

## Arterial calcifications

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### Abstract

Arterial calcifications as found with various imaging techniques, like plain X-ray, computed tomography or ultrasound are associated with increased cardiovascular risk. The prevalence of arterial calcification increases with age and is stimulated by several common cardiovascular risk factors. In this review, the clinical importance of arterial calcification and the currently known proteins involved are discussed. Arterial calcification is the result of a complex interplay between stimulating (bone morphogenetic protein type 2 [BMP-2], RANKL) and inhibitory (matrix Gla protein, BMP-7, osteoprotegerin, fetuin-A, osteopontin) proteins. Vascular calcification is especially prevalent and related to adverse outcome in patients with renal insufficiency and diabetes mellitus. We address the special circumstances and mechanisms in these patient groups. Treatment and prevention of arterial calcification is possible by the use of specific drugs. However, it remains to be proven that reduction of vascular calcification in itself leads to a reduced cardiovascular risk.

**Keywords:** arterial calcification • proteins • cardiovascular risk • renal insufficiency • diabetes mellitus

### Introduction

Arterial calcification is often seen in studies using various imaging techniques. Although radiological techniques to visualize calcification are emerging, observing calcification is usually a coincidence on images performed for indications other than detecting calcified arteries. These calcifications are not as innocent as doctors tend to believe. They usually harbour an increased vascular risk [1]. Deposits of calcium in the vascular wall can be detected with X-ray. When vascular structures on X-ray images of the chest, the abdomen or extremities have similar density compared to bone, calcification is diagnosed. These images, however, are not valid for quantification of calcium. Using computed tomography (CT), the density of pixels is expressed as Hounsfield units and calcium is usually suspected when a pixel exceeds 130 Hounsfield units. At least in the coronary arteries, this technique is currently the only validated technique for quantification of vascular calcification

[2]. Calcification of the vascular wall has been shown to predict increased cardiovascular risk, independent of the classical cardiovascular risk factors [3–6].

Vascular calcification consists of calcium salt precipitates, mostly in apatite form, similar to the hydroxyapatite found in bone. Several risk factors which are associated with the presence or progression of vascular calcification have been identified [3, 7]. In the past vascular calcification was seen as an inert end-point of atherosclerosis, however, recently it has become clear that it is an actively regulated process already occurring in the early stages of atherosclerotic lesions [8–10].

Many new pathways regulating calcification are being discovered. Recent research suggests important roles for activation of receptor tyrosine kinase Ax1, transglutaminase pathways or stimulation of the calcium receptor on vascular smooth muscle cells

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(VSMCs) [11–13]. In this review, we will focus on the currently known proteins involved in arterial calcification. These findings advance our understanding of the arterial calcification process and provide us with possible future interventions.

## Prevalence of calcifications and relation to cardiovascular outcome

Calcification of the arteries is usually detected with plain X-ray or CT. Prevalence is highly dependent upon the studied population. In a population-based cohort of over 100,000 men and women (mean age 47 years; 30–89 years range), who had a chest X-ray for screening purposes, prevalence of calcification in the aorta was 1.9% in male patients and 2.6% in female patients [3]. Calcification results in an increase of aortic stiffness and hence contributes to systolic hypertension and left ventricular hypertrophy, coronary insufficiency, ischemia and congestive heart failure [14–17]. Although calcification of the thoracic aorta commonly increases with age, the presence of calcifications increases the risk for cardiovascular events independently of age [3]. Most of the risk factors associated with calcification of the aortic arch are also well known cardiovascular risk factors (Table 1). Despite this similarity, aortic arch calcification itself is an independent predictor of cardiovascular risk [3]. Recent research showed changes in the mechanical properties of the atherosclerotic lesion and increased inflammation in response to calcification, which may increase the risk of plaque rupture [3, 18, 19]. In contrast to intimal calcification, medial calcification (also known as Mönckeberg's sclerosis) exclusively involves VSMCs in the absence of inflammation and lipid infiltration. Electron-beam computed tomography (EBCT) and multi-slice computed tomography (MSCT) are reproducible methods to measure coronary calcifications [2, 20]. Among a healthy American population aged 40 to 45 years, prevalence of coronary artery calcifications (CAC) was 19.2% in white patients and 10.3% in Afro-American patients [21]. In another study among healthy persons older than 40 years, CAC prevalence was 29% among men and 19% among women [22]. Strikingly, the prevalence of vascular calcification was higher in men in every decade except between 70 and 80 years. Firstly this difference can be explained by the more favourable risk profile in women in the premenopausal phase causing calcification to develop at an older age. Secondly, men with excess coronary calcification at a younger age might already have died due to their increased cardiovascular risk. The presence of CAC in asymptomatic individuals has been shown to be associated with an increased risk for cardiovascular end-points. Over a variable follow up period from 3 to 4.3 years and depending on the characteristics of the studied population, the relative risk for any coronary event varied from 2.6 in low-risk females to 11.8 in healthy men aged 40 to 50 years [4, 5, 23, 24]. Calcification of the abdominal aorta as seen with CT is more frequently observed in the presence of older age, hypertension, coronary artery disease and peripheral vascular disease [25]. Additionally, the area of calcification on X-ray films of the abdominal aorta is positively correlated with age, systolic blood pressure

