Calcification and C-reactive protein in atherosclerosis : effects of calcium blocking and cholesterol lowering therapy

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Chapter 1

General introduction

Cardiovascular disease (CVD) is a major cause of mortality and morbidity in Western society [1]. Atherosclerosis is the primary etiologic factor underlying CVD. Atherosclerosis is characterized by the presence of atherosclerotic lesions in large and medium-sized elastic and muscular arteries, which may lead to narrowing of the vessel lumen and restriction of blood flow. Ultimately, clinical manifestations may occur, such as angina pectoris, myocardial infarction, stroke, and peripheral vascular disease. Risk factors for developing atherosclerosis include elevated low-density lipoprotein (LDL)-cholesterol levels, low high-density lipoprotein (HDL)-cholesterol levels, elevated triglyceride levels, obesity, hypertension, smoking, diabetes and several genetic factors [2].

Pathogenesis of atherosclerosis

Atherosclerosis is a chronic and multifactorial disease, which is characterized by different stages: I) the fatty streak, in which cholesterol is deposited in the vessel wall and macrophage-derived foam cells develop, II) the fibrofatty lesion that is characterized by migration and proliferation of vascular smooth muscle cells (VSMCs), III) the advanced lesion, which contains a lipid core and fibrous cap, and IV) the ruptured lesion, that often causes clinical complications such as myocardial infarction and stroke.

A high concentration of plasma cholesterol, in particular LDL cholesterol, is one of the principal risk factors for atherosclerosis, and the process of atherosclerosis is often associated with oxidative modification of LDL in the vessel wall. For many years atherosclerosis has been regarded as a degenerative process, which occurs naturally with age. However, in recent years the process of atherosclerosis has been described as an active, more complex, process in which chronic inflammation is an important component [3].

The vessel wall

The vessel wall consists of three layers, tunica intima, media and adventitia. The intima is the innermost layer of the vessel wall and consists of a single layer of endothelial cells. The intima functions as a semi-permeable barrier between the blood and the tissues. The intima is separated from the media by the lamina elastica interna. The media consists mainly of VSMCs. The outer layer of the vessel wall, the adventitia, surrounds the media and is composed of adipocytes, connective tissue, and elastic fibres.

Initiation of atherosclerosis

The earliest changes that precede the formation of atherosclerotic lesions take place in the endothelium. The response-to-injury hypothesis of atherosclerosis proposes that "injury" to the endothelium is the initiating event in atherosclerosis [3-5]. Possible causes of endothelial injury include the accumulation of atherogenic lipoproteins, such as LDL, in the arterial wall as a consequence of elevated plasma lipoprotein concentrations. Free radicals, generated by, for instance, cigarette-smoking convert LDL in the arterial wall into oxidized LDL (oxLDL), which is pro-atherogenic. Other causes of injury to the endothelium are infection with pathogens, and hypertension [6-8]. Endothelial dysfunction, which is the result of this injury, leads to compensatory (inflammatory) responses that alter the normal properties of the endothelium. The endothelium becomes procoagulant, loses fibrinolytic and antioxidant activity and produces insufficient amounts of nitric oxide (NO). Moreover, endothelial dysfunction is characterized by increased adhesiveness and permeability of the endothelium, allowing for increased adhesion and transmigration of monocytes into the subendothelial space [fig. 1A], where they differentiate into macrophages and develop into foam cells.

Initial atherosclerotic lesion

Recruitment of monocytes and T-lymphocytes to lesion-prone sites of the arterial wall is regulated by adhesion molecules, which are expressed on endothelial cells in response to inflammatory stimuli, such as described in the previous paragraph. Normally, circulating leukocytes have a slight interaction with the endothelium. Binding of leukocytes to endothelial P-and E-selectin allows these cells to tether and roll along the vessel wall. Rolling leukocytes are slowed down, which allows for subsequent firm adhesion to the endothelium, if adhesion molecules are present. Firm adhesion is mediated by binding to intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1). The functional importance of these adhesion molecules has been demonstrated by knockout studies in mouse models. Mice that lacked VCAM-1 had a marked reduction in the development of atherosclerosis [9]. Knockout studies have also indicated involvement of P- and E- selectins [10] and ICAM-1 [11]. After adhesion, the leukocyte becomes flattened and migrates into the arterial wall through endothelial cell-cell junctions (diapedesis). Once monocytes have entered the subendothelial space, they differentiate into macrophages. These macrophages accumulate modified forms of LDL, including oxLDL, thus forming foam cells. This initial atherosclerotic lesion is called a fatty streak [fig. 1B].

