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

Trion, A.

# Chapter 2

# Vascular smooth muscle cells and calcification in atherosclerosis

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## Abstract

Vascular calcification is a prominent feature of atherosclerosis, but the mechanisms underlying vascular calcification are still obscure. Since the detection of bone associated proteins such as osteonectin, osteocalcin and matrix Gla protein in calcified vascular tissues, calcification is considered to be an organized, regulated process similar to mineralization in bone tissue. Vascular smooth muscle cells (VSMCs) are currently considered to be responsible for the formation of vascular calcifications. Apoptosis of VSMCs appears to be a key factor in this process, while other factors including cell-cell interactions (macrophages and VSMCs), lipids, and plasma inorganic phosphate levels modulate the calcification process. The focus of this review is on the role of VSMCs in the development of calcifications in atherosclerotic plaques.

Keywords: atherosclerosis, vascular smooth muscle cells, calcification

## Introduction

Cardiovascular disease due to atherosclerosis is the major cause of mortality and morbidity in the Western hemisphere. Atherosclerosis is characterized by the presence of atherosclerotic lesions in the arterial intima leading to narrowing of the vessel lumen. 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 genetic factors [1].

Given the high prevalence of cardiovascular disease, research has focussed for a long time on the severity of luminal coronary stenosis, its risk factors and its clinical consequences [2]. However, atherosclerosis may develop without causing significant stenosis due to compensatory vessel widening [3], and even then plaques can bear significant risks. In fact, most acute coronary events occur in patients with non-obstructive luminal disease [4].

The presence of calcium deposits in the vessel wall is indicative 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 [5,6]. Vascular calcification causes a reduction in elasticity of the vessel wall and reduced compliance. Calcification of blood vessels and heart valves generally occurs with advanced age. In high-risk asymptomatic adults the presence of calcification in one or more coronary arteries was shown to increase the risk of a coronary death or nonfatal infarction [7]. Vascular calcification may occur in the intima, as is the case in atherosclerosis, and/or media (medial or Mönckeberg's sclerosis) of the vessel wall. Vascular calcification due to Mönckeberg's sclerosis has been reviewed elsewhere and will not be discussed in this paper.

Several cell types are involved in different stages of the atherosclerotic process. Initially the endothelium and monocytes are involved. In later stages VSMCs contribute to the development of an atherosclerotic lesion by migration, proliferation, and secretion of matrix components. VSMCs are currently considered to be responsible for the formation of vascular calcifications.

## Characteristics of vascular smooth muscle cells

VSMCs are highly specialized cells that maintain vascular tonus by contraction or relaxation. Accelerated proliferation of VSMCs is known to play an integral role in atherosclerotic lesion formation and in postangioplasty restenosis, and it is a characteristic feature in arteries of hypertensive patients and animals [8,9]. Even though many factors have already been identified that may play a role in the regulation of VSMC growth, such as growth factors (EGF, FGF, PDGF), interleukins, angiotensin II and thrombin (for review see [10]), little is known regarding the mechanisms and factors that regulate changes in the differentiated state of the VSMCs in vascular disease. There is considerable interest in the cellular and molecular regulation of VSMC differentiation, since it is critical to the understanding of atherosclerosis.

Mature VSMCs have been shown to express unique isoforms of a variety of contractile proteins that are important for their differentiated function. Smooth muscle α-actin (sm-α-actin) is the single most abundant protein in VSMCs [fig. 1A]. Smooth muscle myosin heavy chain (sm-MHC) has also been found to be a highly specific marker of the SMC lineage [fig. 1B] [11,12]. Calponin is a 28-34 kDa protein that has been postulated to function as a regulator of SMC contraction. Rat aortic VSMC express calponin [fig 1C].

