



## Review

# Cellular and molecular mechanisms that underlies the formation of atherosclerotic plaque and plaque rupture-review

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### Abstract

Atherosclerosis (AS) is the main risk factor for CVD and manifested by lipid accumulation, extracellular matrix protein deposition, and calcification in the intima and media of the large to medium size arteries promoting arterial stiffness and reduction of elasticity. It is initiated by endothelium activation and, followed by a cascade of events (accumulation of lipids, fibrous elements, and calcification), triggers the vessel narrowing and activation of inflammatory pathways. This review focuses on the different stages of AS development, ranging from endothelial dysfunction to plaque rupture and the role of genetic abnormalities in AS development. In addition, the correlation of monocyte recruitment and atherogenesis, cytokine involvement with the role of phagocytosis in AS, fundamental signaling pathways in multiple stages of AS, and genetics of AS and the molecular mechanisms of plaque rupture and cap formation are covered here to provide a global view of the disease.

**Keywords:** Atherogenesis, cytokines, endothelial dysfunction, oxidative stress and inflammation

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Atherosclerosis (AS) is a chronic arterial disease and the leading causes of CVD which results in a high rate of morbidity and mortality [1]. AS is a disease that is characterized by the accumulation of lipids, fibrous elements, and calcification within the large arteries. This process is initiated by endothelium activation, followed by a cascade of events, which implies the vessel narrowing and activation of inflammatory pathways leading to atheroma plaque formation. Altogether, these processes result in cardiovascular complications that remain as the leading cause of death worldwide [2]. It has been recognized that dyslipidemia participates in the initiation of AS development [1]. First, lipoproteins accumulate in dysfunctional endothelial cell, which causes the initial lipid deposition and inflammatory response in the intima [3]. Second, additional lipids are deposited in the intima, which attracts apoptotic or necrotic cell debris, and all of these deposits are within the vessel wall [4]. Dyslipidemia promotes oxidative stress and the inflammatory response in plaques, such as the secretion of inflammatory cytokines and chemokines [1]. AS is caused by genetic and non-genetic factors and complex gene-environment interactions [5]. Although clinical complications of AS can arise from

plaques causing flow-limiting stenoses, the most severe clinical events follow the rupture of a plaque, which exposes the prothrombotic material in the plaque to the blood and causes sudden thrombotic occlusion of the artery at the site of disruption [5]. To this date, the molecular mechanisms underlying the pathogenesis of AS remain largely unknown. Understanding the molecular and cellular mechanisms that lead to the development of AS are critical for identifying strategies to limit disease progression before it leads to clinical consequences. The aim of this study is to review the mechanism of development of AS, including endothelial dysfunction, fatty streak formation, fibrous plaque formation, and plaque rupture.

## Cellular Processes in Atherosclerotic Lesion

### Initiation of AS

By position and large surface area exposed to the blood, EC are the first cells to experience the impact of any minute perturbation occurring in the blood or interstitial fluid homeostasis [6]. The endothelium has a pivotal role in maintaining vascular homeostasis and in providing a physical barrier between the

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blood components and the subendothelial space [7, 8]. Importantly, endothelial dysfunction represents a common link among all known cardiovascular risk factors (Fig. 1), including dyslipidemia, smoking, diabetes, hypertension, obesity, and mental stress [9]. Several features can characterize endothelial dysfunction, including a reduced bioavailability of nitric oxide (decreased eNOS expression), increased expression of adhesion molecules, increased levels of proinflammatory and prothrombotic factors, oxidative stress, and abnormal modulation of vascular tone [6]. When ECs lose their ability to maintain homeostasis, vessel walls are predisposed to vasoconstriction, lipid infiltration, leukocyte adhesion, platelet activation, and oxidative stress, among other things [2]. Together, these induce an inflammatory response that is considered the first step of atheromatous plaque formation: the fatty streak [10]. In addition, endothelial dysfunction also plays a remarkable role in subsequent steps of AS by participating in plaque development and in its rupture in the last steps of AS [10]. Therefore, an increased endothelial dysfunction is considered an early indicator of atherogenesis [11].

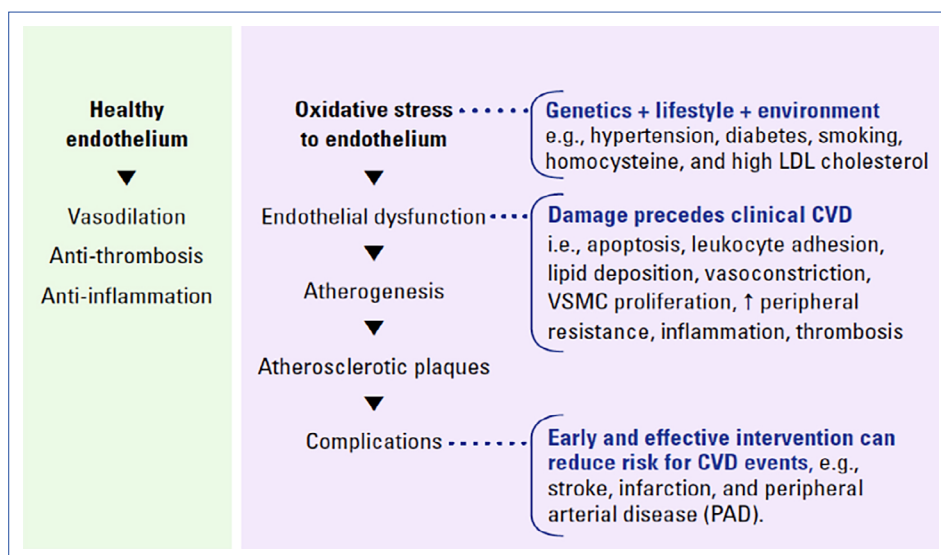
AS initiates on endothelial dysfunction accompanied by low-density lipoprotein (LDL) retention and its modification in the intima [12]. Modified LDLs, together with additional atherogenic factors, promote the activation of ECs, leading to monocyte recruitment within the intima. Modified LDLs are avidly captured by differentiated monocytes and vascular smooth muscle cell (VSMC), which promote foam cell formation [13]. In addition, several inflammatory signaling pathways are activated, allowing the fatty streak formation, which represents the first sign of AS and is characterized by a substantial accumulation of lipids both within the cells (macrophages and VSMC) and the extracellular media [2]. Following endothelial injury, the lesion develops to a fatty streak consisting mainly of lipid-laden macrophages and progresses to AS silently over many years before the disease is manifested by rupture or erosion of the lesion [14]. Fully oxidized LDL (OxLDL) can contribute to atherogenesis, and macrophages

in the artery wall take up OxLDL via scavenger receptors, leading to cholesterol ester accumulation and resulting in the formation of foam cells, the hallmark of the arterial fatty streak, which is recognized as the earliest atherosclerotic lesion [15].