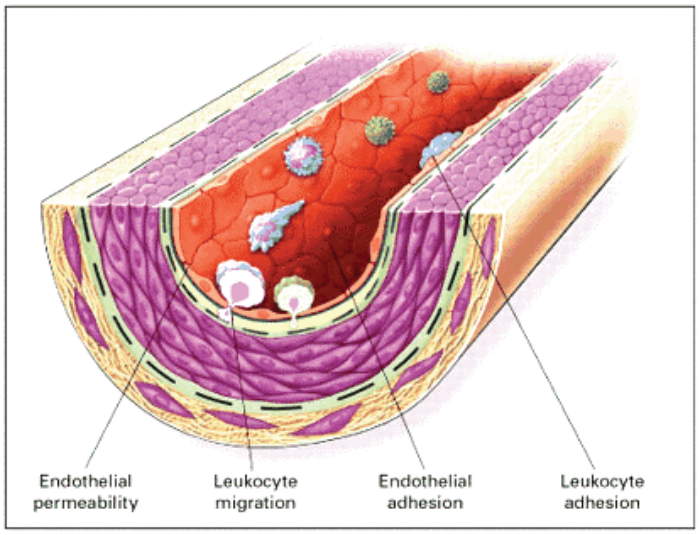

Figure 1A. Endothelial dysfunction and atherosclerosis. The earliest changes that precede the formation of atherosclerotic lesions take place in the endothelium. These changes include increased endothelial permeability to lipoproteins and other plasma constituents, and up-regulation of adhesion molecules.

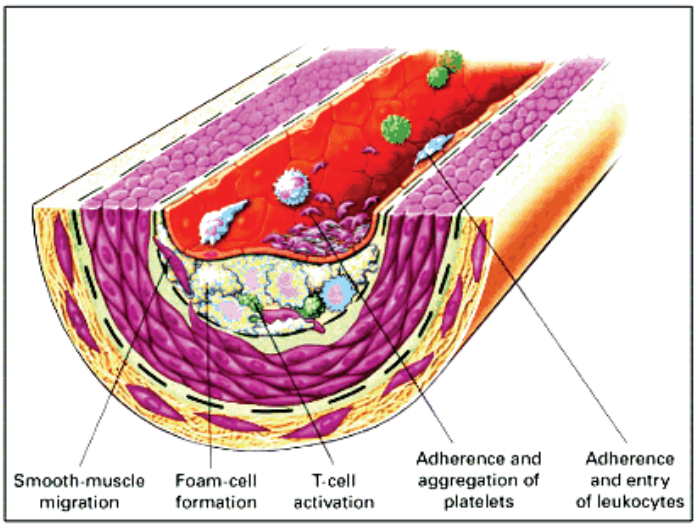

Figure 1B. Fatty streak formation in atherosclerosis. Fatty streaks initially consist of lipid-laden monocytes and macrophages (foam cells) together with T lymphocytes. Later they are joined by various numbers of smooth muscle cells [3].

The fatty streak is the earliest lesion of atherosclerosis, and can be found even in infants and young children. It is a lipid-rich, inflammatory lesion, which hardly has any effect on the luminal diameter of the vessel. The fatty streak consists mainly of lipid-laden macrophages (foam cells), but some lipid-laden VSMCs may also be present [12].

Lesion progression

In the normal vessel wall VSMCs exist as a contractile phenotype. In the atherosclerotic lesion activated macrophages and endothelial cells secrete chemoattractants (including cytokines and chemokines) that induce migration of VSMCs from the media to the intima. Subsequently VSMCs start to proliferate, may become foam cells upon uptake of modified LDL, and produce extracellular matrix components such as collagen and proteoglycans [13]. The transition of the phenotype of VSMCs from a contractile to a synthetic phenotype marks the progression of fatty streaks into fibrofatty and advanced lesions.

The synthetic VSMC phenotype is responsible for the formation of a collagen-rich fibrous cap on top of the atheromatous lipid pool. These lesions are called atheromas or fibrofatty plaques, and may, depending on their size and accompanying enlargement or shrinkage of the vessel wall, impede the blood flow, leading to ischemia of the distal regions [fig. 2A] [14,15].