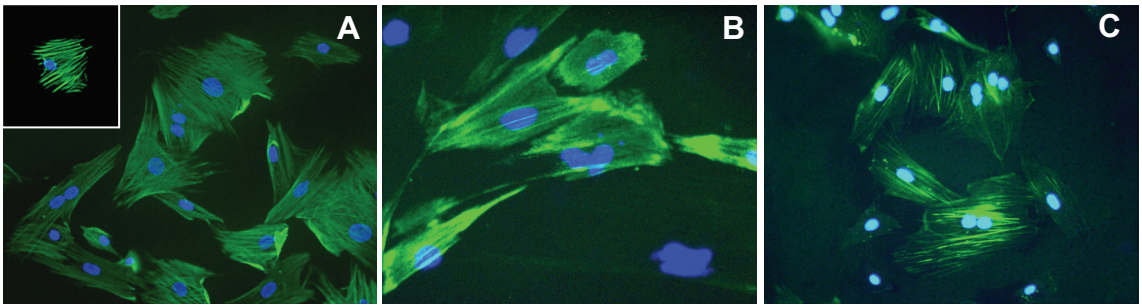


**Figure 1.** VSMCs were isolated by outgrowth from rat aortic explants and characterized by immunocytochemistry. Antibodies used; anti-smooth muscle α-actin (A and insert), anti-smooth muscle myosin (B), and anti-calponin (C). Original magnification 200x. VSMC = vascular smooth muscle cell.

Apparently subpopulations of VSMCs exist in the vessel wall. Lemire et al. have identified at least two types of SMCs isolated from the rat aorta vessel wall [13]. Studies in porcine artery confirm that subpopulations of SMCs are present in the vessel wall [14]. When normal porcine coronary artery SMCs were grown in culture, two subpopulations with distinct phenotypes were noticed. These different SMC phenotypes may explain why some media-derived SMCs

start to migrate and become the "dedifferentiated" synthetic phenotype in response to "atherosclerotic and calcifying stimuli", whereas other SMCs in the media remain contractile SMCs. In neointimal SMCs cultured from rat aorta injured with a balloon catheter two distinct phenotypes have been found that vary with regard to the presence and quantity of proteins such as sm-α-actin, sm-MHC and desmin [15]. The isolation of a clone of calcifying vascular cells (CVC) [16], cells that are particularly prone to calcification, suggests that besides a contractile and a synthetic phenotype, a calcifying SMC phenotype may exist in the vessel wall.

## Calcification of vascular smooth muscle cells

Vascular calcification is a prominent feature of atherosclerosis. Vascular calcification refers to the deposition of calcium phosphate mineral, most often in the form of hydroxyapatite, in the vessel wall. Calcification may occur in the intima (atherosclerosis) and media (medial or Mönckeberg's sclerosis) of the vessel wall, as well as in heart valves, and is associated with ageing, diabetes and uremia.

Arterial calcification was previously thought to be a degenerative, end-stage process of vascular disease. However, the presence of proteins that are associated with bone formation such as bone morphogenetic proteins [17,18], osteonectin, osteocalcin and matrix Gla protein in calcified vascular tissues suggests that calcification is an organized, regulated process similar to mineralization in bone tissue. Also supporting this hypothesis is the presence of vesicle-like structures in the matrix of the atherosclerotic plaque [19]. Matrix vesicles are membrane-bound structures which bud-off from chondrocytes and osteoblasts. Matrix vesicles are commonly found in cartilage and bone and are thought to serve as nucleation sites for calcium crystal growth [20].

The characteristics of calcifying cells and the roles of several factors in the process of calcification have been, and are, studied in atherosclerotic plaques obtained from arteries of humans and experimental animals, as well as in VSMCs cultured *in vitro*. To study the factors responsible for phenotypical changes in calcifying VSMCs, we developed an in vitro model for VSMC calcification using a rat or mouse aortic explant method. After optimising the culture conditions we are now able to reproducibly produce calcifications within 21 days [fig. 2].