### Monocyte recruitment to the intima is linked with atherogenesis

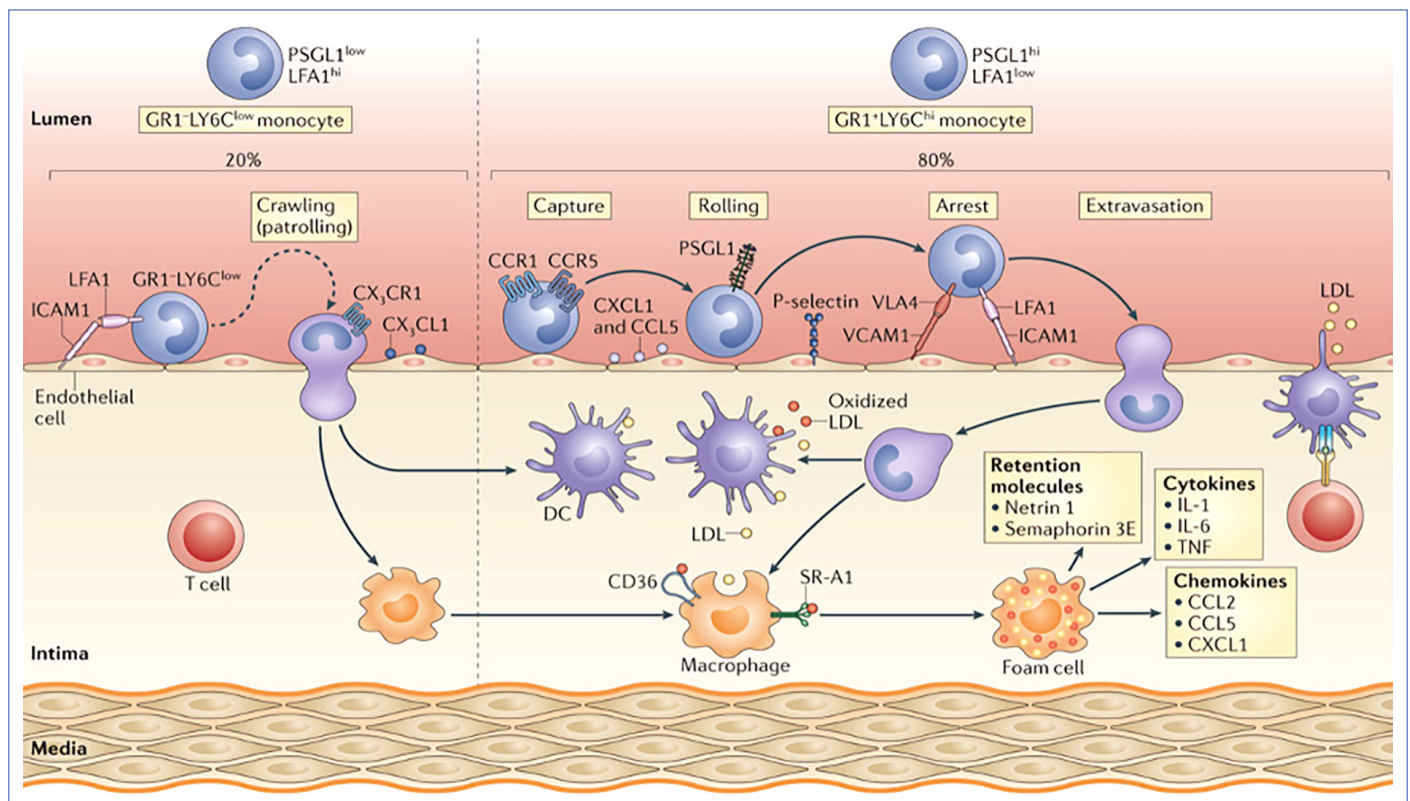
Monocyte recruitment starts with monocyte capture and rolling over the endothelium, which is mainly mediated by P-selectin [2, 17]. Monocyte-rolling is then reduced, and monocytes remain firmly attached to the endothelium [5], a process mediated by the binding of monocytes integrins to VCAM-I and ICAM-I of ECs [2]. In addition, while rolling over the endothelium, monocytes are activated by endothelial surface-bound chemokines, such as CXCL1, CXCL2, CXCL4, and CCL5, and this increases monocyte adhesiveness [18]. Afterward, monocytes transmigrate into the intima space. This movement comprises the crossing throughout the EC barrier, its basement membrane, and the pericyte layer and the migration process is held by chemokines, which have been previously secreted in response to proinflammatory signals (Fig. 2) [2].

Regarding monocyte recruitment, monocyte chemoattractant molecule-1 (MCP-1) (also named as CCL2) is the most frequent chemokine mediating monocyte transmigration which is produced mainly by ECs, smooth muscle cells, and monocytes and macrophages of the intima, and its expression is upregulated after proinflammatory stimulus or tissue injury, favoring the trans-endothelial migration of circulating monocytes from the plasma to the intima [19]. This process is mediated by the paracellular and trans-cellular routes [20]. In the paracellular route, monocyte migration is favored through EC junctions, due to the redistribution of junctional molecules in the inflamed endothelium. In addition, some endothelial junction molecules actively mediate this type of migration [2]. On the other hand,



**Figure 1.** Risk factors and process of atherosclerosis development [16].

LDL: Low-density lipoprotein; CVD: Cardiovascular disease; VSMC: Vascular smooth muscle cell.



**Figure 2.** Monocyte recruitment and accumulation in plaques.

PSGL1: P-selectin glycoprotein ligand-1; LFA1: Lymphocyte function-associated antigen 1; ICAM1: Intercellular adhesion molecule 1; CX<sub>3</sub>CR1: C-X<sub>3</sub>-C motif chemokine receptor 1; CCR: Chemokine receptor; VLA4: Very late antigen-4; VCAM1: Vascular cell adhesion molecule 1; LDL: Low-density lipoprotein; DC: Dendritic cell; SR-A: Macrophage Scavenger protein A; IL: Interleukin; CXC-chemokine ligand 1.

in the transcellular route, cells migrate through the body of ECs. Finally, monocytes cross the EC basement membrane, which is composed of a network of laminin and collagen, and the pericyte sheath, which is found in most venules [2, 20].