Fibrofatty plaques may develop into advanced lesions, which contain a large lipid core as a consequence of ongoing foam cell accumulation, cholesterol crystals, necrotic and apoptotic cells and calcification. Vascular calcification is a prominent feature of advanced atherosclerosis, and the extent of coronary calcification ("calcium score") has been found to add incremental prognostic significance to conventional risk factors for coronary artery disease [16,17]. Vascular calcification causes a reduction in elasticity and compliance of the vessel wall. Calcification of blood vessels and heart valves generally occurs with advanced age. Whether calcifications destabilize the plaque causing plaque rupture and thrombosis or stabilize the plaque and prevent rupture is under debate. Echolucent plaques, i.e. plaques that contain a lipid-rich core without calcifications, have been associated with increased risk of stroke and cerebrovascular events [18]. Compared to unstable plaques, stable plaques contain smaller atheroma size with lower density of macrophages and higher density of VSMCs covered by a thicker fibrous cap [19]. Even though the presence of calcium in the plaque indicates the presence of advanced atherosclerosis, it apparently does not decrease the stability of the atherosclerotic plaque as calcification does not increase fibrous cap stress in ruptured or stable human coronary atherosclerostic lesions [20]. Furthermore, patients with extensive calcification of carotid artery plaques are less likely to have symptomatic disease than patients with less calcification [21].

Plaque rupture

The development of atherosclerosis is a slow process, and decades can pass before the lesions are extensive enough to impede blood flow and cause symptoms. However, atherosclerosis may also develop without causing significant stenosis due to compensatory vessel widening [14]. Most acute coronary events occur in patients with non-obstructive luminal disease [22]. Acute clinical events, such as myocardial infarction and stroke, are often the result of sudden rupture of the atherosclerotic lesion causing thrombus formation. Lesions that are prone to rupture usually have a thin, collagen-poor fibrous cap overlaying a large lipid core containing a high number of inflammatory cells, and relatively few smooth muscle cells [23]. Rupture of the atherosclerotic lesion generally occurs at the shoulder regions of the lesion, where the concentration of lipid-filled macrophages is high and the fibrous cap is weak. Plaque rupture exposes lipids and procoagulant tissue factor to the circulation, resulting in thrombus formation [fig. 2B] and subsequent clinical events. Cardiovascular events generally appear around the age of 55 in men, and beyond the age of 60 in women.

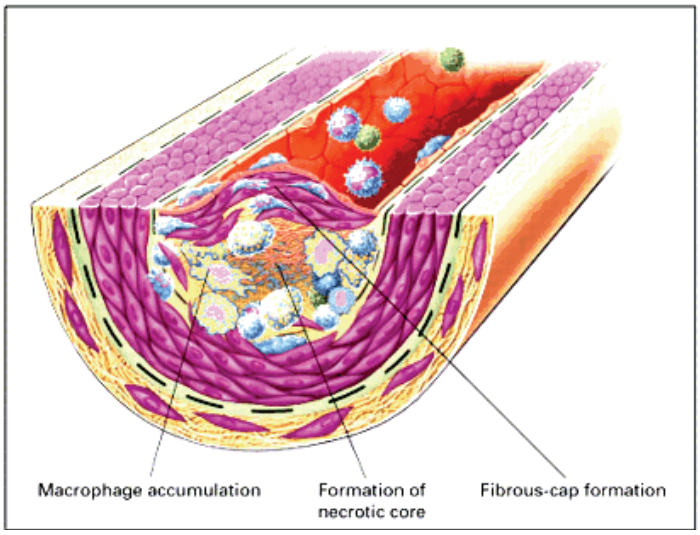

Figure 2A. Formation of an advanced, complicated lesion of atherosclerosis. As fatty streaks progress to intermediate and advanced lesions, they tend to form a fibrous cap covering the lesion. The fibrous cap covers a mixture of macrophages, lipid, and debris, which may form a necrotic core.

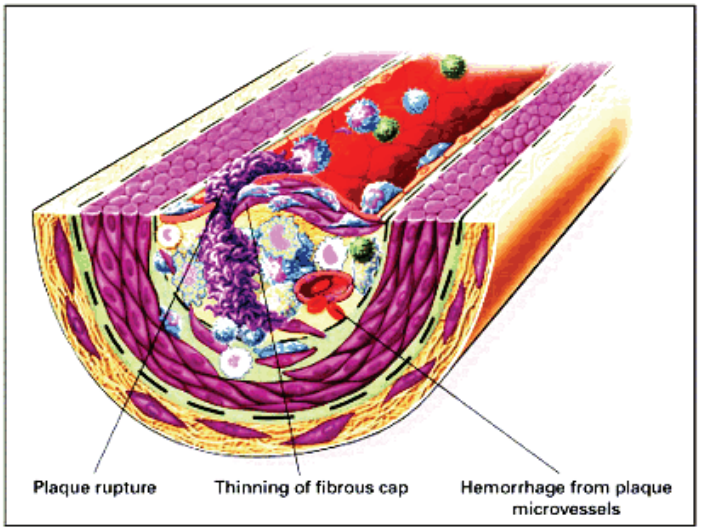

Figure 2B. Unstable fibrous plaques in atherosclerosis. Rupture of the fibrous cap or ulceration of the fibrous plaque leads to thrombosis and usually occurs at sites of thinning of the fibrous cap that covers the advanced lesion [3].