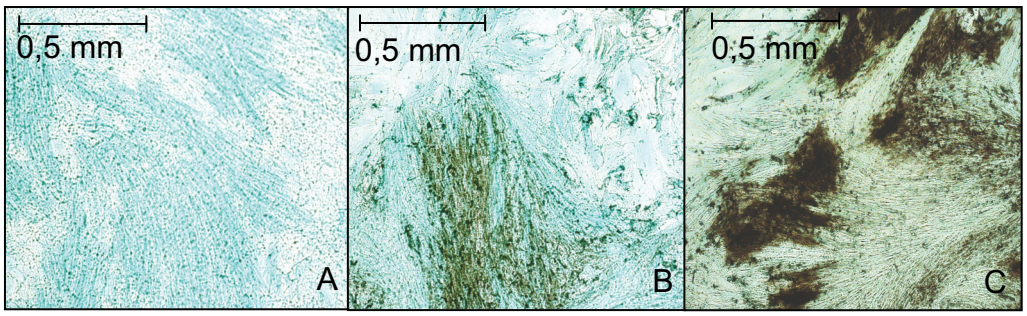


**Figure 2**. Von Kossa staining for detection of calcification (black = positive staining) of rat VSMCs. VSMCs were cultured in calcification medium consisting of DMEM (high glucose, 4.5 g/L) containing 15% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), 6 mmol/l $CaCl_2$, 10 mmol/L sodium pyruvate, $10^{-6}$ mol/L insulin, 50 μg/ml ascorbic acid, 10 mmol/L β-glycerophosphate and $10^{-7}$ mol/L dexamethazon for 3 weeks (B) or 4 weeks (C). Panel A depicts cells that were cultured in control medium which consists of DMEM (high glucose), penicillin, streptomycin and 15% fetal calf serum. DMEM = Dulbecco's modified Eagle's medium; VSMC = vascular smooth muscle cell.

Besides VSMCs, pericytes have been linked to vascular calcification [21]. Normally pericytes are located in the microvasculature where they share a basement membrane with endothelial cells and contribute to the 'tightness' of capillary permeability. Pericytes are also involved in angiogenesis. Evidence is accumulating that pericytes can function as progenitor cells and are capable of differentiating into osteoblasts, chondrocytes, adipocytes and SMCs. Pericyte-like cells have been found in the inner intima, outer media and vasa vasorum of arteries. Although pericytes may play a role in the calcification process observed during atherosclerosis, our review will focus on the involvement of VSMC in this process.

### *Bone formation and bone associated proteins in atherosclerotic lesions*

Given the similarities between vascular calcification and bone formation, it is useful to briefly outline the process of bone formation. Bone formation is an active process and it involves the production of an extracellular matrix that is permissive of mineralisation. Chondrocytes produce a layer of cartilage. As the layer thickens, older chondrocytes degenerate leaving spaces that osteoblasts invade. Osteoblasts are specialised cells which produce a bone matrix on top of the original cartilage base. Collagen I is an important constituent of the extracellular matrix that facilitates calcification. The production of bone is influenced by growth hormone and insulin-like growth factors.

$Ca^{2+}$ is a major building block of bone and $Ca^{2+}$ is essential in cell biology. The plasma $Ca^{2+}$ concentration is regulated by three hormones that influence the movement of $Ca^{2+}$ between bone, kidney and intestines. These hormones are parathyroid hormone, vitamin $D_3$

and calcitonin. Osteoclasts are large multinucleated cells derived from monocytes, are responsible for bone resorption, and can help restore the $Ca^{2+}$ concentration in the blood when it becomes too low.

In human atherosclerotic calcification the expression of mineralization-regulating proteins has been documented. In human plaque examined by in situ hybridization, bone morphogenetic protein 2-a (BMP2-a) mRNA was found to be expressed [17]. BMP2-a is a potent factor for osteoblastic differentiation and its presence in atherosclerotic plaque suggests that arterial calcification is a regulated process similar to bone formation.

Another protein that may be involved in vascular calcification is osteopontin (OPN). OPN is an arginine-glycine-aspartate (RGD) containing acidic phosphoprotein normally restricted to bone matrix and it has been implicated in bone morphogenesis. OPN is present focally in human atherosclerotic plaques but absent in non-diseased arteries [22]. OPN is synthesized by cells within the plaque, i.e. SMCs, endothelial cells, and macrophages [23]. Moreover, OPN is co-localized with sites of early calcification in the plaque, providing support for the role of OPN in vessel wall calcification [23].

Several bone-associated proteins that are constitutively expressed at low levels in the healthy vessel (e.g. matrix Gla protein) can become up-regulated in association with vascular calcification. Moreover, the expression of a number of bone-associated proteins not normally found in the vessel wall was also increased in association with vascular calcification [24]. The latter proteins include alkaline phosphatase (ALP), bone sialoprotein, and bone Gla protein. In atherosclerotic lesions both VSMCs and macrophages express OPN and matrix Gla protein [25]. It has been hypothesised that in the healthy vessel wall calcification is inhibited by inhibitor proteins but if inhibition is lost, e.g. by the presence of activators, or when the equilibrium between inhibition and initiation of calcification is otherwise disturbed, the vessel wall will calcify [26,24].