Once in the intima, monocytes are differentiated into macrophages that can be polarized to the M1 (pro-inflammatory) or M2 (anti-inflammatory) phenotype [21]. Nonetheless, macrophages show sensitivity to the changes in inflammatory environment, and, in response to new signals, they are able to switch their phenotype from pro-inflammatory to anti-inflammatory [22]. Macrophage plasticity is fundamental for a successful response with M1 predominating in disease progression and M2 in regression [2, 21]. M1 macrophages release inflammatory cytokines and chemokines and produce NO and reactive oxygen species (ROS), which promote monocyte recruitment and inflammatory response propagation [23]. In addition, macrophages express a battery of receptors that mediate the internalization of modified and non-modified LDLs [23]. Retained lipoproteins in the intima are prone to suffer modifications due to the inflammatory environment, allowing their internalization through CD36, SRA-I, and LOX-I scavenger receptors [24]. It is important to underline that the expression of those receptors is not downregulated by cholesterol uptake but when oxLDL content is significantly enhanced, cells internalize higher amounts of oxLDLs [2]. Within the cells, oxLDLs are degraded in the lysosomes, and the lipoprotein-contained

cholesterol is esterified by acyl CoA:cholesterol acyltransferase (ACAT) in the endoplasmic reticulum. Cholesterol esters are stored as lipid droplets located both in the cytoplasm or linked to the ER [25]. Hydrolysis of these packed cholesterol esters mediated by neutral cholesterol ester hydrolases, such as nCEH and NCEH1, generates free cholesterol that is transferred from macrophages to apoA1 or high-density lipoprotein (HDLs), an important step for the removal of cholesterol excess from peripheral tissues [26]. This process is mediated by ABCA1 and ABCG1 ATP-binding cassettes and SR-B1, cholesterol transporters that play an important role mediating cholesterol efflux from the cells and preventing foam cell formation [2, 24]. However, the pro-inflammatory microenvironment of atherosclerotic lesions impairs the ABCA1 efflux system, both in M1 and M2 macrophages, and promotes foam cell accumulation [2].

In addition, the excess of lipid uptake by macrophages perpetuates the inflammatory response, and oxLDLs induce signaling cascades that activate NF- $\kappa$ B targets, which maintain EC activation, monocyte recruitment, and foam cell formation [27, 28]. The uptake of oxLDLs by macrophages could be considered a protective mechanism, as they remove cytotoxic elements from the intima [28]. However, the increased migration of monocyte to the intima and the subsequent differentiation into macrophages lead to a large number of foam cells inducing the growth of the atherosclerotic lesion; therefore, cholesterol accumulation is considered

**Table 1. The role of key cytokines in atherosclerosis**

Cytokine	Outcome of studies using mouse model systems
IFN- $\gamma$	Pro-atherogenic. Deficiency of the cytokine or its receptor decreased atherosclerosis associated with reduction of lesion cellularity and lipid accumulation. Injection of the cytokine augmented disease development. Post-natal blocking of cytokine function by expression of a soluble mutant decoy receptor attenuated lesion formation and produced a stable plaque phenotype.
TNF- $\alpha$	Pro-atherogenic. Deficiency of the cytokine or its receptor (p55) reduced atherosclerosis with attenuated expression of several pro-inflammatory cytokines and adhesion molecules along with decreased uptake of modified LDL.
IL-1 $\beta$	Pro-atherogenic. Deficiency of the cytokine or its receptor reduced atherosclerosis associated with decreased arterial inflammation and oxidative stress. Administration of recombinant IL-1 receptor antagonist or its overexpression reduced atherosclerosis and modulated lipoprotein metabolism and foam cell formation.
IL-18	Pro-atherogenic. Deficiency of the cytokine decreased atherosclerosis, reduced IFN- $\gamma$ action and produced a more stable plaque phenotype. Administration of the cytokine increased atherosclerosis via an IFN- $\gamma$ dependent manner. <i>In vivo</i> electrotransfer of an expression plasmid for IL-18 binding protein reduced lesion development and produced a stable plaque phenotype.
IL-10	Anti-atherogenic. Deficiency of the cytokine increased atherosclerosis associated with augmented inflammatory response, LDL levels, MMP and tissue factor activity, and markers of systemic coagulation. Local or systemic overexpression of the cytokine attenuated atherosclerosis and reduced inflammation, oxidative stress, cholesterol levels, and Th1 response.
TGF- $\beta$	Anti-atherogenic. Disruption of signaling accelerates atherosclerotic development associated with increased inflammation and decreased collagen content. Disruption of TGF- $\beta$ signaling in T cells also accelerates atherosclerosis and is associated with increased T cells, activated macrophages and reduced collagen content. TGF- $\beta$ -mediated plaque stabilization was mediated through an IL-17 dependent pathway. Overexpression of TGF- $\beta$ attenuates atherosclerosis, oxidative stress and inflammation and stabilizes plaques.
IL-33	Anti-atherogenic. Administration of the cytokine reduced atherosclerosis associated with increased levels of IL-4, IL-5 and IL-13, higher levels of anti-OxLDL antibodies and a Th1 to Th2 shift. Inhibition of cytokine action using a soluble decoy receptor increased plaque development. The cytokine inhibited macrophage foam cell formation <i>in vitro</i> and <i>in vivo</i> .

IFN: Interferons; TNF- $\alpha$ : Tumour necrosis factor- $\alpha$ ; LDL: Low-density lipoprotein; MMP: Matrix metalloproteases; TGF- $\beta$ : Transforming growth factor- $\beta$ ; oxLDL: Oxidized low-density lipoprotein.

a hallmark of atherosclerotic lesions [2, 29]. An accumulation of cholesterol in the subendothelial compartment also promotes the formation of cholesterol crystals both inside and outside the cells and contributes to the development of atherosclerotic plaques [30]. Although cholesterol crystals are a common feature of advanced atherosclerotic lesions, they are present also in early plaques and can be used as a marker of early AS development [2].

Hyperlipidemia increases the number of GR1<sup>+</sup>LY6C<sup>hi</sup> monocytes, use different chemokine–chemokine receptor pairs to infiltrate the intima, which is facilitated by endothelial adhesion molecules, including selectins, ICAM1 and VCAM1. The recruited monocytes differentiate into macrophages or DCs in the intima, where they interact with atherogenic lipoproteins. Macrophages avidly take up native and modified LDL through macropinocytosis or scavenger receptor-mediated pathways (SR-A1 and CD36), which results in the formation of the foam cells that are a hallmark of the atherosclerotic plaque. These foam cells secrete pro-inflammatory cytokines (interleukins [IL]-1, IL-6, and tumor necrosis factors [TNF]) and chemokines (CCL2, CCL5 and CXC-chemokine ligand 1 [CXCL1]), as well as macrophage retention factors (netrin 1 and semaphorin 3E) that amplify the inflammatory response. CX<sub>3</sub>CL1, CX<sub>3</sub>C-chemokine ligand 1; CX<sub>3</sub>CR1, CX<sub>3</sub>C-chemokine receptor 1; LFA1, lymphocyte function-associated antigen 1; PSGL1, P-selectin glycoprotein ligand 1; VLA4, very late antigen 4 [17].