Inflammation and atherosclerosis

Recognition of the significant role of inflammation in the development of atherosclerosis has dramatically changed our understanding of the pathophysiology of CVD in the last decade [3,24]. Basic research has established an elementary function of inflammation in all stages of atherosclerosis development, starting from endothelial dysfunction and fatty streak formation to advanced complex lesions, ruptured plaques and, subsequently, thrombotic involvement [3].

Even though plasma lipoprotein levels are a major risk factor for the development of CVD, plasma total cholesterol levels poorly predict risk of CVD: many cardiovascular events occur in individuals with below-average plasma cholesterol levels [25]. Plasma levels of inflammatory markers and soluble cell adhesion molecules can be quantified and are nowadays used as predictors of cardiovascular risk [26-28]. Of the inflammatory markers studied, the acute phase

marker C-reactive protein (CRP) and fibrinogen have recently emerged as sensitive markers of CVD risk [29-31].

CRP is a member of the highly conserved pentraxin family, and is produced mainly by hepatocytes in response to infection or inflammation. Its production is stimulated by cytokines such as interleukin-6 (IL-6), interleukin-1 (IL-1) and tumor necrosis factor-α (TNFα). CRP binds to phosphatidylcholine in cell membranes and plasma lipoproteins, in a Ca^{2+}-dependent manner. CRP has a role in opsonization of infectious agents and damaged cells [32,33]. The plasma level of CRP is considered to reflect the inflammatory condition of the patient and/or the vessel wall. In the general population, presymptomatic, baseline plasma CRP levels < 1 mg/L are associated with a low risk for CVD, levels between 1 to 3 mg/L indicate average risk, and CRP levels > 3 mg/L are associated with an increased risk of myocardial infarction and stroke [34]. Among patients with acute coronary syndromes plasma CRP values > 3 mg/L are associated with increased risk of coronary events. However, CRP values > 10 mg/L can reflect a wide range of pathological states. Thus, if patients are presenting with CRP levels exceeding 10 mg/l, CRP can no longer be used to predict their risk of atherothrombotic events [34].

Moderately elevated CRP levels are now an established predictor of an increased risk of CVD. Whether CRP is actively contributing to the process of atherosclerosis, the primary etiologic factor underlying CVD, remains under debate. Several *in vitro* data indicate that CRP may have a causal role in atherosclerosis. CRP activates endothelial cells to produce adhesion molecules and induces production of monocyte chemoattractant protein-1 (MCP-1) that facilitates leukocyte adhesion and diapedesis. CRP also contributes to the migration of smooth muscle cells, enhances uptake of native LDL by macrophages, and activates complement [35-41]. Furthermore, cells in the atherosclerotic lesion have been reported to produce CRP [42]. However, several *in vitro* studies have yielded contradictory results. In apolipoprotein E deficient mice CRP, has been reported to both accelerate atherosclerosis [43], and to have no effect at all on atherosclerosis development [44]. In cholesterol-fed and Watanabe heritable hyperlipidemic (WHHL) rabbits CRP levels strongly correlate with the extent of atherosclerosis, and CRP is ubiquitously present in the atherosclerotic lesions of these rabbits. However, the CRP found in these lesions is mostly derived from the circulation and not produced within the lesion [45]. Therefore, further studies to investigate the role of CRP in atherosclerosis are warranted.

Treatment of atherosclerosis

Elevated levels of plasma lipoproteins are a risk factor for the development of atherosclerosis. Therefore, lipoprotein metabolism has been one of the main focuses of research aiming at the prevention of CVD.

Lipoprotein metabolism

Cholesterol and triglycerides are the most important lipids in the circulation. The body obtains cholesterol and triglycerides via the diet and through endogenous synthesis. Cholesterol is a constituent of cell membranes, and essential in the endogenous synthesis of bile acids and steroid hormones. Triglycerides are either stored in adipose tissue, or are lipolysed to glycerol and free fatty acids and used as an energy source for cardiac and skeletal muscle. Since cholesterol and triglycerides are hydrophobic molecules, they are packaged into lipoproteins to be transported in hydrophilic environments such as the blood and lymph.

