no impact on the lesion area [34]. In other studies, myriocin treatment of apoE-deficient mice was reported to lower plasma cholesterol and triglyceride levels in a dose-dependent manner, to reduce VLDL- and LDL- cholesterol, to increase HDL-cholesterol, and to enhance the level of apoAI and LCAT in the liver [26]. Recently, a series of miRNAs have been shown to control  $\beta$ -amyloid level by regulation of SPT levels, providing a link between the upregulation of SPT and sporadic Alzheimer's disease [35]. Therefore, there is still enough therapeutic potential to be explored by inhibiting SPT.

### Sphingomyelin Synthase

Sphingomyelin synthase (ceramide:phosphatidylcholine cholinephosphotransferase; SMS; EC 2.7.8.27) catalyzes the last step of SM de novo biosynthesis, through the reaction between ceramide and phosphatidylcholine, with the formation of sphingomyelin and 1,2 diacylglycerol. Two SMS genes, SMS1 and SMS2, have been cloned and characterized [36]. The proteins differ by their localization, with human SMS1 in the Golgi apparatus and SMS2 at the plasma membrane, and they also exhibit different tissue expression profile. SMS role in cell survival can be appreciated by the fact that it consumes the pro-apoptotic ceramide, while generating the anti-apoptotic diacylglycerol. SMS2 overexpression in apoE-deficient mice correlated with an enhanced endothelial dysfunction and aggravated atherosclerotic plaque instability [37]. Hepatic SMS2 is essential for plasma SM level regulation, as demonstrated with SMS2-deficient and SMS2 liver-specific transgenic mice. Thus, SMS2-deficient mice had decreased SM levels, while the transgenic animals overexpressing SMS2 had increased SM level. Additionally, atherogenic lipoproteins from the transgenic mice displayed a significantly stronger tendency toward aggregation after treatment with mammalian sphingomyelinase, compared with controls. Moreover, SMS2 knockout significantly raised plasma apoE level, whereas liver-specific SMS2 overexpression decreased it. Plasma of SMS2-deficient mice promoted cholesterol efflux from macrophages, whereas plasma of SMS2 transgenic mice prevented it [38]. SMS2 deficiency attenuates the activation of the pro-atherogenic factor NF- $\kappa$ B, as proved in murine SMS2 knockout macrophages mice exposed to lipopolysaccharide (LPS) and in SMS2 siRNA-treated HEK-293 cells exposed to TNF  $\alpha$  [39]. Another group reported that SMS2/apoE double knockout mice showed a significant decrease in aortic atherosclerotic lesions [40]. Also, transplantation of bone marrow alone from SMS2 knockout mice into LDLR-deficient mice led to a reduction of atherosclerosis [41]. However, in macrophages, SMS2 seems to be responsible only for less than 20% of the total SMS activity. SMS1 is responsible for most SMS activity of macrophages and is capable of rescuing growth of SMS2-deficient murine lymphoid cells [42]. SMS1 knockout mice have significantly decreased level of sphingomyelin and highly increased levels of glucosylceramide and GM3 in the plasma, liver, and macrophages, an effect not seen for SMS2 deficiency. As for SMS2 knockout, SMS1 deficiency attenuated LPS-induced Nk-FB activation and SMS1-deficient mice bone marrow

transplantation into LDLR-deficient mice led to a reduction of atherosclerosis [43]. Recent data showed that, besides the alteration in lipid profile, SMS1 null mice exhibited increased pro-atherogenic oxidative stress [44]. Despite the valuable data accumulated via genetic manipulation of Sms genes, the pharmacological approaches lagged behind. Only recently, small molecule SMS inhibitors in the micro molar range that could be used in atherogenesis or related pathologies as insulin resistance, were described [45]. Pharmacological reduction of SMS has also proved that SMS could be a target of anti-atherogenic therapy. Dy105, a SMS inhibitor, reduced SM levels in SM-rich microdomains on cell membranes, decreased apoB secretion in a human hepatoma cell line and impaired the activation of NF- $\kappa$ B and p38 in bone marrow derived macrophages [46]. It appears that tissue-specific SMS targeting could lead to new developments in anti-atherosclerotic therapies.

### Sphingomyelinase

Sphingomyelinase (Sphingomyelin phosphodiesterase; SMase; EC3.1.4.12) catalyzes sphingomyelin (SM) breakdown into phosphocholine and ceramide. A few types of SMases have been described so far, depending on their optimal pH and cation requirements for activity. Among these, important SMases in cardiovascular pathology are (i) secreted and lysosomal acidic zinc-dependent isoforms, and (ii) membrane magnesium-dependent SMase [47]. Cellular stress activates these SMases with the subsequent generation of ceramide; however, the final effects depend on the SMase type and its cellular locations [48]. The two forms of acidic SMase balance their level on the account of each other, by diverting a common precursor toward Golgi or lysosomes upon receiving specific signals [49]. Most reports point toward a pro-atherogenic role of secretory SMase: it is present in the atherosclerotic lesions and binds specifically to certain subendothelial extracellular matrix constituents, stimulating subendothelial accumulation of atherogenic lipoproteins [50]; increased activity of secretory SMase and specific ceramides in the aorta of apoE-deficient mice during aging were reported [51]; SMase treatment of lipoproteins from apoE-deficient mice enhanced their potential as inducers of macrophage foam cell formation [52]. Additionally, acidic SMase deficient mice on a apoE<sup>-/-</sup> or LDLR<sup>-/-</sup> atherosclerosis-prone genetic background showed significantly reduced lipid retention and atherosclerotic lesions, despite no alteration in plasma cholesterol or lipoproteins, supporting a causative role of SMase in atherogenesis [53]. Surprisingly, adeno-associated virus-mediated expression of acidic SMase in apoE-deficient mice reduced atherosclerosis [54]. Secretory SMase is released by endothelial cells in vitro upon signaling by inflammatory cytokines. Its level is also increased in LPS treated wild-type mice via IL-1, as demonstrated by abrogation of this increase in IL-1 converting enzyme knockout mice or further enhancement in IL-1-receptor antagonist knockout mice [55]. Macrophages deficient in lysosomal SMase exhibited an impaired cholesterol traffic and efflux [56]. Generation of ceramide via hydrolysis of membrane SM by acidic SMase results in lipid rafts clustering and promotes oxidative stress-induced endothelial dysfunction [57]. Neutral SMase 2 has been also implicated in the evolution of atherosclerosis, mainly in relation to plaque remodeling and fibrous cap degradation [58]. Thus, apoCI and apoCI-enriched HDL activate the neutral SMase-ceramide signaling pathway, leading to apoptosis in human aortic SMCs, an event that may promote plaque rupture in vivo [59]. Other reports of the involvement of neutral SMase in the cardiac pathology are summarized in ref. [60]. A recent study reports that palmitate-induced

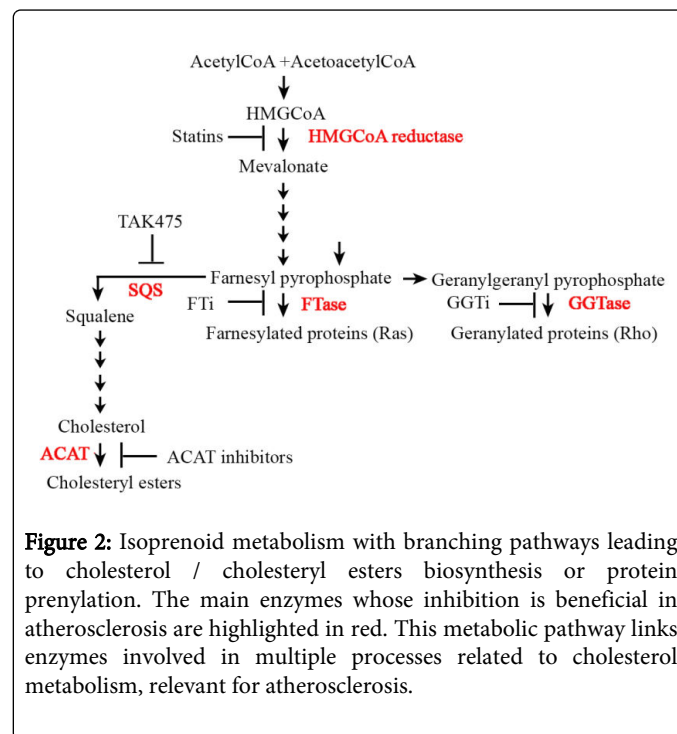
inflammatory response of microvascular endothelial cells to LPS involves neutral SMase [61]. Recently, alkaline sphingomyelinase, which was less related to atherogenic processes, has been implicated as a physiological factor promoting cholesterol absorption by reducing SM levels in the intestinal lumen, as demonstrated in a murine knockout model [62]. Despite the extensive exploration of SMase mechanisms of action, design of specific SMase-type inhibitors is still a challenge and they have not received yet clinical validation [47].