### Cytokine involvement in AS

Cytokines are a diverse group of low-molecular weight proteins with over 100 identified so far and clustered into several classes such as the interleukins (IL), chemokines, colony-stimulating factors (CSF), TNF, the interferons (IFN) and transforming growth factors (TGF) [31]. Many cytokines are expressed in atherosclerotic plaques and all cells involved in the disease are capable of producing cytokines and responding to them and play a key role at every stage of AS (Table 1), from early events involving dysfunction of the endothelium and lipid metabolism, and later phase actions such as enhancing matrix metalloproteases (MMP) secretion [32]. Within the disease state, the production of many cytokines is auto-inducible through autocrine and paracrine signaling, which helps to augment and sustain inflammation [32, 33]. Extracellular GFs and cytokines are highly expressed during AS and mediate the proliferation and survival of cells involved in plaque formation [33, 34].

### AS-associated thrombosis

Atherosclerotic plaques that are prone to disruption and acute luminal thrombosis are characterized by the combination of large necrotic areas, fibrous cap thinning, centralized apoptosis around the necrotic core, and high levels of inflammatory cytokines and matrix proteases [2]. Accumulation of apoptotic cells and debris within the lipid core is a major feature of advanced human atherosclerotic lesions and has been

**Table 2. Overview of Molecules involved in atherogenesis**

Molecule	Nature of function
Selectin-E, selectin-L, selectin-P	Up regulation of leukocyte and endothelial adhesion molecules Platelet adhesion and aggregation
Integrins	Up regulation of leukocyte adhesion molecules, platelet adhesion and aggregation
Interleukins (IL-1, IL-2, IL-8)	Migration of leukocytes into arterial wall, T-cell activation, formation of foam cells, formation of fibrous cap
Adhesion molecules (platelet endothelial cell adhesion molecule, ICAM-1, VCAM-1)	Up regulation of leukocyte and endothelial adhesion molecules
Monocyte chemotactic protein-1	Migration of leukocytes into arterial wall macrophage accumulation
Macrophage colony stimulating factor	Migration of leukocytes into arterial wall, formation of foam cell, macrophage accumulation
Platelet derived growth factor	Migration of leukocytes into arterial wall, Stimulation of smooth muscle migration, formation of fibrous cap
Tumour necrosis factor- $\alpha$	T-cell activation, formation of foam cell formation of fibrous cap
Transforming growth factor- $\beta$	Stimulation of smooth muscle migration formation of fibrous cap
Osteopontin	Migration of leukocytes into arterial wall formation of fibrous cap
Fibroblast growth factor II	Stimulation of smooth muscle migration
Granulocyte macrophage colony-stimulating factor	T-cell activation
Fibrin, thromboxane, tissue factor	Platelet adhesion and aggregation

IL: Interleukin; ICAM-1: Intercellular adhesion molecule 1; VCAM-1: Vascular cell adhesion molecule 1.

associated with increased susceptibility to thrombotic plaque complication [35]. The magnitude of the thrombotic response on ruptured or eroded plaques is extremely variable, and only occasionally does a major and life-threatening luminal thrombus evolve. Probably the determinants are: (1) Thrombogenicity of the exposed plaque material, (2) local flow disturbances, and (3) systemic thrombotic propensity [36]. The time relationship between plaque rupture and syndrome onset is not easily assessed because rupture in itself is asymptomatic and the following thrombotic process is highly unpredictable. The vulnerable plaque is used sometimes as a concept comprising plaques at high-risk of thrombosis by any mechanism (rupture, erosion) and sometimes to describe a set of histological features that by association are assumed to increase the risk of imminent rupture and thrombosis [2, 35, 36].

Endothelial cells covering the fibrous cap become either extremely thin or loaded with lipid droplets turning into EC-derived foam cells; in either case, they are fragile and susceptible to erosion [36]. Ultimately, the EC are injured and their disruption exposes the ECM (rich in pro-inflammatory and pro-coagulant molecules) to the circulating blood cells, which initiate the thrombus formation [35, 37]. The majority of acute coronary events are related to disruption of a "Vulnerable" atherosclerotic plaque, leading to the exposure of subintimal contents and the formation of a platelet-rich thrombus. Plaque rupture is the most frequent cause of thrombosis [36]. In plaque rupture, a structural defect - a gap - in the fibrous cap exposes the highly thrombogenic core to the blood. Dislodged plaque material is sometimes found within the thrombus, indicating that rupture and thrombosis coincided and thereby supporting its causal relationship [36]. Local platelet activation (by tissue factor

[TF]-mediated thrombin generation or by collagen) stimulates further thrombus formation, which leads to additional platelet recruitment caused by cell-surface thrombin formation and the release of serotonin, ADP, and thromboxane A<sub>2</sub> [35]. After activation, thrombus forms as platelets aggregate through the binding of bivalent fibrinogen to glycoprotein IIb/IIIa [17].

Platelets have a major role in the thromboembolic complications of the vulnerable plaque. When the plaque ruptures, platelets adhere to the exposed extracellular matrix (ECM) rich in pro-inflammatory factors, become activated, aggregate and form a thrombus on the surface of the disrupted lesion [2]. The overlying thrombus is often in continuity with the underlying necrotic core rich in macrophages [38]. Acute thrombosis is predominantly characterized by layered platelet aggregates with variable amounts of fibrin, RBCs and acute inflammatory cells [37]. Treatment with lipid-lowering agents over time stabilizes plaques, probably by depletion of plaque lipid and through a reduction in inflammatory cell activity. These changes, in turn, appear to lead to a reduction in thrombosis by decreasing platelet activity and TF expression [2].

## Molecular Mechanism of AS Pathogenesis

To this date, the molecular mechanisms underlying the pathogenesis of AS (Table 2) remain largely unknown.

### Molecular mechanisms of plaque rupture and cap formation

The fibrous cap is a sub-endothelial barrier between the lumen of the vessel and the atherosclerotic necrotic core consisting of VSMCs that have migrated to the luminal side of the artery and ECM derived from VSMCs [39]. The role of the

fibrous cap is to serve as a structural support to avoid the exposure of prothrombotic material of the core that otherwise would trigger thrombosis [2].