So, VSMCs express many of the calcification-regulating proteins commonly found in bone. The precise role of these proteins in the calcification process is not clear yet. However, many of these proteins have calcium and apatite binding properties and accumulate in areas of vascular calcification, where they may serve a variety of functions including regulation of apatite crystal nucleation and growth.

### *Matrix vesicles, apoptosis and calcification*

Matrix vesicles have been shown to be the initial site of calcification in cartilage, bone and other tissues. A matrix vesicle is a submicroscopic extracellular membrane-invested particle that serves as the initial site of calcification in all skeletal tissues, including growth plate cartilage, embryonic bone and adult bone.

The biogenesis of matrix vesicles is accomplished primarily by polarized budding and pinching-off from the plasma membranes of chondrocytes, osteoblasts and odontoblasts. Their diameters range from 30 nm at the smallest to 1 μm or more (approx. 200 nm) [27]. After polarized release from the cell, the matrix vesicles soon become encased and immobilized in the extracellular matrix. Due to continuous synthesis and secretion of extracellular matrix, the matrix vesicles move away from the cell and become centers of calcium crystal growth. The regulation of matrix vesicle production is not yet fully understood but it is thought to be associated with apoptotic cell death.

Apoptosis and apoptotic bodies have also been linked to vascular calcification. Human VSMCs spontaneously form multicellular nodules and deposit calcium crystals after approximately 28 days in culture [28]. Also, vesicle-like structures can be identified within these nodules. It has been suggested that VSMC-derived apoptotic bodies are able to concentrate and crystallize calcium in a form that is found *in vivo* [29]. Subsequently the hypothesis that apoptosis initiates vascular calcification has been tested. Inhibition of apoptosis also inhibited vascular calcification and stimulation of apoptosis promoted vascular calcification, providing a strong link between apoptosis and initiation of vascular calcification [29].

In carotid endarterectomy specimens with advanced atherosclerosis small membrane-bound vesicles of varying size were detected, shed from apoptotic VSMCs, and these remnants were enclosed by a cage of thickened basal lamina. These vesicles resemble the matrix vesicles seen in bone, and subsequent calcification of these vesicles may be considered *"the graves of SMCs that have died in the plaques"* [19].

### *Cytokines and growth factors*

Tumor necrosis factor α (TNF-α) is a pleiotropic cytokine, which has been shown to play a role in both vascular and bone pathophysiology. TNF-α is mainly secreted by macrophages in response to factors such as oxidized or acetylated LDL, physically damaged extracellular matrix, or bacterial infection. TNF-α influences many aspects of atherosclerosis by increasing the permeability of endothelial cells, promoting monocyte adhesion, inducing macrophage differentiation, and promoting foam cell formation. Since TNF-α is a regulator of bone formation and since vascular calcification resembles bone formation, the role of TNF-α in in vitro vascular calcification has been investigated [30]. Calcifying vascular cells (CVCs) (a cloned subpopulation of bovine aortic SMCs previously shown capable of osteoblastic differentiation) were treated with TNF-α and the effects of TNF-α on differentiation and mineralization of CVCs were determined. TNF-α caused CVCs to change from an elongated to a cuboidal shape. Treatment with TNF-α also induced a dose-dependent increase in ALP activity, a marker of osteogenic differentiation of osteoblastic cells. Further investigation

provided evidence that in CVCs TNF-α signals via the cAMP pathway [30]. So, TNF-α promotes CVC mineralization by increased expression and activity of ALP, an enzyme that has been shown to be important for matrix mineralization.

Vitamin $D_3$ is a fat-soluble hormone present in the diet. It can also be produced in the skin after exposure to sunlight. Vitamin $D_3$ is a precursor of the hormone 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$). This hormone enhances the intestinal absorption of calcium. It supports mobilization of $Ca^{2+}$ from the bone and can also exert a direct effect on VSMCs that express vitamin D receptors. $1,25(OH)_2D_3$ stimulates $Ca^{2+}$ influx into VSMCs and inhibits proliferation of VSMCs. In a study about the effect of $1,25(OH)_2D_3$ on in vitro calcification of VSMCs it was demonstrated that $1,25(OH)_2D_3$ promotes calcium deposition by depressing endogenous parathyroid hormone-related peptide (PTHrP) expression. Parathyroid hormone (PTH) and PTHrP have been shown to inhibit bovine VSMC calcification through depression of ALP activity [31].