## Lipoprotein Metabolism Related Enzymes

### Acyl-coenzyme A: cholesterol acyltransferases (ACAT)

Acyl-coenzyme A: cholesterol acyltransferase (ACAT; EC 2.3.1.26) catalyzes the conversion of cholesterol to cholesteryl esters (CE) [63], as illustrated in Figure 2. ACAT is part of a family containing also DGAT (diacylglycerol acyltransferase) [64], and MGAT (monoacylglycerol acyltransferase) [65]. The two isoforms of ACAT identified in mammals are differentially expressed: ACAT1 is ubiquitously expressed while ACAT2 is secreted by the liver and small intestine [66]. Increased ACAT1 expression was detected in macrophage-derived foam cells in human atherosclerotic lesions, revealing its contribution to the foam cell formation during atherogenesis [67]. Considering that ACAT1 inhibition prevented the macrophages foam cells formation and thus exerted protective effects against atherosclerosis, several ACAT inhibitors have been synthesized. It was reported that CI-976 ACAT inhibitor was effective in reducing the accumulation of CE in the atherosclerotic plaques of Yucatan micropig [68]. Likewise, the ACAT inhibitor avasimibe reduced atheroma in rabbits [69] and in apoE3-Leiden mice [70] and F-1394 inhibitor markedly decreased early or advanced atherosclerotic plaques in apoE-deficient mice [71,72]. Despite the promising results noticed in animals, negative results have been obtained in clinical trials. Thus, in A-PLUS (Avasimibe and Progression of Lesions on UltraSound) trial, avasimibe caused a mild increase in LDL-cholesterol and did not positively alter coronary atherosclerosis [73]. In another study, pactimibe treatment of patients with coronary atherosclerosis also failed to reduce the volume of atherosclerotic plaques [74]. These negative results may be due to the lack of selectivity for ACAT1 or ACAT2 of avasimibe and pactimibe. To evaluate whether selective ACAT1 inhibition would lead to beneficial effects against atherosclerosis, ACAT1<sup>-/-</sup>/apoE<sup>-/-</sup> and ACAT1<sup>-/-</sup>/LDLR<sup>-/-</sup> mice were generated. The results showed that in both strains of double transgenic mice, ACAT1 deficiency had detrimental effects, resulting in dry eye syndrome, extensive xanthomatosis [75], as well as deposition of unesterified cholesterol in skin and brain [76]. Then, the question of whether macrophage-specific ACAT1 inhibition would be effective in treating atherosclerosis was raised. Using LDLR<sup>-/-</sup> mice lacking ACAT1 in macrophages, Fazio and coworkers noticed that the inability of these cells to esterify cholesterol resulted in a toxic accumulation of free cholesterol and even accelerated atherogenesis [77]. On the other hand, ACAT2-deficient mice were protected against diet-induced hypercholesterolemia and cholesterol gallstone formation due to the lack of CE synthesis in the intestine and consequently a restricted capacity to absorb cholesterol [78,79]. Moreover, these mice were also protected from atherosclerosis when they were crossed with apoE-deficient mice [80]. Interestingly, in ACAT2<sup>-/-</sup>/apoE<sup>-/-</sup> mice a compensatory increase in LCAT (lecithin cholesterol acyltransferase) activity was noticed, explaining the high levels of HDL-CE. Additionally, mice lacking both ACAT2 and LCAT on LDLR<sup>-/-</sup> background were highly resistant to atherosclerosis [81].

The complete absence of CE from the plasma of these ACAT2<sup>-/-</sup>/LCAT<sup>-/-</sup>/LDLR<sup>-/-</sup> mice confirmed that there are only two enzymes that possess the ability to synthesize plasma CE, LCAT and ACAT2, but not ACAT1. Another approach for ACAT2-specific inhibition in the liver was the use of antisense oligonucleotides, which resulted in atheroprotection [82].



**Figure 2:** Isoprenoid metabolism with branching pathways leading to cholesterol / cholesteryl esters biosynthesis or protein prenylation. The main enzymes whose inhibition is beneficial in atherosclerosis are highlighted in red. This metabolic pathway links enzymes involved in multiple processes related to cholesterol metabolism, relevant for atherosclerosis.

The results of the studies in mouse models raised the question of whether ACAT2-specific inhibition will protect against atherosclerosis in humans. Hence, the development of selective ACAT2 inhibitors is of utmost importance, but remains a challenge.

### Lecithin cholesterol acyltransferases (LCAT)

Lecithin cholesterol acyltransferase (LCAT; EC 2.3.1.43) is a plasma enzyme playing a key role in the production of plasma CE and in the formation and maturation of HDL particles. LCAT promotes the reverse cholesterol transport (RCT), by which excess cellular cholesterol is removed from peripheral tissues by HDL and transported to the liver for excretion [83]. Mutations in the LCAT gene can lead to two very rare autosomal recessive disorders: familial LCAT deficiency (FLD), in which the complete lack of LCAT activity affects both  $\alpha$ -LCAT activity (the esterification on HDL) and  $\beta$ -LCAT activity (the esterification on LDL) [84], and fish-eye disease (FED), in which only  $\alpha$ -LCAT activity is reduced [85]. Both disorders are characterized by very low plasma HDL levels and cholesterol deposits in the cornea which cause corneal opacities. Patients with FLD may develop hypertriglyceridemia, as well as renal dysfunctions. Different studies have reported increased [86] or decreased [87] LCAT activity in patients with coronary atherosclerosis confirmed by angiography. The importance of LCAT in the protection against atherosclerosis, as well as the impact of LCAT mutations on HDL-cholesterol levels is highlighted by the study of Hovingh et al. that found enhanced atherosclerosis in heterozygotes for LCAT gene mutations as compared to controls [88]. Increasing the LCAT activity was proposed as a promising anti-atherosclerotic strategy, at least in subjects with

low LCAT levels. In a 25 year follow-up study, vascular abnormalities and an atherogenic lipid profile were detected in heterozygotes for LCAT deficiency [89]. In contrast, Calabresi et al. reported that low HDL levels in the Italian carriers of LCAT mutations, with total or partial LCAT deficiency, did not result in premature atherosclerosis [90]. An interesting clinical case supporting the paradoxical association between LCAT deficiency, low HDL levels, and absence of subclinical atherosclerosis was recently reported [91]. An explanation for these contradictory findings has been suggested, such as possible involvement of other proteins that could alter the effect of LCAT deficiency on atherosclerosis. As a potential therapy for FLD, Rousset et al. demonstrated the feasibility of the infusion of human recombinant LCAT into LCAT-deficient mice which increased cholesterol efflux and restored the normal lipoprotein phenotype [92]. Despite the numerous studies in animal models and humans focusing on LCAT, well reviewed in ref. [93], its precise role in atherogenesis remains to be clarified. Hence, recombinant LCAT as well as recently developed activators of LCAT may help to answer the question whether increasing LCAT activity is a valuable anti-atherosclerotic strategy.

### Lipoprotein-Associated Phospholipases A2

Lipoprotein-Associated Phospholipase A2 (Lp-PLA2; EC 3.1.1.47) is an enzyme mainly synthesized by the inflammatory cells, and it is transported in the blood mostly on circulating LDL particles and to a lesser extent on HDL and remnant lipoprotein particles (reviewed in ref. [94]). Lp-PLA2 was discovered as platelet-activating factor (PAF) acetyl-hydrolase due to its ability to catalyze the degradation of PAF by hydrolysis of the acetyl group at the sn-2 position, yielding the biologically inactive products, lyso-PAF and acetate [95]. Due to the degradation of proinflammatory PAF and indirect inhibition of platelet activation, Lp-PLA2 was initially considered as an anti-inflammatory protein. Thus, it was reported that overexpression of human PAF acetyl-hydrolase (induced by adenovirus-mediated gene transfer) significantly reduced the macrophages recruitment to the atherosclerotic lesion in the aortic root of apoE<sup>-/-</sup> mice [96]. Likewise, human PAF acetyl-hydrolase gene transfer in apoE<sup>-/-</sup> mice decreased the oxidized lipoproteins accumulation, inhibited neointima formation and atherosclerosis [97]. However, increasing evidence support a pro-atherogenic role for this enzyme that hydrolyzes oxidized LDL particles generating lyso-phosphatidylcholine and pro-inflammatory oxidized fatty acids, which can amplify the atherogenic process. Increased expression of Lp-PLA2 by macrophages was detected in human and rabbit atheroma [98]. An association of Lp-PLA2 expression in vulnerable and ruptured atherosclerotic plaques was also revealed [99]. Moreover, increased Lp-PLA2 activity was positively associated with coronary heart disease [100], suggesting the modulation of the enzyme activity as a potential target in cardiovascular disorders.