In response to injury, VSMCs switch their phenotype to the synthetic one in which migratory and proliferation activities prevail and neighboring cells activate the healing process by producing several growth factors (GFs), which include epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor, platelet-derived growth factor (PDGF), TGF- $\beta$ , and vascular endothelial growth factor (VEGF) [2, 40]. In AS, in response to the GFs produced by foam cells (VSMC- or macrophage-derived) or ECs of the intima, VSMCs from the tunica media migrate to the intima [39]. Moreover, IL-1 produced by macrophages enhances the endogenous production of PDGF by VSMC, and, once in the intima, it autocritically leads to their proliferation [2]. In addition to migration and subsequent proliferation, VSMCs with a synthetic phenotype also increase the production of ECM components, such as interstitial collagen, elastin, and proteoglycans [41]. These proliferating VSMCs, along with ECM production, generate a fibrous cap that will cover the developing atherosclerotic plaque, thus surrounding the lesion and preventing its rupture [2]. Interestingly, if the mitogen production does not cease, VSMCs do not switch back to the contractile phenotype, which facilitates lesion development [42]. Fibrous-cap features, such as thickness, cellularity, matrix composition, and collagen content, are important determinants of plaque stability [43].

The exact mechanism of plaque rupture is not known, but it includes cap thinning, excess inflammatory cytokines and proteases that mediate digestion of the matrix, decreased collagen synthesis and the presence of injured or apoptotic cells within the necrotic core. All the cells that contribute to the formation of the atherosclerotic plaque are also implicated in the plaque rupture and the consequent thrombosis [37]. Molecular mediators associated with atherogenesis could alter collagen metabolism in ways that could thin or weaken the plaque's fibrous cap and IFN- $\gamma$  strikingly inhibited the ability of the smooth muscle cell to express the genes encoding procollagens which indicated as an important mechanistic link between inflammation and impaired synthesis of collagen in atheromata [44]. The inflammatory cells, through the factors they secrete within the plaque, send molecular messages: macrophage-derived-foam cells secrete cytokines, GFs, TF, IFN- $\gamma$ , MMPs, and produce ROS; lymphocytes secrete among others CD-40L [37].

Accumulation of free cholesterol within the plaque is a potent inducer of apoptosis of macrophage derived-foam cells; in addition, within the lesions, SMC and T-cells may also go through apoptotic cell death [45]. Apoptotic cells release their content initiating the formation of the necrotic core. The defining feature of this stage is a lipid rich necrotic core encapsulated by fibrous tissue [37]. Excess extracellular unesterified cholesterol nucleates into cytotoxic crystals and the atherosclerotic plaque evolves to complicated atheroma, eventually causing the total occlusion of coronary artery branches [2].

The continued inflammatory response ultimately leads to the destabilization of atherosclerotic plaques via the action of proinflammatory cytokines. Indeed, studies have shown that IFN- $\gamma$ , IL-18, GDF-15, and TWEAK destabilize plaques whereas TGF- $\beta$  causes stabilization [31]. IFN- $\gamma$ , TNF- $\alpha$ , and IL-1 $\beta$  promote apoptosis of macrophages along with foam cells leading to enlargement of the lipid core. In addition, such cytokines stimulate apoptosis of SMCs leading to thinning of the fibrous cap [36]. Pro-inflammatory cytokines also inhibit the synthesis of plaque stabilizing components of the ECM produced by SMCs. For example, IFN- $\gamma$  inhibits the synthesis of collagen by SMCs [31].

Macrophages infiltrate the thinned fibrous cap which express and secrete a large number of inflammatory cytokines and proteases (MMPs), which digest the stabilizing matrix, thus having a key role in the weakening and ultimate rupture of the atherosclerotic plaque [37]. The same reported that, necrosis of the vulnerable plaque is due to a combination of macrophages death and defective phagocytic clearance of apoptotic cells that accelerates or induces plaque disruption by releasing inflammatory cytokines and matrix proteases [2]. The mechanical stress caused by the necrotic core to the overlying cap may also produce the plaque rupture [46].

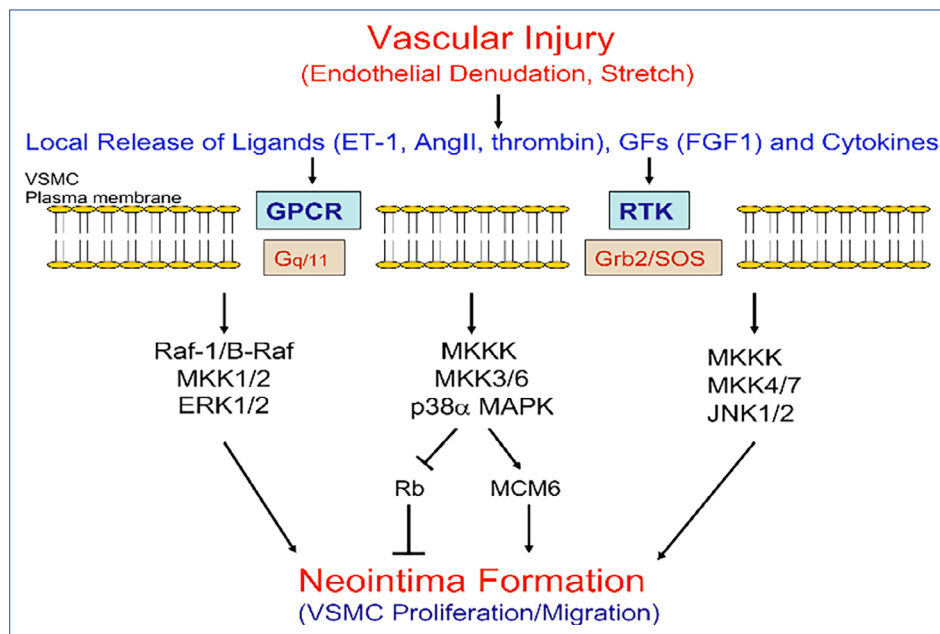
### **Fundamental cellular signaling pathways relevant to multiple stage of atherogenesis**

Understanding of several basic signaling pathways (in relation to inflammation) is a prerequisite to the study of atherogenesis which are linked to various intracellular signaling pathways through adaptor proteins or other transducers.

#### **Receptor Tyrosine Kinases (RTKs)**

RTKs are transmembrane glycoproteins that are activated by the binding of their cognate ligands, and they transduce the extracellular signal to the cytoplasm by phosphorylating tyrosine residues on the receptors themselves (autophosphorylation) and on downstream signaling proteins [47]. They are key regulators of critical cellular processes, such as proliferation and differentiation, cell survival and metabolism (Fig. 3), cell migration, and cell-cycle control [48]. The RTK family includes the receptors for insulin and for many GFs, such as EGF, FGF, PDGF, VEGF, and nerve growth factor and mutations in RTKs and aberrant activation of their intracellular signaling pathways have been causally linked to cancers, diabetes, inflammation, severe bone disorders, arteriosclerosis, and angiogenesis [48].

The major feature of INSR and other RTK is inhibition of apoptosis together with stimulation of growth and mitogenesis, and its anti-apoptotic effects in endothelium seem to generally translate to protection against AS based on endothelial and results from manipulation of downstream signaling molecules (Fig. 3). On the other hand, impaired insulin signaling in macrophages often led to greater apoptosis, impaired proliferation, and smaller plaques in earlier stages, but potentially more complex and inflamed plaques at later stages of plaque development [47].