*Macrophages and vascular smooth muscle cell calcification*

Inflammation is an important aspect of atherosclerosis. Inflammatory cells such as macrophages and T lymphocytes are frequently found in atherosclerotic plaques. Interaction between inflammatory cells, VSMCs, endothelial cells and extracellular matrix may contribute to the development of plaque calcification.

The interaction between macrophages and VSMCs is considered to modulate the phenotypic changes of VSMCs resulting in atherosclerotic calcification [32]. One of the factors involved in this interaction is the production of $1,25(OH)_2D_3$ by macrophages since monocytes and tissue macrophages are capable of synthesizing $1,25(OH)_2D_3$ when stimulated with interferon-γ. Since $1,25(OH)_2D_3$ promotes VSMC calcification [31], it is likely that local production of $1,25(OH)_2D_3$ by macrophages is involved in atherosclerotic calcification.

Activated monocytes produce osteoinductive factors that stimulate the differentiation of neighbouring CVCs [33]. The osteoinductive activity of monocyte-conditioned media could be blocked partially by a neutralizing antibody to TNF-α, indicating that besides $1,25(OH)_2D_3$, TNF-α produced by monocytes is an osteoinductive factor [32].

Apoptosis may be another mechanism by which macrophages influence VSMC calcification. Boyle et al. [34] found that macrophages are potent inducers of VSMC apoptosis. In this process cell-cell contact was required and was at least in part mediated by Fas/FasL interactions. Since apoptosis has been demonstrated to promote VSMC calcification and macrophages induce VSMC apoptosis, macrophage-VSMC interaction might be a mechanism by which atheroscleotic calcification is enhanced.

*Lipids*

Several lines of evidence suggest a role for lipids in atherosclerotic calcification. Histological studies have demonstrated a close association of lipids with calcification in atherosclerotic lesions. Recently it was reported that hyperlipidemia in LDL-receptor knockout mice fed a high fat diet was associated with active induction of vascular calcification [35]. Delipidation of bioprosthetic valves significantly reduces their calcific degradation when implanted in rats [36], which supports the view that lipids play a significant role in the process of calcification.

Oxidized lipids and lipoproteins induce fatty streak formation, and evidence for their presence in the lesion has been clearly demonstrated [37]. The effects of several atherogenic oxidized lipids on differentiation and mineralization of calcifying vascular cell (CVC) cultures have been tested. Minimally oxidized LDL (MM-LDL) was shown to promote calcification of CVC. This was accompanied by an increase in ALP activity and collagen I production, both markers of osteoblastic differentiation. Also, a change from spindle to cuboidal cell morphology and decreased proliferation was observed. These effects of MM-LDL were due to products of lipid oxidation since native LDL did not cause these effects [38]. So, lipid oxidation products are important regulators of atherosclerotic calcification *in vitro*, and probably also *in vivo*.

*Phosphate regulation and vascular calcification*

Bone formation requires the precipitation and attachment of calcium phosphate crystals to the extracellular matrix. The most common form of calcium phosphate is hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). Therefore, inorganic phosphate (Pi) is essential for the formation and the mineralization of bone. A positive correlation exists between the plasma Pi level and the rate of bone growth and/or mineralization. Calcification of the media of peripheral arteries (Mönckeberg's sclerosis) commonly occurs in aged and diabetic patients. High Pi levels are thought to influence the development of medial calcification and therefore research has focused on the effect of high Pi levels on VSMCs.