Based on the hypothesis that the inhibition of Lp-PLA2 activity may have beneficial effects, a lentiviral-mediated RNAi approach was used to knock-down Lp-PLA2 expression in apoE-deficient mice [101]. The results showed that Lp-PLA2 RNAi reduced inflammation and attenuated the development of atherosclerotic plaques [101]. In diabetic and hypercholesterolemic swine, darapladib, a selective inhibitor of Lp-PLA2, significantly reduced plasma and lesion Lp-PLA2 activity and inhibited the progression of advanced coronary atherosclerosis [102]. Also, A-002 (Varespladib), an inhibitor of secretory Lp-PLA2, reduced lipid accumulation in the aorta and the development of diet-induced atherosclerosis in guinea pigs, despite

any changes in plasma lipids [103]. In spite of the positive effects of Lp-PLA2 inhibitors in mice and pigs, clinical trials that evaluated the darapladib efficiency gave disappointing results. Thus, in the STABILITY study (“Stabilization of Atherosclerotic Plaque by Initiation of Darapladib Therapy”), darapladib failed to diminish the risk of cardiovascular death in patients with stable coronary heart disease [104]. In the SOLID-TIMI 52 study, this pharmacological inhibitor failed to reduce the risk of major coronary events in patients with recent acute coronary syndrome events [105]. In another trial, varespladib treatment increased the risk for myocardial infarction, leading to early termination of the VISTA-16 trial [106].

Recently, the exomes of 6325 participants were sequenced in the “Atherosclerosis Risk in Communities” study in order to find genetic variants of PLA2G7 gene encoding for Lp-PLA2 that reduce its activity and the results of this study showed no association between loss-of-function variants and cardiovascular risk [107].

### Proprotein convertase subtilisin/kexin type 9 (PCSK9)

Proprotein convertase subtilisin/kexin type 9 (PCSK9; EC 3.4.21.111) is a serine protease important for cholesterol homeostasis via hepatic degradation of LDLR [108]. Mutations of PCSK9 are responsible for autosomal dominant hypercholesterolemia, the third hypercholesterolemia-associated genetic locus, after LDLR and ApoB. PCSK9 binds to the epidermal growth factor-like repeat A (EGF-A) domain of LDLR, inducing its degradation, with subsequent decreased metabolism of LDL-cholesterol and hypercholesterolemia [109]. Interestingly enough, the gene was initially identified as being involved in the differentiation of cortical neurons [110]. Since its discovery, a little over a decade, inhibitors of this enzyme have already entered Phase III trials as therapeutic agents for lowering cholesterol level [1]. These inhibitors belong to several classes: monoclonal antibodies (alirocumab, evolocumab), peptide mimetics of the EGFA domain of the LDLR, gene silencers, and small molecules. Safety issues are under research.

### Oxidative Stress Related Enzymes

Reactive oxygen species (ROS) production in limited amounts is required to maintain the physiological redox balance. Oxidative stress appears when ROS levels exceed the detoxifying capability of the cells, and it has deleterious effects on the cellular components, leading to abnormal physiological function and cell death. Oxidative stress exacerbates atherosclerosis. In the vasculature, the major ROS-producing enzymes are represented by NADPH oxidases, cyclooxygenases, lipoxygenases, dysfunctional - “uncoupled” enzymes - endothelial NO synthase (eNOS) and xanthine oxidase, and the mitochondrial respiratory chain [111]. NADPH oxidase, besides its primary function in ROS production, can induce eNOS uncoupling as well as xanthine oxidase activation [112]. Thus, dysfunctional xanthine oxidase and eNOS become ROS-generators only when they are provided with an external source of ROS (e.g. NADPH oxidases), becoming secondary providers of ROS [113]. Besides the ROS producers, there are the enzymatic ROS scavengers, represented by superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), heme oxygenase, paraoxonase (PON), thioredoxin, glutaredoxin, and peroxiredoxin, etc. These enzymes play important roles in maintaining the normal redox status, and thus may be targeted in atherosclerosis. In this review, we will focus on the main ROS producers (NADPH oxidase and Nitric Oxide Synthase), and on some ROS scavengers (SOD, catalase, GPx, heme oxygenase and PON). Cyclooxygenase and

lipoygenase will be discussed in section 6, since their function is mainly related to the biosynthesis of inflammatory mediators with important role in atherosclerosis development.

### NADPH oxidase

NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase, EC 1.6.3.1) is a membrane-bound enzyme complex. There are five isoforms of NADPH oxidase, named from NOX1 to NOX5. NOX is formed by five subunits: a membrane-associated cytochrome b558, containing p22phox and gp91phox, and three cytosolic regulatory subunits, p40phox, p47phox, and p67phox; there are also two different homologs: Nox organizer 1 (Noxo1), a p47phox analog, and Nox activator 1 (Noxa1), a p67phox analog. NOX activation also involves GTP-binding protein, Rac1/2 or Rap 1A. The structure and function of NADPH oxidases are presented in detail by Manea [114]. The cells involved in atherosclerosis express NADPH oxidases. Endothelial cells express four NADPH oxidase isoforms, out of which NOX1, 2, and 5 trigger endothelial dysfunction and vascular disease, and NOX4 generates hydrogen peroxide and exerts protective effects on the vessel wall [115]. In macrophages, Nox1 and Nox4 are inducible and mediate LDL oxidation [116]. Recently, Nox5 was revealed to be constitutively expressed in human monocytes and induced in macrophages by IFN $\gamma$  and oxidized LDL, with implications on atherogenesis [117]. SMCs express Nox1, Nox4, and Nox5 and a low Nox2 level and adventitial fibroblasts contain primarily Nox2 and Nox4 [118].

Pharmacological data showed that NOX1- and NOX4 inhibitor (GKT136901) decreased ROS production and atherosclerosis [119,120]. Noxa1 represents a potential pharmacological target for the modulation of ROS production in atherosclerotic arteries [119]. Studies of pharmacological inhibition NOX1/2 using apocynin showed that aortic atherosclerotic lesions of apoE $^{-/-}$ /LDLR $^{-/-}$  double transgenic mice were decreased, especially on descending aorta, but not in the aortic arch [121].

These data corroborated with other studies on two double knockout mice models: apoE $^{-/-}$ /p47phox $^{-/-}$  and apoE $^{-/-}$ /Nox2 $^{-/-}$  double knockout mice [122-124] which indicated that the atherosclerotic lesion formation on the aortic sinus was not affected. These data suggest that NOX2 effect on atherogenesis is different in various regions of the vasculature. However, other data from the literature showed that apoE $^{-/-}$ /p47phox $^{-/-}$  mice were protected from atherosclerosis as compared to the apoE-deficient mice, and the atherosclerotic lesions of the whole aorta (including the aortic arch) were diminished [120,123,125].

Contradictory data were also reported for Nox1 $^{-/-}$ /apoE $^{-/-}$  mice maintained on a high-fat diet. Sheehan and co-workers reported that these double knockout mice developed fewer and smaller atherosclerotic lesions [126], while another group determined that the lesions were even larger than those found on apoE $^{-/-}$  mice [115].

### Nitric Oxide Synthase

Nitric Oxide Synthase (NOS; EC 1.14.13.39) catalyzes the nitric oxide (NO) production from L-arginine. The endothelial NOS (eNOS) and the neuronal NOS are constitutively expressed and the inducible NOS (iNOS) is biosynthesized in pathological states such as heart failure [127]. At the level of the vasculature, NO is involved in homeostasis, having vasodilator, antiproliferative and antiplatelet actions, as recently reviewed in ref. [128]. Endothelial NOS is mainly located in endothelial vesicles, named caveolae [129], localization that

confers a controlled activation, through the interaction with caveolin-1 [130]. Our previous data showed that eNOS gene polymorphism (eNOSG894T) is a risk factor for cardiovascular diseases and hypertension, but also in other maladies accompanied by endothelial dysfunction such as Fabry's disease [7].