There are five different classes of lipoproteins; chylomicrons, very low-density lipoproteins (VLDL), low density lipoproteins (LDL), intermediate density lipoproteins (IDL) and high-density lipoproteins (HDL). Lipoproteins consist of a lipid-rich core of triglycerides and esterified cholesterol and a surface layer of free cholesterol, phospholipids and apolipoproteins. Most of the cholesterol in the plasma is contained in LDL.

Besides high LDL-cholesterol concentrations, elevated levels of VLDL and IDL predict risk for developing of atherosclerosis. The oxidation of LDL in the vascular wall is considered to be an important pro-atherogenic process. The reverse cholesterol transport from the periphery to the liver by HDL is an important anti-atherosclerotic process. HDL also counteracts the oxidative modification of LDL [46].

HMG-CoA-reductase inhibitors

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase catalyzes the rate-limiting step in cholesterol synthesis [fig 3] and has been an important target for the development of cholesterol-lowering drugs. By inhibiting the endogenous cholesterol synthesis in the liver, HMG-CoA reductase inhibitors (statins) stimulate the upregulation of LDL receptors by the liver, thereby enhancing clearance of LDL cholesterol from the circulation, resulting in a reduction of plasma cholesterol levels by 25-55%. Statins were first introduced into clinical practice in the end of the 1980s, and can be grouped into two subtypes: 1) the fermentation-derived or natural statins (lovastatin, pravastatin, and simvastatin) and 2) the synthetic statins (atorvastatin, cerivastatin, fluvastatin, and rosuvastatin). The different statins vary in their potency to lower serum cholesterol levels [47].

The beneficial effects of statins on cardiovascular diseases, in terms of both primary and secondary prevention, are now widely recognized. Most of these effects can be attributed to strong LDL-cholesterol lowering effects, however, non-lipid effects (so-called pleiotropic effects) have also been described [23,48,49]. These pleiotropic effects include positive modification of endothelial function, anti-inflammatory effects, increased plaque stability, and reduced thrombogenic response (for review [50-52]).