**Figure 3.** Smooth muscle cell proliferation in neointima formation after vascular injury.

ET-1: Endothelin-1; GFs: Growth factors; FGF1: Fibroblast Growth Factor 1; VSMC: Vascular smooth muscle cell; GPCR: G-protein-coupled receptors; RTK: Receptor Tyrosine Kinases; Gq: G protein sub-family q; Grb2/SOS: Growth factor receptor-bound protein 2/Son of Sevenless; Raf-1: Proto-oncogene, serine/threonine kinase; B-Raf: Proto-oncogene B; MKK: MAP kinase kinases; ERK: Extracellular signal-regulated kinases; MKKK: MAPK kinase kinase; MAPK: Mitogen-activated protein kinase; JNK: c-Jun N-terminal kinase; MCM: Minichromosome maintenance complex.

In response to arterial injury that includes endothelial denudation and stretch, there is a local release of ligands such as endothelin-1, angiotensin II and thrombin, GFs, and cytokines that bind to transmembrane receptors. These receptors include G protein-coupled receptors, RTKs and cytokine receptors that activate intracellular signaling proteins such as G proteins (Gq/11) and the Grb2/SOS complex. In turn, intracellular mitogen-activated protein kinase (MAPK) cascades are activated that ultimately leads to VSMC migration into the intima and proliferation, resulting in neointima formation. The activation of p38 $\alpha$  MAPK in vascular smooth muscle cells promotes the hyperphosphorylation of Rb and the expression of MCM6 that both contribute to cell proliferation [49].

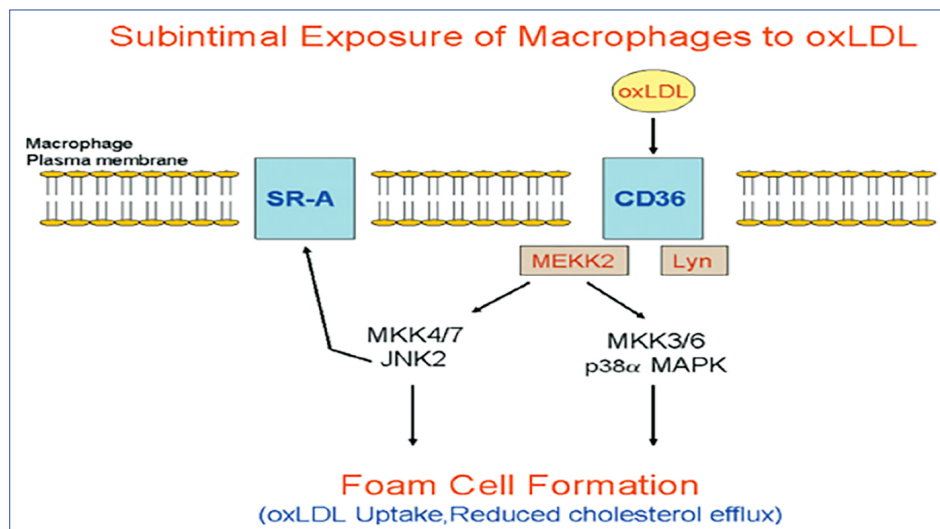
### MAPK signaling

Cellular behavior in response to extracellular stimuli is mediated through intracellular signaling pathways such as MAPKs pathways, which are a conserved proline-directed Ser/Thr protein kinases enzymes connecting cell-surface receptors to critical regulatory targets within cells [50], the activation of which requires dual phosphorylation on the Thr-X-Tyr motif that is catalyzed by MAP2K kinases (Fig. 4) [51]. After activation, MAPKs phosphorylate specific serine and threonine residues of target substrates, which include other protein kinases and many transcription factors [51]. They respond to chemical and physical stresses, thereby controlling cell-survival and adaptation.

MAPK cascades are triple kinase pathways that include a MKKK (MAPK kinase kinase), a MAP kinase kinases (MKK) (MAPK kinase, also called MEK kinase) (Fig. 4) [52]. MAPKs modulate atherosclerotic lesion formation through the regulation of macrophage foam cell formation and a terminal MAPK [49]. p38 MAPK has been strongly implicated in the development of AS and promotes AS by, p38MAPK to stimulate secretion of MCP-1 and IL-8, which attract monocytes to vascular endothelial cells and mediates the MCP-1-dependent trans-endothelial migration, integrin activation, and chemotaxis [53]. p38 MAPK can be activated in monocytes/macrophages, vascular endothelial cells, and vascular smooth muscle cells by a variety of stimulants, including ROS; chylomicron remnants; free fatty acids; cholesterol; and proinflammatory cytokines, such as TNF- $\alpha$  and GFs [53].

JNK2 and p38 $\alpha$  MAPK activity are required for the uptake of oxLDL by macrophages in culture, and JNK2 is required for atherosclerotic lesion development *in vivo* because of its ability to facilitate the transformation of macrophages into foam cells [49]. MAPK cascades play a pivotal role in the formation of neointima after vascular injury (Fig. 4). IL-4 might play a pivotal role in protection against AS through inhibition of the MAPK signaling pathway and p38 MAPK has been shown to be a potentially important mediator in promoting AS, and p38 pathway kinases may function as anti-inflammatory drug targets for inflammatory diseases [54].

In response to vascular injury, monocytes are recruited to the intimal surface of arteries where they adhere and invade the vessel, differentiating into macrophages. In the subinti-



**Figure 4.** The role of MAPK in macrophage foam cell formation.

oxLDL: Oxidized low-density lipoprotein; SR-A: Scavenger receptor; CD36: Cluster of differentiation36; MEKK: Mitogen-activated protein kinase kinase kinase; JNK: c-Jun N-terminal kinase; MAPK: Mitogen-activated protein kinase.

mal space, macrophages are exposed to oxLDL that binds to the transmembrane protein CD36. Binding of oxLDL to CD36 triggers the activation of Src-family kinases such as Lyn and MEKK2. Activation of Lyn and possibly MEKK2 leads to the activation of JNK2 and also p38 $\alpha$  MAPK. Activation of JNK2 and p38 $\alpha$  MAPK promote the internalization of oxLDL through CD36, SR-A or through other scavenger receptors. JNK2 promotes the phosphorylation of SR-A, and this may lead to SR-A internalization while it is associated with modified LDL. The activation of MAPK cascades in macrophages may also modulate cholesterol efflux pathways, MAP kinase kinases (MKKs) [49].