Dysfunctional eNOS ("uncoupled" eNOS) was observed in different pathological states associated with the oxidative stress. In this state, the oxygen reduction is uncoupled from NO synthesis as recently explained by Heltianu et al. and Takimoto et al. [131,132]. Many experimental trials were done to recouple NOS in cardiovascular diseases including: administration of tetrahydrobiopterin BH4 (to block upstream ROS production), folic acid (to recycle dihydrobiopterin into BH4) and L-arginine (to supplement the NOS substrate) [132-134].

Experimental studies were performed on animal models using adenovirus-mediated iNOS gene transfer at the site of vascular lesion. The consequent local NO synthesis induced significant proliferation of smooth muscle cells and intimal hyperplasia in rats, processes with important impact on atherosclerosis progression [135].

### Superoxide dismutases

Superoxide dismutases (SOD; EC 1.15.1.1) catalyze the dismutation of the superoxide radical into molecular oxygen and hydrogen peroxide. SOD family includes three isoforms expressed in different cellular compartments: cytosolic Cu/Zn SOD1, mitochondrial Mn SOD2 and Cu/Zn extracellular SOD3 [136]. Despite the fact that extracellular SOD genetic deficiency was found to have no influence on atherogenesis in apoE deficient mice [137], SOD local delivery decreased the cuff-induced arterial neointima [138], accelerated the endothelial recovery and inhibited in-stent restenosis in the aorta of Watanabe hyperlipidemic rabbits [139]. Both knockout [140] or overexpression [141] studies showed that mitochondrial SOD protected apoE-deficient mice against atherosclerosis. Dietary supplements up-regulated SOD expression in apoE-deficient mice and attenuated atherosclerosis [142]. Utilization of synthetic SOD mimetics over the native Cu/Zn SOD enzyme alleviated endothelial dysfunction in apoE deficient mice. The search for improved cell permeability and stability is expected to lead to the development of more potent drugs [143].

### Catalases

Catalase (CAT, EC 1.11.1.6) is located in peroxisomes where it reduces H $_2$ O $_2$  to H $_2$ O and O $_2$ . In apoE $^{-/-}$  mice, systemic overexpression of catalase retarded the development of atherosclerosis, which is further decreased by combined overexpression of Cu/Zn-SOD and catalase [141]. In apoE-deficient mice, dietary supplements upregulated CAT expression and reduced atherosclerosis [144,145]. CAT is also powerful in the attenuation of non-dietary atherosclerosis in apoE-deficient mice. Thus, its overexpression in apoE mice exposed to the polycyclic aromatic hydrocarbon benzopyrene, alone or in association with Cu/Zn-SOD, led to increased protection as compared with those overexpressing only Cu/Zn-SOD [146].

Although antioxidant enzymes are important therapeutic agents for relieving the vascular oxidative stress, their rapid elimination from the bloodstream compromises their effects. Therefore, various chemical

and gene delivery methods were envisioned [147,148], to maximize their anti-atherogenic potential.

### Glutathione peroxidases

Glutathione peroxidases (GPx; EC 1.11.1.9) are enzymes with peroxidase activity, playing a protective role against the oxidative damage. GPx reduce lipid hydroperoxides to the corresponding alcohols and catalyze the conversion of hydrogen peroxide to water. Until present, eight isoforms (GPx-1 to GPx-8) were identified in humans. In the vasculature, GPx deficiency has implications in atherogenesis. The double knockout mouse model (GPx-1<sup>-/-</sup>/apoE<sup>-/-</sup>) developed significantly more pronounced atherosclerotic plaques compared to the apoE<sup>-/-</sup> control [149]. However, GPx-1 deficiency could not induce atherosclerosis in high-fat diet fed C57BL/6 mice [150]. In humans, decreased GPx-1 activity in red blood cell was found to be related to multivascular atherosclerosis and an augmented cardiovascular risk [151]. Diphenyl diselenide, an organoselenium compound with GPx mimetic abilities was found to inhibit human LDL oxidation *in vitro* [152] and to reduce the formation of atherosclerotic lesions in hypercholesterolemic LDLR knockout mice [153]. Therefore, it is considered that diphenyl diselenide has antiatherogenic actions by modulating intracellular signaling pathways related to antioxidant and anti-inflammatory responses. GPx-1 role in many diseases and speculations on potential future therapies is reviewed Lubos et al. [154].

### Heme oxygenase-1

Heme oxygenase (HO; EC 1.14.99.3) is an enzyme that catalyzes, in a rate-limiting reaction, the NADPH-dependent degradation of heme, with the production of biliverdin, iron, and carbon monoxide. The beneficial roles exerted by these products of heme catabolism, once believed to be toxic, are nowadays brought to light: CO is a regulator of vascular tone, bilirubin is a potent anti-oxidant, while iron increases the production of the cytoprotective ferritin [155]. Three isoforms of the enzyme were described so far: HO-1, HO-2 and HO-3. Of these isoforms, HO-2 and HO-3 are constitutively expressed, while HO-1 is inducible as a stress response to insults as hypoxia, hyperoxia, endotoxins. Reports of the rare and lethal genetic HO-1 deficiency in humans described severe dysfunctions as intravascular hemolysis and endothelial and renal damage [156] or asplenia and inflammation [157]. HO-1 null mice are characterized by retarded growth, anemia, susceptibility to stress, either oxidative or LPS-induced [158,159]. Hypoxia induces apoptosis in cardiomyocytes from heme oxygenase-1 null mice [160] and also compromises the post-ischemic reperfusion response in heterozygous HO-1 mice [161]. HO-1 has become an especially interesting target for atheroprotection since the ablation of heme-binding transcription repressor Bach1 in apoE-deficient mice, which leads to the overexpression of HO-1 in endothelium, macrophages, and vascular SMCs, suppresses atherosclerosis in Bach1/apoE double knockout mice [162]. This effect was abrogated by Sn protoporphyrin, an inhibitor of HO activity [163]. HO-1 induction in LDLR-deficient mice by intraperitoneal injections of hemin (H group) or hemin and desferrioxamine (HD group) led to significantly smaller atherosclerotic lesions in the proximal aorta upon feeding a high-fat diet, whereas the group treated with Sn-protoporphyrin presented larger lesions compared with the control group, which received saline only [164]. Another inducer of HO-1, the synthetic phenolic compound butylated hydroxyanisole, was shown to reduce the proliferation of vascular SMCs via p38/MAPK pathway, being thus a

therapeutic compound useful in atherosclerosis [165]. Certain less toxic dietary antioxidants also induce HO-1. Besides the pharmacological modulation by inducers of HO-1 gene expression, which sometimes may be affected by certain polymorphisms in the promoter region, alternative strategies may be used for increasing HO-1 activity, such as gene delivery or exogenous delivery of HO-1 products (CO and bile pigments). For a comprehensive overview of HO-1 targeting, the reader is directed to Durante [166].

### Paraoxonases

Paraoxonases (PONs; EC 3.1.8.1) are antioxidant enzymes that degrade bioactive oxidized lipids, playing a clear antiatherogenic role. PONs is present in three forms: PON1 and PON3, which are structurally and functionally related to HDL particles in the plasma, and PON2, an intracellular enzyme with a wide cellular distribution [167]. Besides antioxidant properties, PONs have important cardioprotective characteristics, including positive effects on macrophage cholesterol metabolism (PON1), reduction of macrophage triglyceride accumulation (PON2), as well as improvement of bile acids metabolism (PON3). The most studied paraoxonase is PON1, which has been shown to exert protective effects against lipoprotein oxidation, in particular HDL oxidation [168]. PON1 prevents the accumulation of oxidized LDL and promotes the cholesterol efflux from macrophages. Interestingly, PON1 was found to be able to freely exchange between HDL particles and cell membranes, exerting its antioxidant effects in the new location [169]. A landmark study indicating the atheroprotective role of PON1 is the generation of PON1-deficient mice, which are more susceptible to atherosclerosis than their wild-type littermates [170]. Additionally, the transgenic expression of human PON1 in mice pointed out the ability of this enzyme to retard or reverse atherosclerosis [171,172]. The proposed mechanisms for their atheroprotective role are the following: a reduction in circulating and aortic oxidized-LDL, a decrease in macrophage oxidative stress and foam cell formation [173], a stimulation of reverse cholesterol transport and a normalization of the endothelial function [174]. However, oxidative stress, as well as high levels of cholesterol, triglycerides and glucose can inactivate PON1. In humans, low levels of serum PON1 increase the susceptibility to coronary heart disease, representing a cardiovascular risk factor [175-177]. Immunostaining studies in human aortas indicated a progressive increase of PON1 expression during atherosclerosis development [178]. A natural substrate for PON1 was found to be homocysteine thiolactone, which is associated with atherogenesis related to hyperhomocysteinemia [179]; therefore an important atheroprotective mechanism of PON1 was proposed to take place through metabolizing homocysteine thiolactone and limiting N-homocysteinylolation.