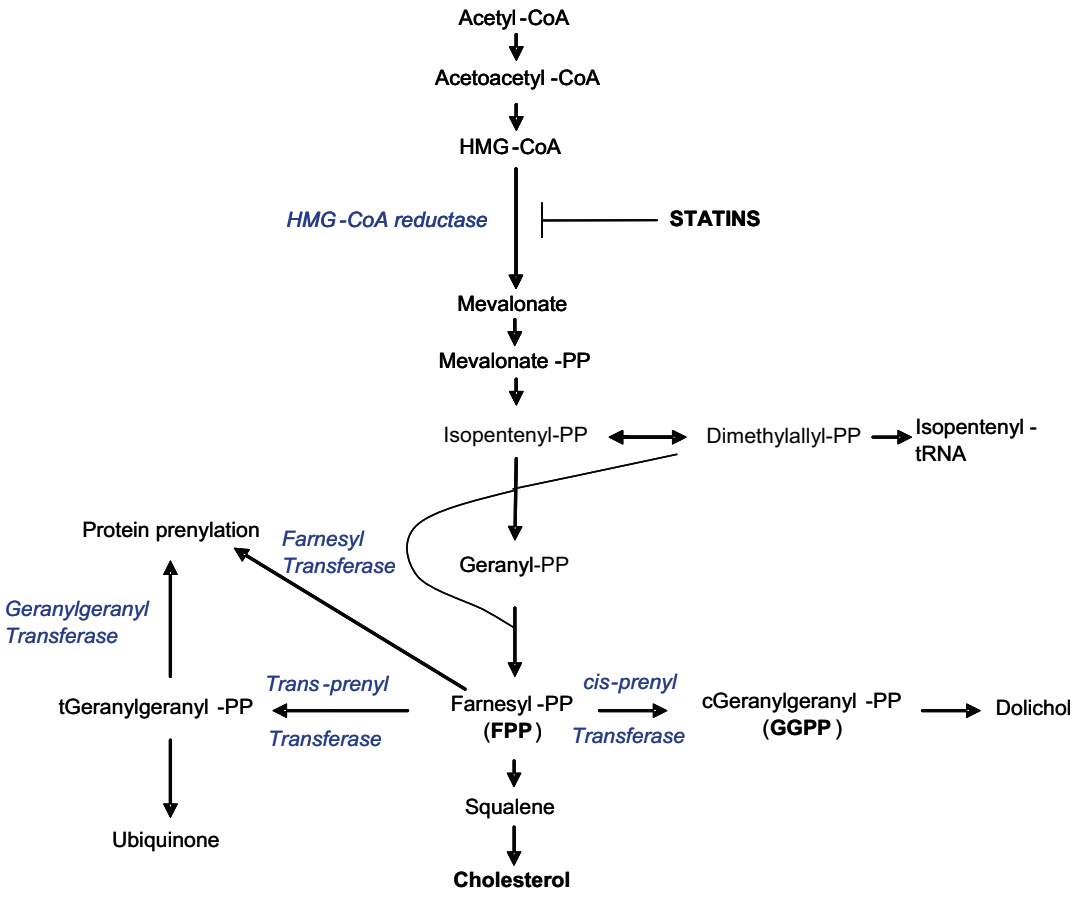

Figure 3. Schematic representation of the pathway of cholesterol synthesis, including the mevalonate pathway, the main enzymes (italics), mevalonate metabolites, and the site of action of the HMG-CoA reductase inhibitors. PP = pyrophosphate.

The pathway leading to cholesterol synthesis also includes other, less well-known, compounds such as the polyisoprenoids, farnesyl phosphate and geranylgeranyl phosphate [fig. 3]. These polyisoprenoids can bind to proteins (prenylation), thereby altering their functions in several ways. Isoprenoid intermediates such as the aforementioned farnesyl- or

geranylgeranyl-phosphates are important in activation of small GTP-binding proteins including Rho, Ras and Rac through isoprenylation [48,53]. Inhibition of protein prenylation by statins may have direct effects on cells which are independent of lipid-lowering [48,53], thereby contributing to the pleiotropic effects observed with statin therapy.

Other lipid-lowering drugs include fibrates, bile acid sequestrants and nicotinic acids. Fibrates are a widely used class of lipid-lowering drugs, which substantially decrease plasma triglycerides and are usually associated with a moderate decrease in LDL cholesterol and an increase in HDL cholesterol concentrations [54]. Bile acid sequestrants interrupt the enterohepatic circulation of bile acids. By non-specific binding of bile acid sequestrants to bile in the intestines, resorption is inhibited. This decrease in bile acid resorption causes an increase of hepatic bile acid synthesis, at the expense of the hepatic cholesterol pool. Subsequently LDL receptors are upregulated to supply this extra demand in cholesterol leading to lowering of plasma cholesterol levels [55]. Nicotinic acid is a vitamin B that has been shown, in high doses, to lower plasma total cholesterol, LDL-cholesterol and VLDL-triglycerides, while raising HDL-cholesterol levels. Its exact mechanism of action is not known, but nicotinic acids appear to inhibit hepatic VLDL production [56].

Calcium antagonists

The primary action of calcium channel blockers, also called calcium antagonists (CAs), is to reduce blood pressure by blocking calcium transport into the VSMC. This is achieved by inhibiting Ca^{2+} influx through voltage-gated transmembrane channels.

Free Ca^{2+} ions are required for contraction of the myocardium and the arterial wall. Driven by an enormous gradient of roughly 10,000:1 Ca^{2+} ions immediately enter the cell whenever suitable membrane Ca^{2+} channels open. CAs inhibit this process, thus inhibiting smooth muscle contraction. Several types of Ca^{2+} channels are present in the membrane of VSMCs. The L-type voltage-gated Ca^{2+} channel (L-VGCC) is the major transsarcolemmal Ca^{2+} pathway opened by depolarisation. The L-VGCC exists in 3 states – resting, open and activated, closed and inactivated – that are controlled by depolarisation and phosphorylation. Other types of calcium channels are: T-type VGCC, receptor–operated channels (ROC) and non-selective cation channels (NSC). Stretch-activated Ca^{2+} channels (SAC) have been postulated [57,58]. The L-type VGCC is the target of the CAs used in this thesis.

Calcium ions play critical roles in physiological and pathophysiological signal transduction in VSMCs. Therefore it is reasonable to assume that CAs influence the process of atherosclerosis and vascular calcification. The lipophilic CA amlodipine has been shown to restore cholesterol-induced membrane bilayer abnormalities in VSMCs derived from the atherosclerotic rabbit aorta [59,60], thereby restoring normal calcium homeostasis. Other proposed mechanisms through which CAs may affect atherosclerosis development include

inhibition of proliferation and migration of VSMCs [61-64], and inhibition of lipoprotein oxidation [65,66]. In addition, CAs modify binding of monocytes to the endothelium and activate synthesis of matrix components [67]. The effects of CAs on atherosclerotic calcification have not been widely studied however.