Because 1) vascular calcification is a common finding in atherosclerosis, 2) vascular calcification is frequently observed in diabetic and uremic patients, and 3) hyperphosphatemia is correlated with vascular calcification, the ability of Pi levels to regulate mineralization of human aortic smooth muscle cell (HSMC) cultures was examined [39]. HSMCs cultured in media containing physiological levels of Pi (1.4 mmol/L) did not mineralise. However, HSMCs cultured in media containing Pi levels comparable to those observed in hyperphosphatemic individuals (>1.4 mmol/L) showed a dose-dependent increase in mineral deposition. Elevated levels of Pi also enhanced the expression of

osteoblastic differentiation markers, like osteocalcin and Osf2/Cbfa-1 in these cells. The effect of elevated phosphate levels on HSMCs was shown to be mediated by a sodium-dependent phosphate co-transporter. Phosphate uptake by a sodium-dependent phosphate co-transporter leads to an increased expression of the bone specific transcription factor Cbfa-1. So, elevated Pi concentrations may directly stimulate HSMCs to undergo phenotypic changes that predispose to calcification, which may explain why hyperphosphatemic conditions predispose for vascular calcification.

Vascular calcification is considered to be regulated by Pi transport and by Pi released by the action of ALP [40]. Pi directly increases HSMC calcification in a concentration- and time-dependent manner [39].

Another mechanism by which elevated Pi levels might stimulate calcification is through the induction of apoptosis in vascular cells. The hypothesis that Pi activates osteoblast apoptosis was tested on isolated osteoblast-like cells from explants of human bone [41]. These cells showed an osteogenic phenotype in culture, exhibiting raised ALP activity, expressing osteogenic mRNA transcripts and depositing biological mineral. When these cells were treated with 1-7 mmol/L Pi, there was a dose- and time-dependent decrease in cell viability. It was confirmed that cell death was caused by apoptosis [41]. So, since 1) high levels of Pi induce apoptosis in osteoblasts-like cells, 2) VSMCs in atherosclerotic plaque exhibit many properties resembling osteoblasts, and 3) apoptosis has been linked to calcification, apoptosis may be a mechanism by which high Pi levels influence vessel wall calcification. Whether high Pi levels are indeed able to induce apoptosis in calcifying VSMC remains to be investigated.

## Discussion

The detection of calcification is still improving, and the actual mechanisms underlying vascular calcification are slowly emerging. It is obvious that atherosclerotic calcification is a multifactorial process which involves many different cell types and signalling proteins. The process of vascular calcification appears to be a regulated process similar to bone formation as demonstrated by the presence of bone-associated proteins, such as BMP2a, matrix Gla protein and osteocalcin, in the atherosclerotic plaque. On the systemic level, circulating lipids and Pi levels are involved in the development of atherosclerotic calcification. On the cellular level, interactions between macrophages and VSMCs in the plaque may promote calcification.

It is unclear whether vascular calcifications act as a plaque destabilizer by facilitating plaque rupture and the development of thrombosis, or whether calcifications actually stabilize the plaque and prevent rupture. Calcification does not increase fibrous cap stress in typical

ruptured or stable human coronary atherosclerostic lesions. Lipid pools however were shown to dramatically increase stresses within the plaques [42]. In patients with coronary artery disease (CAD) who have been treated intensively with lipid-lowering drugs (niacin, lovastatin and colestipol) for 10 years, magnetic resonance images of carotid atherosclerotic plaques showed a smaller lipid deposit area (a measure of atheroma size) and higher calcium cluster area (a measure of calcification) compared to patients with CAD who were matched for age and baseline levels of LDL-cholesterol and triglycerides but who have never been treated with lipid-lowering drugs [43]. Carotid plaque size did not differ between the two groups of patients. Given the plaque-stabilizing effects of HMG-CoA reductase inhibitors (statins) reported by several groups (see reviews [44,45]), an increase of calcification at the expense of atheroma is apparently beneficial in terms of less major adverse cardiovascular events during follow-up of statin-treated patients [46]. Supporting these findings is the observation that patients with extensive calcification of carotid artery plaques are less likely to have symptomatic disease [47]. Echolucent plaques, i.e. plaques that contain a lipid-rich core were associated with increased risk of stroke and cerebrovascular events [48]. 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 [49]. A mechanism by which statins stabilize plaques may include apoptosis of macrophages and proliferation of VSMCs that produce extracellular matrix. The presence of calcium in the plaque indicates the presence of advanced atherosclerosis, but apparently it does not decrease the stability of the atherosclerotic plaque. The ways by which vascular calcification is modified pharmacotherapeutically are not fully explored yet, and deserve further study.

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