The possible relationship between PON genetic polymorphisms and coronary artery disease has just been discussed recently [180]. A meta-analysis of the Q192R, L55M, and T(-107)C polymorphisms in the PON1 gene, as well as of the S311C polymorphism in the PON2 gene found a weak association of coronary heart disease with Q192R polymorphism, but no with the other polymorphisms [181].

Different substrates and assays were proposed for serum PON1 measurement, as a biomarker for diagnosis of diseases related with oxidative stress and inflammation, but there are still problems regarding the variety of substrates and the lack of reference methods [182,183].

## Vascular Remodeling Related Enzymes

### Matrix Metalloproteases

Matrix metalloproteinases (MMPs) are cell surface or secreted zinc-dependent endopeptidases that hydrolyze extracellular matrix (ECM) components. Implicated in many biological processes, these proteinases play a central role both in normal tissue functioning (embryogenesis, tissue remodeling, wound healing, angiogenesis, etc.), as well as in various diseases (atheroma, arthritis, cancer, tissue ulceration, etc.). All MMPs have a common structure containing Zn<sup>2+</sup> ion at the catalytic active site. Their activity is controlled by specific endogenous inhibitors called tissue inhibitors of metalloproteinases (TIMPs). In humans, MMP family comprises over 20 endopeptidases, which were classified according to Raffetto and Khalil [184] in the following subgroups:

1. MMP-1, MMP-8, MMP-13 and MMP-18 collagenases cleave interstitial collagens I, II and III and other ECM and non-ECM molecules;
2. MMP-2 and MMP-9 gelatinases digest denaturated collagens, as well as native collagen types I, IV, fibronectin and laminin; MMP9 was previously found to be involved in mammalian bone formation by promoting vascular invasion into the growth plate;
3. MMP-3, MMP-10, and MMP-11 digest ECM components;
4. MMP-7 and MMP-26 matrilysins digest cell surface molecules and ECM components;
5. MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25 membrane-type act on ECM molecules and collagen I, II and III;
6. other MMPs, with a role in macrophage migration and digestion of other proteins.

Some of the MMPs family members were identified to be associated with the atherosclerotic lesions both in human and animal models. Moreover, their contribution in the weakening of the vascular wall of the atherosclerotic lesion is well known. Upon MMP activation, matrix degradation leads to plaque rupture causing unstable angina, myocardial infarction and stroke (via destruction of ECM proteins). The stability of the atheromatous plaque depends on the equilibrium between the biosynthesis and the degradation of ECM. MMPs-1, -2, -3, -7, -9, -11, -12, -13, -14, and -16 are upregulated in macrophages found in the human plaques, while in animals, numerous studies showed that MMPs are also upregulated in the atheromatous plaques of cholesterol-fed rabbits and apoE-deficient mice [185,186]. MMP-1, whose inhibition leads to accumulation of collagen in plaques (correlated with resistance to plaque rupture in humans), was found to be linked to human acute coronary diseases. Poor collagen content is found in the fibrous cap of the ruptured atherosclerotic plaque. MMP-9 expression was enhanced in untreated diabetic *apoE*<sup>-/-</sup> mice and absent in insulin-treated mice [187]. Moreover, MMP-9 was associated with dysfunctional HDL, which are lipoproteins with antiatherogenic functions [188]. MMP-12 is involved in plaque progression and instability, while MMP-14 expression is enhanced in a distinct subpopulation of foam cell macrophages which have TIMP-3 downregulated [189]. EMMPRIN, an extracellular MMP inducer is expressed in human atherosclerotic plaque and up-regulated in *apoE*<sup>-/-</sup> mice, playing a role in the plaque rupture [190,191].

To test whether MMP inhibition has a beneficial effect in atherosclerosis, double-knockout mice of various MMPs on apoE

background were generated. Data obtained on *apoE*<sup>-/-</sup>/*MMP*-2<sup>-/-</sup> mice showed that the area of calcified lesions in aortic intima of double-knockout mice was significantly reduced compared to *apoE*<sup>-/-</sup> mice [192]; in addition, the analysis of aortas from *apoE*<sup>-/-</sup>/*MMP*-2<sup>-/-</sup> mice demonstrated a reduction of osteocalcin and BMP2 expression, which are highly expressed in calcified lesions in *apoE*<sup>-/-</sup> mice. Another study demonstrated that adenoviral overexpression of TIMP-1 in *apoE*<sup>-/-</sup> mouse model reduced the atherosclerotic lesions [193]. A comprehensive study of Rouis and co-workers [193] analyzed the double knockouts *apoE*<sup>-/-</sup>/*MMP*-3<sup>-/-</sup>, *apoE*<sup>-/-</sup>/*MMP*-7<sup>-/-</sup>, *apoE*<sup>-/-</sup>/*MMP*-9<sup>-/-</sup>, and *apoE*<sup>-/-</sup>/*MMP*-12<sup>-/-</sup> double versus *apoE*<sup>-/-</sup> single knockout. The results showed that in *apoE*<sup>-/-</sup>/*MMP*-3<sup>-/-</sup> and *apoE*<sup>-/-</sup>/*MMP*-9<sup>-/-</sup> double knockouts, the brachiocephalic artery plaques were larger than in controls. Lesion size was reduced in *apoE*<sup>-/-</sup>/*MMP*-12<sup>-/-</sup> double knockouts as compared with the controls. Moreover, *apoE*<sup>-/-</sup>/*MMP*-12<sup>-/-</sup> presented increased SMCs number and fewer macrophages in the plaque suggesting a stable phenotype of the atheroma. *ApoE*<sup>-/-</sup>/*MMP*-7<sup>-/-</sup> mice displayed a higher content of SMCs in the atherosclerotic plaques than controls, but did not present a change in the plaque stability. MMP-3 and -9 deficient mice indicated the protective role for these MMPs, while MMP-12 determined increased lesion and plaque destabilization.

The development of therapeutic drugs specifically targeting MMPs is relevant for atherogenesis prevention, but also for the stabilization of the mature plaques. Most of the exogenous synthetic inhibitors generally contain a chelating group designed to bind to the catalytic zinc atom in the enzyme active site. RS-130830, a non-selective MMP inhibitor, showed no positive effect on atherogenesis or on the stability of the plaques in *apoE*<sup>-/-</sup> mice [194]. However, doxycycline, which inhibits MMPs activity, is capable of decreasing gelatinase activity on atherogenesis in apoE-deficient mice [195]. Inhibition of MMP-3, MMP-12, and MMP-13 in LDLR<sup>-/-</sup> mice by the broad-spectrum MMP inhibitor CGS 27023A led to a delay in intimal lesions formation, but did not prevent them. A phosphinic peptide (RXP470.1), which inhibits MMP-12, significantly reduced atherosclerotic plaque and stopped the evolution of the lesions in the brachiocephalic arteries in *apoE*<sup>-/-</sup> mice [196]. It was reported that MMP13i-A, an inhibitor of MMP-13 collagenase, administered in *apoE*<sup>-/-</sup> mice did not diminish the size of the atherosclerotic plaques, did not affect the accumulation of macrophages or smooth-muscle cells in the lesion site, but substantially increased interstitial collagen content in the intima and fibrous cap of the atheroma [197]. Novel MMPs inhibitors are also being considered and recently reviewed in ref. [198].

Another approach targeting MMPs was based on the use of specific antibodies. EMPIRIN antibody-therapy showed an amelioration in the atheroma of *apoE*<sup>-/-</sup> mice, through a mechanism including the reduced migration of monocytes and their accumulation in the plaque [199].

### Inflammation Related Enzymes

Cyclooxygenase and lipoxygenase are involved in the arachidonic acid metabolism, the precursor of the potent lipid mediators, prostaglandins and leukotrienes. These are critical modulators of inflammation, sometimes controversial for atherosclerosis progression [200].