Besides causing vasodilatation through inhibition of calcium channels, long-acting CAs, such as amlodipine, have been demonstrated to produce clinical benefits in patients with coronary artery disease that might be independent of changes in blood pressure [66,68,69]. Several studies have demonstrated that the anti-atherosclerotic effect of CAs was limited to the first stages of atherosclerosis [64,70]. Pre-existing lesions were not influenced by CA therapy as far as angiographic progression or regression was concerned. More recent data have demonstrated that the CA amlodipine had no effect on the progression of atherosclerosis or cardiovascular events, but was associated with a reduction in cardiovascular morbidity [71]. These data failed to support the hypothesis that amlodipine alters the development or progression of minimal coronary artery lesions [71]. However, the recently published CAMELOT study has demonstrated that administration of amlodipine to patients with coronary artery disease (CAD) and normal blood pressure resulted in less cardiovascular events, and assessment of atherosclerosis progression with intravascular ultrasound showed that treatment with amlodipine slowed progression of atherosclerosis [72]. Therefore, the anti-atherosclerotic effects of CAs remain under debate.

Novel actions of amlodipine have been described which suggest that some of its atheroprotective effect may be due to its unique physical and pharmacokinetic properties. Amlodipine is highly lipophilic which enables this drug to partition into the cell membrane. In VSMCs derived from the atherosclerotic rabbit aorta amlodipine restores cholesterol-induced membrane bilayer abnormalities [59,60]. Because of the possible anti-atherosclerotic effects of CAs, in this thesis we studied the effects of the CA amlodipine on VSMC calcification and early atherosclerotic lesion development.

Other anti-hypertensive drugs include angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, beta-blockers and diuretics. Besides lipid-lowering and anti-hypertensive agents, anti-thrombotic drugs (including aspirin) are also an important treatment in the reduction of the risk of CVD or recurrent events.

Coronary interventions

Since pharmacotherapy is not always effective in relieving a patient from his or her symptoms, several vascular interventions have been developed. These vascular interventions include percutaneous transluminal coronary angioplasty (PTCA), i.e. balloon dilation of a coronary artery often in combination with stent placement, and coronary artery

bypass grafting (CABG) where a piece of autologous, healthy vessel is used to bypass the coronary artery seriously affected by atherosclerosis. These procedures are proven to be very successful, although restenosis after PTCA and thrombosis after CABG may occur during follow-up. (In-stent) Restenosis is caused by excessive proliferation of VSMCs after balloon-induced vascular injury and may even block implanted stents or vein grafts. In 15 to 60% of all patients treated with PTCA or CABG restenosis occurs [73], although the use of stents that slowly release novel drugs that inhibit proliferation of VSMCs reduces restenosis considerably [74,75].

Models of atherosclerosis

In vitro models

There are two major advantages of using cultured cells; the first advantage is the control of experimental variables, and the second advantage is the reliable, manipulatable, and consistent source of relatively large quantities of biological material that is often needed for cellular and molecular studies. Three major cell types are involved in atherosclerosis development, namely endothelial cells, monocytes/macrophages and VSMCs. All three cell types have been successfully isolated and cultured *in vitro*, and are used in atherosclerosis research. In this thesis we have used neonatal VSMCs, as this cell type resembles the VSMCs with the synthetic phenotype that are generally present in atherosclerotic lesions [76].

In vivo models/mouse models

Animal models are a useful tool for research, since genetic variability and differences in environmental factors can be minimized by using inbred strains and similar housing conditions. In humans, genetic variability and differences in life-style often interfere with the disease process studied.

Several animal models for atherosclerosis development have been described. These include cholesterol fed monkeys, rabbits, rats and mice [77,78]. However, monkeys are legally protected for being used in animal experiments in many countries. Rabbits develop atherosclerosis upon cholesterol feeding, but they do not exhibit complex lesions. The use of mice is favoured over the use of rats because of the fact that mice can be made transgenic more easily. The use of mice has more advantages, such as easy breeding and short generation time.