### NF- $\kappa$ B activation

Signaling pathways related to NF- $\kappa$ B are exceedingly complex and which is one of the main signaling pathways activated in response to proinflammatory cytokines, including TNF- $\alpha$ , IL-1, and IL-18, as well as following activation of the Toll-like receptors by the pattern recognition of pathogen-associated molecular patterns. Activation of this pathway plays a central role in inflammation through the regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, GFs, and inducible enzymes such as cyclooxygenase-2 and inducible nitric oxide synthase [55].

Activated NF- $\kappa$ B has been identified in SMC, macrophages, and EC of human atherosclerotic lesions, which controls the expression of proinflammatory cytokines TNF- $\alpha$ ; IL-6 and IL-8; MMP-1, -3, and -9; and TF. In endothelial cells, active NF- $\kappa$ B induces transcription of cell adhesion molecules such as ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and E-selectin, as well as several chemokines and cytokines including MCP-1, M-CSF, GM-CSF, IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$ . In general, pro-inflammatory gene targets of NF- $\kappa$ B, many elements of upstream signaling leading to NF- $\kappa$ B activation have been found to be atherogenic [55].

### Proinflammatory mediators

#### OxLDL

oxLDL plays a pivotal role through the induction of foam cell formation, alteration of nitric oxide signaling, initiation of endothelial activation, and expression of adhesion molecules that accelerate leukocyte homing to the site of AS. And, OxLDL directly affect the migration of monocytes to the aortic wall by switching from CCR2 to CX<sub>3</sub>CR1 expression using a peroxisome proliferator-activated receptor  $\gamma$ -dependent pathway [5].

#### C-reactive protein (CRP)

Elevated plasma CRP is associated with increased risk of AS, since it is found within atherosclerotic plaques close to LDL and macrophages but the mechanisms have not been fully identified [5]. Similar reports demonstrated that CRP can modulate endothelial functions and leukocyte activities in the meantime it induces the production of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, CXCL1, and CXCL8 by human monocytes *in vitro* [5]. CRP binds to minimally modified (mm) LDL and prevents the formation of foam cells from macrophages [56].

#### ROS

Extensive production of ROS has been implicated in AS by inducing the chronic activation of the vascular endothelium and components of the immune system. In humans, higher expression of NADPH oxidase subunit proteins is associated with increased superoxide (O<sub>2</sub><sup>-</sup>) production and severity of AS [5]. Superoxide production from both monocytes/macrophages and vascular cells plays a critical role in atherogenesis [57]. One of the mechanisms by which superoxide affects atherogenesis is the activation of SMC mitogenic signaling pathways [5, 57]. Platelets also produce ROS, and NADPH induced superoxide production results in enhanced availability of released ADP and amplified platelet recruitment [58].



## Inflammation-regulating enzymes in AS

### 5-lipoxygenase (5-LO)

The 5-LO pathway is responsible for the production of leukotrienes, inflammatory lipid mediators that have a role in innate immunity but that can also play a proatherogenic role [59]. Expression of 5-LO and leukotriene A4 hydrolase in atherosclerotic segments correlates with plaque instability. Elena and Klaus [5] reports the decreased lesion in 5-LO-deficient *Ldlr*<sup>-/-</sup> mice and bone marrow transplantation experiments suggest that macrophage 5-LO is mainly responsible for atherogenesis.

### 12/15-LO

The proatherogenic effect of 12/15-LO has been established in 12/15-LO-deficient *Apoe*<sup>-/-</sup> mice that showed reduced lesions throughout the whole aorta [5]. Unexpectedly, these mice also had diminished plasma IgG autoantibodies to oxidized LDL, which suggests that the 12/15-LO pathway affects not only lipid peroxidation but also the adaptive immune response [5]. Overexpression of 12/15-LO leads to the formation of fatty streak lesions, at least partially through the elevated adhesion of monocytes to endothelium and 12/15-LO induces the production of IL-6, TNF- $\alpha$ , and CCL2 and therefore connects the metabolic and immune branches of AS [60].

### Heme oxygenase (HO)-1

HHO catalyzes the rate limiting step of heme catabolism. The inducible form of the HO-1 is an endogenous anti-inflammatory pathway induced in response to inflammatory stimuli and the induction of HO-1 reduced monocyte chemotaxis in response to LDL oxidation [5]. HO-1 can be upregulated in human EC by TNF and IL-1, and HO-1 possesses potent antiapoptotic and anti-inflammatory properties and its deficiency in humans is associated with the presence of severe and persistent endothelial damage [5]. The anti-inflammatory properties of HO-1 seem to be related to an inhibitory action on P- and E-selectin expression on EC [55]. The absence of HO-1 exacerbated AS in HO-1-deficient *Apoe*<sup>-/-</sup> mice, and macrophages expressing HO-1 are crucial players in this process [61].

### Paraoxonases

The paraoxonase family consists of three members (PON1, PON2, and PON3) that share structural properties and enzymatic activities, among which is the ability to hydrolyze oxidized lipids in LDL. PON1 prevents oxidation of LDL as well as HDL, with which PON1 is associated in the serum. HDL of *Pon1*<sup>-/-</sup>*Apoe*<sup>-/-</sup> mice is predisposed to oxidation, and as a consequence lesions in *Pon1*<sup>-/-</sup>*Apoe*<sup>-/-</sup> mice are larger compared with controls [5]. Over-expressing PON1, the role of PON1 as inhibitor of lipid oxidation [62].

## Genetics of AS in humans

Genetic factors contribute importantly to AS in human populations, with a heritability estimated to be approximately 50% [63]. Although there has been considerable success in identifying genes for rare disorders associated with AS (Table 3) and the modes of inheritance of AS are complex and multifactorial, that is, the disease phenotype is a consequence of interactions between genetic and environmental factors [8].

Several genes and cytokines have been identified as risk factors for AS, and the microRNAs (miRNAs) may play a role in regulating the atherosclerotic process. Several miRNAs have been reported to be related with endothelial dysfunction; like the significantly upregulated expression of miR-21 in atherosclerotic plaques [64] that can inhibit the expression of peroxisome proliferator-activated receptor- $\alpha$ , leading to enhanced expressions of VCAM-1, and monocyte chemoattractant protein-1 [65].