## Cyclooxygenase

Cyclooxygenase (COX), named also prostaglandin-endoperoxide synthase (EC 1.14.99.1) is the enzyme responsible for the formation of prostanoids: prostaglandins and thromboxane A<sub>2</sub> (TxA<sub>2</sub>). There are two COX isoforms (constitutive COX-1 and inducible COX-2), of different genetic origins, which are about two-thirds identical and have nearly identical catalytic sites, but exhibit differences which allowed the development of specific inhibitors [201]. Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit production of prostaglandins by acting on COX-1 and/or 2 COX-2. Nonselective NSAIDs inhibit both isoenzymes (e.g., indomethacin, ibuprofen, naproxen), while selective NSAIDs act on COX-1 (e.g., aspirin) or COX-2 (coxibs, e.g., celecoxib), but their use appeared to be to a certain extent a double-edged sword. While relieving the pain and inflammation, which they were primarily designed for, they also promoted undesired cardiovascular side effects, mainly by compromising the balance between the constitutive vasoconstricting TxA<sub>2</sub> produced by platelet COX-1 and the vasodilating endothelial prostacyclin [202]. The results coming from genetic studies are also subjected to many variables, and therefore sometimes contradictory. Disruption of COX-1 gene in apoE-deficient mice led to reduced platelet-vessel wall interactions and decreased lesion size upon high fat feeding. These effects are more intense when both COX-1 alleles are disrupted, as compared with single COX-1 gene deletion [203]. Both COX-1 and COX-2 were involved in early atherogenesis, as shown by selective macrophage depletion in apoE<sup>-/-</sup> and LDLR<sup>-/-</sup> mice, respectively [204]. Celecoxib, a selective COX-2 inhibitor, reduced the area of the atherosclerotic plaques in apoE<sup>-/-</sup> mice for the duration of the treatment. The protective effect was lost when drug administration was interrupted [205]. On the same trend, in high fat-fed LDLR deficient mice, pharmacological inhibition of COX-2 with either selective (rofecoxib) or nonselective (indomethacin) or genetic macrophage-specific COX-2 ablation reduced atherosclerosis [206]. In contrast, COX-2 postnatal deletion on an apoE-deficient background accelerated atherosclerosis in a gender-independent manner [207]. COX-2 specific deletion in macrophages attenuated inflammation, but not atherosclerosis in apoE-deficient mice [208]. In an elegant tandem of knockout studies, FitzGerald and collaborators showed that, when specifically targeted, macrophage COX-2 was pro-atherogenic, as demonstrated by its specific deletion in LDLR<sup>-/-</sup> mice [209], while endothelial and vascular SMCs COX-2 reduced atherosclerosis in the same murine model [210]. Therefore, a successful therapy would target an anti-inflammatory drug specifically to the macrophage, avoiding other deleterious systemic side effects. A further enhancement could be provided by the use of certain natural COX inhibitors as hyperforin [211] or various flavonoids [212]. Another strategy is the use of dual inhibitors, as in the case of licofelone, a combined lipoxygenase/cyclooxygenase inhibitor which entered Phase III trials [213]. In the end, it is worthwhile to mention that aspirin, an adjuvant for the main atherosclerotic therapy via its anti-thrombotic action by inhibiting platelet COX-1 synthesis of TxA<sub>2</sub>, is sometimes deprived of its effect in the “aspirin resistance” of multiple etiology [214].

## Lipoxygenase

Lipoxygenases (fatty acid:oxygen oxidoreductase; EC 1.13.11.-) catalyze the dioxygenation of polyunsaturated fatty acids in lipids containing a cis,cis-1,4-pentadienic structure. Among the mammalian arachidonate-related enzymes, 5-lipoxygenase (5-LO), and 12/15-

lipoxygenase (12/15-LO) were thoroughly studied in atherosclerosis context [215] in relation to their predominant expression in macrophages. Deficiency of 12/15-LO in bone marrow cells prevented the high-fat induced atherosclerosis in apoE<sup>-/-</sup> mice to the same extent as complete absence of 12/15-LO, despite a high level of peroxidized lipids. 12/15-LO deficient macrophages were unable to promote endothelial activation in the presence of native LDL and had decreased capacity to form foam cells when exposed to LDL [216]. Similarly, murine atherosclerotic lesions were significantly reduced when 12/15-LO deficiency was superposed on a LDLR<sup>-/-</sup> background [217]. The double 5-LO and 12/15-LO deficiency reduces atherosclerosis in apoE-deficient female mice, while males are rather unaffected by LO deficiency [218], these results being consistent with the gender-dependent regulation of leukotrienes synthesis [219]. In the light of the above and other, unmentioned here, studies that point toward a pro-atherogenic role of lipoxygenases, a degree of uncertainty accompanies other studies pointing toward a cardioprotective role [220]. A partial explanation of the noticed discrepancies was correlated with the genetic complexity of 5-LO genetic locus, since a series of chromosomal congenic subregions were shown to affect the size of aortic lesions independently of 5-LO [221].

## Posttranslational Modifications Enzymes Related To Isoprenoid Metabolism

Prenyltransferases posttranslationally modify proteins by adding an isoprenoid (prenyl) group to the carboxyl terminus of the target protein, such that these proteins become membrane-associated. Most prenylated proteins are involved in cellular signaling where membrane association is essential for their function. Prenyltransferases add a farnesyl or geranylgeranyl group to the cysteine residue of proteins having a C-terminal CAAX box. Farnesyltransferase (FTase) recognizes CAAX boxes where X = Met, Ser, Gln, Ala, or Cys, whereas geranylgeranyltransferase I (GGTaseI) recognizes CaaX boxes with X = Leu or Glu [222]. GGTaseI and FTase I are heterodimers with the same a subunit encoded by the FNTA gene, but with different β Zn-containing subunits. Prenylation occurs as a branching pathway of isoprenoid metabolism, while cholesterol biosynthesis is another. Therefore, the two pathways are expected to influence each other. Pathological aspects of prenylations are found in various conditions such as insulin sensitivity, inflammation, cardiac hypertrophy, hepatic lipogenesis [223]. Therefore, development of inhibitors to FTase and GGTase-I would serve therapeutic purposes. The available inhibitors of FTase and GGTase-I can be classified into four categories: (i) small molecules competing with the isoprenoid substrate; (ii) small molecules competing with the protein substrate; (iii) bisubstrate analogs; (iv) Small molecules uncompetitive with any of the substrates [224].

## Farnesyltransferase (Protein farnesyltransferase)

Farnesyltransferase (FTase, protein farnesyltransferase; EC 2.5.1.58) adds the C15- isoprenoid farnesyl to proteins bearing the four amino acid motif CAAX at the carboxyl terminus of a protein, as depicted in Figure 2. FTase targets Ras superfamily of small GTP-binding proteins critical to cell cycle regulation and it was associated with various types of cancer. In mice, FTase knockout is lethal in the embryonic stage, but it is not essential to adult homeostasis. FTase exclusion during tumor progression had a restricted inhibitory effect [225]. The induction of FTase by hyperinsulinemia may partly account for the proliferative and atherogenic effects of insulin [226]. FTase also plays a

significant role in progeria (Hutchinson-Gilford syndrome), a process of accelerated aging in which patients die prematurely due to atherosclerotic complications. This syndrome is caused by a mutation of the nuclear protein lamin A, which leads to the retention of the farnesyl group on the abnormal lamin A protein, progerin and its accumulation in the nucleus. Progerin accumulation is directly involved in the vascular disease associated with the syndrome [227]. FTase inhibition has proved to be beneficial for the alleviation of the condition [228,229]. In the clinical trials of progeric children, FTase inhibitor lonafarnib improved vascular function, bone structure, and audiological status [230]. Manumycin A, a farnesyltransferase inhibitor, prevented atherosclerosis development in apoE-deficient mice fed a Western diet, independently of cholesterol level. Treated animals displayed decreased Ras activity and reduced oxidative stress [231]. FTase inhibitor R115777 hindered protein prenylation in mice with chronic renal failure, with both local and systemic effects: it decreased aortic atherosclerotic lesion area, calcification, vascular nitrotyrosine, total collagen and collagen type I content, restored the normal serum levels of apolipoprotein IV,  $\alpha$  globin and fetuin A. In vascular smooth muscle cells, R115777 inhibited type I collagen synthesis and reduced mineral deposition through Ras-Raf pathway [232]. FPT III, a FTase inhibitor, was found to reduce in-stent restenosis by a new, Ras-independent pathway in rabbit models [233]. It was found that TR006, an analog of FTase substrate farnesyl pyrophosphate, which strongly inhibited FTase enzyme activity, blocked the human smooth muscle cells in culture and inhibited growth factor-induced DNA synthesis [234]. Combinations of statins and certain FTIs may exert a concerted action in preventing excessive cell proliferation. Thus, lovastatin and the FTase inhibitor 3-allylfarnesol acted synergically to up-regulate and relocate RhoB from the membrane to cytosolic compartments in A10 vascular smooth muscle cells [235]. It is expected that combinations of statins and FTase inhibitors may have therapeutic potential in overproliferation.