There are also some drawbacks to using mouse models for atherosclerosis research. Lipid and lipoprotein metabolism is dissimilar between mice and humans. The main lipoprotein class in mice is the anti-atherogenic HDL. In humans the predominant lipoproteins are the proatherogenic VLDL and LDL. Wild-type mice are therefore highly resistant to

atherosclerosis and do not develop atherosclerotic lesions spontaneously. However, certain mice are susceptible to diet-induced atherosclerosis such as the inbred strain C57Bl/6 [79]. When these mice are fed a cholesterol containing diet, lipoprotein distribution changes from HDL toward VLDL and LDL, inducing fatty streak formation after several months on the diet [80]. By overexpression or knockout of specific genes, mouse models have been generated which are more suitable for the study of hyperlipidemia and atherosclerosis than wild-type mice. These mouse models include LDL-receptor knockout mice, ApoE knockout mice and ApoE*3-Leiden transgenic mice. Of these mouse models the ApoE*3-Leiden transgenic mouse is one of the most useful models for investigating genetic and environmental factors, and the effects of drugs and dietary intervention on hyperlipidemia and/or atherosclerosis [81].

*AopE*3-Leiden transgenic mice*

Apolipoprotein E (ApoE) is a major component of plasma lipoproteins and has a high affinity for the LDL receptor and other receptors such as the LDL receptor related protein (LRP) and VLDL receptor. The ApoE*3-Leiden mutation is a dominant negative mutation of ApoE. This rare mutation is associated with familial dysbetalipoproteinemia in humans. Introduction of this mutation in a mouse results in a model with defective clearance of ApoE containing lipoproteins, such as VLDL and IDL, resembling human familial dysbetalipoproteinemia. These heterozygous ApoE*3-Leiden transgenic mice exhibit significant elevations of plasma cholesterol and triglyceride levels when fed a normal mouse diet. When feeding these mice a semi-synthetic Western-type diet with high fat/cholesterol levels, plasma cholesterol levels rise considerably. Depending on diet composition, plasma cholesterol and triglyceride levels can vary between 3-40 mmol/L and 0.5-4.5 mmol/L, respectively. On a cholesterol-rich diet, ApoE*3-Leiden mice develop atherosclerosis, which is correlated to plasma cholesterol levels and cholesterol exposure [82]. Because these mice develop different types of lesions ranging from fatty streaks to severe lesions depending on the amount of cholesterol exposure [83], this mouse model is very suitable for studying atherosclerosis progression and regression. Furthermore, these lesions resemble the lesions found in human pathology.

Not all aspects of atherosclerosis can be studied in mouse models. In mice, the development of atherosclerosis is not influenced by the coagulation and fibrinolytic systems, and spontaneous plaque rupture does not occur. In humans plaque rupture and subsequent thrombus formation are the most important causes of atherosclerosis-induced cardiovascular events.

Scope of this thesis

The presence of calcium deposits in the vessel wall is indicative of advanced atherosclerosis, and the extent of coronary calcification has been found to add prognostic significance to conventional risk factors of coronary artery disease. Vascular calcification reduces elasticity and compliance of the arterial wall. Calcification of blood vessels and heart valves generally occurs with advanced age.

Vascular calcification is a prominent feature of atherosclerosis. However, the mechanisms underlying vascular calcification are still obscure. The major objective of the work described in the first part of this thesis was to elucidate the mechanisms involved in atherosclerotic calcification. **Chapter 2** summarizes the literature on calcification in atherosclerosis and the involvement of VSMCs in this process. To further study the process of VSMC calcification we developed and characterized an *in vitro* model of neonatal rat VSMC calcification (**Chapter 3**). To investigate whether pharmacotherapy may affect vascular calcifications, we have studied the effect of a calcium antagonist (amlodipine) and a statin (atorvastatin) and their combination (**Chapter 4**) on this process.

Inflammation is an important mechanism in the atherosclerotic process, and prospective and cross-sectional clinical and epidemiological studies have shown that CRP is consistently associated with CVD. In the second part of this thesis we focused on the involvement of the acute-phase marker CRP in atherosclerosis development. In **Chapter 5** the causality of CRP in atherosclerosis is discussed. To enable the study of the effect of CRP on atherosclerosis development *in vivo*, ApoE*3-Leiden/hCRP transgenic mice were generated and studied (**Chapter 6**). In **Chapter 7** the effects of a calcium antagonist (amlodipine), administered either alone or in combination with a statin (atorvastatin), on early atherosclerosis development in ApoE*3-Leiden/hCRP was investigated.

Chapter 8 is a summary of the thesis and discusses future perspectives in this area of research.

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