Recently, several studies have reported that miRNAs play important roles in foam cell formation and inflammation in AS [2]. miRNAs control the expression of inflammatory chemokines such as CCL2 and CXCL1 predominantly indirectly by regulating the expression of signaling molecules of the NF- $\kappa$ B signaling pathway [2]. miR-126 is expressed only in endothelial cells, and thus it is recognized as an endothelium-specific microRNA for regulating angiogenesis, vascular inflammation and possesses the ability to regulate monocyte adhesion by directly targeting VCAM-1, and thus it controls vascular inflammation [8]. Administration of miR-126 in mouse models of AS may reduce macrophage and apoptotic cell content, thereby limiting lesions size and conferring a milder inflammatory reaction [2, 8]. Accordingly, miR-126 may exert an anti-atherosclerotic effect. For instance, miR-181b inhibits the NF- $\kappa$ B-mediated CXCL1 expression by suppressing the expression of importin- $\alpha$ 3, which is required for the nuclear translocation of NF- $\kappa$ B [66]. miR-103 and miR-92a targets the NF- $\kappa$ B inhibitor KLF4 (*Kruppel-like factor 2/4*), thereby increasing CCL2 and CXCL1 expression in ECs [2]. In contrast, miR-21 reduces CCL2 expression by blocking the AP-1 signaling pathway. A direct regulation of CCL2 is reported for miR-495, which induces CCL2 mRNA degradation by binding to its response element in the 3' UTR [66].

Hypoxia can induce the development of AS. The hypoxia-inducible factor pathway is essential for cell survival under conditions of low oxygen and enhances endothelial cell angiogenesis [67]. miR-210 has been shown to be a prominent miRNA that is upregulated by hypoxia. Overexpression of miR-210 in normoxic endothelial cells stimulates the formation of capillary-like structures and VEGF-mediated cell migration through ephrin-A3 downregulation [8]. Anti-miR-210 inhibits cell growth and induces apoptosis in both normoxic and hypoxic conditions (Table 3). Dysregulation of miR-210 is involved in the formation of AS [8]. miR-125a-5p plays a role in endothelial dysfunction as well as in foam cell formation and the five miRNAs-miR-125a-5p, miR-9, miR-146a, miR-146b-5p, and miR-155-were upregulated in oxLDL-stimulated monocytes [8].

**Table 3. Genes associated with atherosclerosis**

Gene	Chromosomal location	Function/phenotypic traits
<b>1. Lipid metabolism</b>		
ApoB	2p	Component of plasma lipoproteins, particularly LDL; mediates binding to LDL receptor
	Arg-3500-Gln	Disorders of hypercholesterolemia known as familial defective apoB-100, due to reduced binding to LDL receptor
	tHR 71-Ile	Possibly associated with increased plasma LDL cholesterol and apoB levels; arg-3531-cys LDL receptor binding defect appears to segregate with Thr allele
ApoCIII	11q T(625)del, C(482)T, T(455)C C3175G (SstI)	Component of plasma proteins Increased plasma triglyceride levels
AapoE	19q	Component of plasma proteins; mediates binding to the LDL and remnant (apoE) receptors
Cholesteryl ester transfer protein (CETP)	16q	Reverse cholesterol transport pathway; possible proatherogenic role in presence of dyslipidemia
Lipoprotein lipase (LPL)	Ile-405-Val, Asp-442-Gly	Increased plasma HDL-C and apoA-I levels
	8p	Hydrolysis of plasma triglycerides
	T(93)G	Increased LPL promoter activity, reduced plasma triglycerides
	T(39)C	Reduced LPL promoter activity
	Asp9-Asn Asn-291-Ser Ser-447-Ter	Increased plasma triglycerides, increased atherosclerotic progression Reduced plasma HDL-C, increased triglyceride levels Increased plasma HDL-C, reduced plasma triglyceride levels; possible impact on responsiveness to blockers
PON	7q	HDL-associated enzyme known to hydrolyze organophosphate poisons; possible contribution to HDL's protective capacity against LDL oxidation
	Gln-192-Arg	Increased enzymatic activity; <i>in vitro</i> , reduced protection against lipid peroxidation
<b>2. Homocysteine metabolism</b>		
Cystathionine-synthase (CBS)	21q	Transulfuration pathway, converting homocysteine to cystathionine, with pyridoxine as cofactor
	Ala-114-Va, Ile-278-Thr, Arg-125-Gln, glu131asp, Gly-307-Ser	Pyridoxine-responsive homocystinuria
Methylene tetrahydrofolate reductase (MTHFR)	1p	Pyridoxine-unresponsive homocystinuria
	C677T (Ala/Val)	Remethylation pathway, generating the 5-methyltetrahydrofolate that serves as the methyl group donor
	C692T	Associated with hyperhomocysteinemia given low dietary folate; increased risk for deep-vein thrombosis in carriers of factor V Leiden Absence of enzyme activity
<b>3. Thrombosis</b>		
Glycoprotein IIIa (GPIIIa)	17q	Component of GPIIb/IIIa platelet adhesion receptor, binding fibrinogen, fibronectin, and von Willebrand factor
	Leu-33-Pro	Interindividual variation in platelet adhesion and/or adhesion; mixed evidence of association with risk of coronary thrombosis
Fibrinogen	4q	Determinant of plasma viscosity, cofactor for platelet aggregation, precursor of fibrin (component of plaques)
	chain G(455)A	Increased plasma fibrinogen levels and progression of atherosclerosis
Factor V	1q	Activated form is procoagulant cofactor in prothrombin activation, inactivated through cleavage by activated protein C
	Arg-506-Gln (Leiden mutation)	Resistance to activated protein C; hypercoagulability
<b>4. Leukocyte adhesion</b>		
Endothelial leukocyte adhesion molecule-1(ELAM)	1q	Adhesion of leukocytes to activated arterial endothelium; also known as E-selectin
	G98T, Ser-128-Arg, Leu-554-Phe	Increased risk for severe atherosclerosis

LDL: Low-density lipoprotein; tHR: Thyroid hormone receptor.

## Conclusion

AS is a multi-focal, slowly progressive process. It occurs due to numerous pathological changes including the disrupted cell and molecular functions in vessel sub-endothelial space. The study of cellular and molecular biology mechanisms of AS has provided remarkable insights into the processes that lead to atheroma development and the clinical manifestations of this disease. Atherosclerotic lesions with the potential to rupture, that is, vulnerable plaques, are the primary cause of luminal thrombosis and consequent clinical episodes. The atherosclerotic plaques include cholesterol-rich core and fibrous cap. Excess lipids and inflammatory reactions (cellular and humoral) are considered the major contributors to plaque development, and the loss of VSMCs and increased intra-plaque hemorrhage are critical steps in necrotic core destabilization and enlargement. Furthermore, the acquired and innate immune agents cause to progress the plaque. Narrowing of arteries caused by the erosion and rupture of plaques slows down blood flow and results in fatal ischemia in the vessels. Thus, knowing the molecular changes and the cellular events in sub-endothelial microenvironment during the plaque growth can help to understand the stenosis in the vessels. As such, better characterization of the immune-modulating role of platelets as well as of miRNAs (endogenous small non-coding RNAs with the ability to regulate gene expression at transcriptional and posttranscriptional levels) may give rise to novel experimental strategies for the prevention and treatment of AS-related diseases.

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