### Geranylgeranyltransferase-I

Geranylgeranyltransferase-I (GGTase I, EC 2.5.1.59) is a heterodimer of two subunits:  $\alpha$  and  $\beta$ , encoded by the genes FNTA and PGGT1B, respectively. GGTase I adds the C20-isoprenoid geranylgeranyl group to CAAX-proteins, one of the targets being the RHO proteins (Figure 2). GGTase I inhibitors are being investigated for their therapeutic value in various pathological conditions as inflammation, multiple sclerosis, atherosclerosis. An extensive review of GGTase-I inhibitors and their classification on structural grounds as natural products, peptide substrate, terpene substrate, etc. is recommended for further information [236]. Cholesterol-fed LDLR<sup>-/-</sup> mice in which GGTaseI was specifically ablated in the macrophages exhibited increased RHO-mediated cholesterol efflux, stimulated macrophage reverse cholesterol transport and reduced aortic atherosclerotic lesions, in spite of a strong inflammatory response [237]. The selective inhibitor GGTI-286 increased the TNF $\alpha$ -mediated overexpression of the endothelial cell adhesion molecule E-selectin, resembling the effect of simvastatin [238]. The same inhibitor GGTI-286 was effective in suppressing phosphatidylcholine hydroperoxide-induced THP-1 cell adhesion to ICAM-1 by interfering with cytoskeletal actin reorganization induced through Rac small GTPase activation [239]. Importantly, inhibition of geranylgeranyltransferase but not inhibition of farnesyltransferase significantly decreased dendritic cells invasion, similarly to atorvastatin. This effect that regulates the interaction between

dendritic and endothelial cell may have relevance to inflammation and atherogenesis [240].

### Epigenetic Modifications Related Enzymes

Epigenetic changes, affecting both DNA and histones, emerged recently as therapeutic targets in the field of atherogenesis [241]. Out of the many types of modifications, only some of them will be reviewed below.

#### Histone deacetylases

Histone deacetylases (HDAC; EC 3.5.1.98) are enzymes that remove the acetyl groups from  $\epsilon$ -N-acetyl lysine residues of the histones. This modification allows a tighter interaction of the histones with the DNA, and, consequently, it suppresses transcription. Their action is opposed by histone acetyltransferases. Since lysine deacetylation also modulates the activity of other non-histone proteins, HDAC proteins are now considered a subclass of lysine deacetylases (KDAC) [242]. To date, there are 18 mammalian HDACs, which are grouped on structural grounds into four classes. Classes I, II and IV form the Zn-dependent family, while class III (sirtuins) are NAD-dependent enzymes. Other differences relate to the subcellular localization, with most of them nuclear, but others with nuclear-cytosolic shuttling capacity [243]. Histone deacetylase inhibitors were mostly developed to target the tumor evolution [244], three inhibitors: vorinostat (SAHA), Romidepsin and Belinostat being already approved by the US-FDA [245]. However, there is an impressive amount of experimental evidence regarding the involvement of HDACs in cardiovascular pathology, which has been recently reviewed in correlation with the stages of atherosclerosis progression [246]. A different reviewing approach has been used by Lehmann et al., which emphasized the murine cardiac phenotype associated with the ablation of specific HDAC class I (high deacetylase activity) and II (low deacetylase activity), since it appears that each HDAC is endowed with a distinct function in cardiac pathophysiological remodeling [247]. For example, homozygous cardiac-specific HDAC 1 and 2 deletion induced cardiac arrhythmias, dilated cardiomyopathy leading to neonatal lethality [248]. In contrast, transgenic cardiac overexpression of HDAC3 promoted hyperplasia [249]. Therefore, targeting HDAC with pan-specific inhibitors may not be a useful approach. HDAC potential in targeting inflammation and other atherogenic events is however increasingly explored, and HDAC pharmacological challenge with inhibitors is claimed to have anti-inflammatory properties and antifibrotic effects in the cerebro- and cardiovascular system [250]. Very recently, it was shown that oral administration of the histone deacetylase inhibitor panobinostat to HIV-infected adults on suppressive antiretroviral therapy in a sub-study of a single-arm, phase I/II clinical trial, led to a significant suppression of HIV-associated inflammation and atherosclerosis. This conclusion was drawn upon evaluation of a panel of cardiovascular risk biomarkers (C-reactive protein, matrix metalloproteinase 9, soluble CD40 ligand, interleukin-6, soluble endothelial selectin) [251]. Cell-specific targeting may increase the chances of therapeutic success through a limitation of the systemic side effects. Thus, endothelial HDAC3 is as an essential prosurvival molecule via Akt activation and conditional Hdac3 knock down in apoE-deficient mice that leads to vessel rupture and advanced atherosclerosis [252]. In contrast, conditional myeloid Hdac3 deficiency promotes collagen deposition in atherosclerotic lesions, stabilizing the plaque and increases macrophage capacity to cope with inflammation and lipid processing [253].

## DNA methylases

DNA methyltransferases (DNMT) catalyze the reaction implying the transfer of a methyl group to DNA. DNA methylation serves a wide variety of biological functions. An in-depth review of the mammalian DNMT family covering all the structural and mechanistic details is highly recommended to the reader [254]. DNMT1 (EC 2.1.1.37) is the key member of the family. All known DNMTs use S-adenosyl methionine as the methyl donor. Increased levels of homocysteine, a marker of cardiovascular disease, lead to an elevation of S-adenosylhomocysteine, a potent inhibitor of methyltransferases throughout the whole genome [255], thus creating a molecular connection between homocysteine and atherosclerosis. Arterial branching points affected by the disturbed blood flow are preferred sites for atherosclerotic plaque development. Recent studies reported that blood flow hydrodynamics controls endothelial gene expression via DNMT1-catalyzed DNA methylation, which suppresses various transcription factors like the cAMP-responsive HoxA5 and KLF3. DNMT inhibitor 5-Aza-2'-deoxycytidine (5Aza) or DNMT1 siRNA reduces shear stress-induced inflammation. Moreover, 5Aza reduces atherosclerosis in apoE<sup>-/-</sup> mice [256]. It was shown that homocysteine-induced apoptosis in human umbilical vein endothelial cells proceeds via DNA methylation mediated by DNMT1, and treatment with the DNMT-1 inhibitors epigallocatechin-3-gallate or 5-Aza prevented cell death [257]. Both histone- and DNA-methylases were implicated in the LDL-induced repression of KLF2 transcription in endothelial cells. LDL-induced endothelial dysfunction effects were prevented by pharmacological inhibition or gene silencing of DNMT [258]. DNA methylation was also implicated in the reduced autophagy of THP-1 macrophages upon exposure to oxidized LDL (oxLDL) [259]. Additionally, it was shown that oxLDL-induced epigenetic suppression of FOXP3 down-regulated T regulatory cells, subsequently increasing the risk of acute coronary syndrome [260]. OxLDL raised significantly MMP-2/MMP-9-mediated migration of human aortic SMCs by epigenetic effects mediated by miRNA [261].

## Histone methyltransferases

Histone methyltransferases (histone-lysine N-methyltransferase and histone-arginine N-methyltransferase; EC 2.1.1.43) catalyze the transfer of one to three methyl groups to lysine/arginine residues of histones [262]. The cofactor S-adenosyl methionine is the methyl donor group for both types of histone methyltransferases. Histones methylation is the main chromatin epigenetic modification responsible for gene expression, genomic stability, development, mitosis. The involvement of histone methylation in atherosclerosis was demonstrated in the pro-atherogenic apoE-deficient mice. Histone methylation is affected both by the genetic environment and exogenous factors such as diet, as revealed by the different pre- and post-natal methylation patterns in vascular endothelial and SMCs of apoE<sup>-/-</sup> mice, as compared with wild-type controls [263]. Reduced H3 histone methylation in peri-renal aortic tissue from all stages of atherosclerotic human patients was recently reported [264]. Accelerated atherosclerosis by high levels of S-adenosyl homocysteine in the plasma was associated with the activation of endoplasmic reticulum stress through modulation of histone methylation [265]. Epigenetic changes involving both histone deacetylases and methyltransferases were involved in the transcriptional control of collagen type I gene in vascular SMCs in response to IFN- $\gamma$ , leading to repression of collagen synthesis, with subsequent impact on plaque

destabilization [266]. In monocytes, the proinflammatory phenotype induced by oxidized LDL also involves epigenetic changes, revertible by the histone methyltransferase inhibitor methylthioadenosine [267].

## Conclusion

In this review, we have tried to outline the main enzymes connected with the evolution of atherosclerosis, based on their association with a particular event or metabolic pathway (summarized in the supplemental Table 1). Definitely, we were unable to cover, even superficially, in a limited space, all the significant enzymes and, not willingly, some may have been left out. This is a huge domain to describe, and, certainly, an open one, as new enzymes may be discovered, like PCSK9, which evolved in a decade as an important therapeutic target with inhibitors in Phase III trials. With the advent of molecular biology, the last decades acknowledged an increased understanding, at the finest structural level, of the mechanistic basis of action and offered the opportunity for exciting pharmacological approaches in the design of inhibitors. Few final remarks are due at this point. The main achievements in the field still emerged from the direct targeting of cholesterol metabolism, either from the biosynthetic pathway (statins) or the transport processes (PCSK9 inhibitors). An important aspect to be reiterated is that the latter enzyme was initially discovered in connection with neurobiology. It is possible in the future to uncover new functions of enzymes already known. A particular attention needs to be paid to certain genetic diseases, in which atherosclerosis develops indirectly, as in the case of progeria. Also, the sphingolipids are coming toward the front of the stage, as critical players, not simple bystanders. From the cellular types involved, macrophage contribution is crucial, both to lipid processing and to inflammation, as macrophage-specific knockouts of certain genes can revert the effect of a general knockout in a murine atherosclerosis model. From the studies reviewed above we can conclude that there is a long and not always straight way for confirming the *in vivo* therapeutic potential of an enzyme modulator proved beneficial *in vitro*. The challenge is enhanced by the multifactorial genesis of atherosclerosis. There seem to be no "magic bullet" to effectively target the disease, but rather a synergy of complementary approaches. As science progresses, novel approaches will emerge. Among these, targeting the main enzymes involved in the atherosclerotic evolution by genetic or pharmacological means remains a source of high expectations. However, neither the genetic nor the pharmacological approach has to be evaluated on its own. A correct appreciation should take both into account and, more importantly, place the experimental data in a significant biological context. From this review and, moreover, from the plethora of data in the literature, one can understand the importance of choosing a proper therapeutic target. To this aim, many aspects should not be neglected, among them: the existence of variants of different or common genetic origin; the spatiotemporal distribution; the existence of natural inhibitors, endogenous and exogenous; the cross-talk between the various metabolic pathways, with their regulation; the lesional stage at which a drug is expected to act. Extrapolation from *in vitro* and *in vivo* models to clinical trials has proved disappointing many times, and in certain cases, never too rare, dangerous. The sooner pitfalls and misinterpretations will be detected, the better both for the benefit of people and to reduce the economic burden associated with death end-clinical trials.

	Enzyme	Athero-protective(+)/ atherogenic (-)	Representative experimental data (with corresponding references)
Cholesterol biosynthesis related enzymes	HMG-CoA reductases	(-)	Inhibited by statins, which reduce LDL-cholesterol [5,6]
	Squalene synthase	(-)	Inhibited by TAK-475, which reduces LDL-cholesterol [20]
Sphingolipid metabolism related enzymes	Serine palmitoyl transferase	(-)	Inhibition by myriocin led to increased HDL, decreased VLDL and LDL in apoE <sup>-/-</sup> mice [26]
	Sphingomyelin Synthase	(-)	Knockout of SMS1 [43] or SMS2 [39] decreased pro-atherogenic NF-κB activation and reduced atherogenic lipoproteins.
	Sphingomyelinase	(-)	Acidic SMase knockout reduced lipid retention and atherosclerotic lesions [53]; neutral SMase knockout reduced palmitate-induced inflammation [61]; alkaline SMase knockout blocked intestinal cholesterol absorption [62]
Lipoprotein metabolism related enzymes	ACAT1, ACAT2	(-)	ACAT2 knockout reduced cholesterol absorption, hypercholesterolemia and gallstones formation [78, 79]; ACAT2 <sup>-/-</sup> /apoE <sup>-/-</sup> mice were highly resistant to atherosclerosis [80]
	LCAT	(+)	Increased LCAT expression by infusion increased cholesterol efflux and normalized lipoproteins in LCAT <sup>-/-</sup> mice [92]
	Lp-PLA2	(-)	Inhibition by RNAi [101] and selective inhibitors [102,103] reduced lipid accumulation and progression of coronary atherosclerosis in various atherogenic experimental models
	PCSK9	(-)	Inhibition by alirocumarib reduced LDL levels [1]
Oxidative stress related enzymes	NADPH oxidase	(-)	NOX1/4 inhibitor GKT136901 reduced ROS [119,120]; NOX1/2 inhibitor apocynin reduced aortic lesions in apoE <sup>-/-</sup> LDLR <sup>-/-</sup> mice [121]
	Nitric Oxide Synthase	(+)	Adenovirus-mediated iNOS gene transfer studies showed that local NO synthesis induced significant proliferation of SMCs and intimal hyperplasia in rats [135]
	Superoxide dismutases	(+)	SOD local delivery decreased the cuff-induced arterial neointima [138], accelerated endothelial recovery and inhibited in-stent restenosis in the aorta of Watanabe rabbits [139]; mitochondrial SOD protected apoE <sup>-/-</sup> mice against atherosclerosis [140,141]
	Catalases	(+)	Overexpression retarded atherosclerosis development in apoE <sup>-/-</sup> mice [141]; dietary supplements upregulated CAT expression in apoE <sup>-/-</sup> mice and reduced atherosclerosis [144,145]; CAT attenuated non-dietary atherosclerosis in apoE <sup>-/-</sup> mice [146]
	Glutathione peroxidases	(+)	Mimetic diphenyl diselenide reduced atherosclerotic lesions in LDLR <sup>-/-</sup> mice [153]
	Heme oxygenase-1	(+)	Inducers hemin:desferrioxamine reduced atherosclerotic lesions in LDLR <sup>-/-</sup> mice [164]
	Paraoxonases	(+)	Reduced protein N-homocysteinylated, prevented accumulation of oxLDL, promoted cholesterol efflux from macrophages [173], improved endothelial function [174]
Vascular remodeling related enzymes	Matrix Metalloproteases	(-)/(+)	Area of aortic calcified lesions was reduced in apoE <sup>-/-</sup> /MMP2 <sup>-/-</sup> mice compared to apoE <sup>-/-</sup> mice ([192]); apoE <sup>-/-</sup> /MMP-3 <sup>-/-</sup> and apoE <sup>-/-</sup> /MMP-9 <sup>-/-</sup> mice had larger brachiocephalic artery plaques [193], while lesion size was reduced in apoE <sup>-/-</sup> /MMP-12 <sup>-/-</sup> mice [193]
Inflammation related enzymes	Cyclooxygenase	(-)	COX-1 knockout in apoE <sup>-/-</sup> mice reduced platelet-vascular wall interactions and decreased lesions [203]
	Lipoxygenase	(-)	12/15 LO knockout in macrophages impaired oxLDL-induced foam cell formation and endothelial activation [216]
Posttranslational modifications enzymes related to isoprenoid metabolism	Farnesyltransferase	(-)	Inhibition by manumycin A reduced Ras activity and oxidative stress [231]; FPTIII reduced in-stent restenosis in a Ras-independent pathway in rabbit model [233]; 3-allyl farnesol and lovastatin promoted relocation of RhoB to the cytosolic compartment in vascular SMC [235]
	Geranylgeranyl transferase-I	(-)	GGTI-286 inhibitor blocked interaction between monocytes and endothelium [239]
Epigenetic modifications related enzymes	Histone deacetylases	(-)	Panobinostat inhibitor reduced HIV-associated inflammation and atherosclerosis [251]

	DNA methylases	(-)	5Aza, a DNMT inhibitor, reduced shear-stress induced inflammation and atherosclerosis in apoE <sup>-/-</sup> mice [256]
	Histone methyltransferases	(-)	Homocysteine-induced ER-stress can be inhibited by the histone methylase inhibitor methylthioadenosine [267]

**Table 1:** Enzymes used as therapeutic targets in atherosclerosis.

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