**Table 1** Risk factors associated with aortic arch calcification as determined in 116,309 patients [3]

Risk factor	Odds ratio (95% confidence interval)*
Age	Men 2.74 (2.58–2.91)
	Women 3.52 (3.32–3.74)
African descent	Women 1.35 (1.11–1.63)
No college education	Men 1.17 (1.00–1.36)
	Women 1.31 (1.14–1.50)
Total cholesterol > 6.6 mmol/l	Women 1.28 (1.06–1.55)
Current smoking	Men 1.30 (1.10–1.53)
	Women 1.16 (1.01–1.33)
Hypertension <sup>†</sup>	Men 1.27 (1.11–1.46)
	Women 1.38 (1.23–1.54)

\*Only significant correlations are depicted.

<sup>†</sup>This might also be caused or aggravated by vascular calcification.

and aortic stiffness [26]. Limited data exist on the association between femoral artery calcification and cardiovascular mortality. However, in patients with type 2 diabetes, femoral artery calcification is an independent predictor of cardiovascular morbidity and mortality [27, 28].

At present it is not clear whether measuring vascular calcification with EBCT, MSCT or plain X-ray can be used as a cost-effective strategy for cardiovascular risk stratification. A disadvantage of these techniques is the considerable amount of radiation to which patients or, indeed, asymptomatic individuals are exposed.

## Pathobiology of arterial calcification

Experimental animal as well as *in vitro* studies have revealed several proteins playing a role in the calcification process [29–33]. Unravelling the function and the mechanism of action of these proteins has been a topic for many researchers in the past few years. Some proteins have been identified as inhibitors of calcification, whereas others promote vascular calcification. VSMCs migrated from the media to the intimal layer of the vasculature lose their contractile phenotype, and change into so-called synthetic VSMCs. When these VSMCs become apoptotic in the atherosclerotic lesion they may form the nidus for calcification [34, 35]. Moreover, VSMCs can change their phenotype upon calcification and develop features of osteoblast- or chondrocyte-like cells with respect to gene expression [36]. Table 2 shows proteins involved in calcification subdivided according to their calcification-inhibiting or -promoting properties. Below we discuss these proteins and their biological properties.

**Table 2** Proteins involved in arterial calcification

Inhibitor proteins of arterial calcification	Promoter proteins of arterial calcification
MGP	BMP-2
BMP-7	RANKL
OPG	
Fetuin-A	
OPN	

### Matrix Gla protein (MGP)

MGP is a 10 kD vitamin K-dependent protein, first discovered by Price *et al.* [37]. It is produced by VSMCs and chondrocytes, and accumulates at sites of calcification. Its production is stimulated by an increase in local calcium levels [38]. There is an active and an inactive form depending on whether or not the protein has been carboxylated (*i.e.* activated) by a vitamin K-driven  $\gamma$ -glutamyl carboxylation. The function of MGP is believed to be a regulator of bone morphogenetic protein type 2 (BMP-2), but it can also bind directly to calcium crystals in the vascular matrix, thereby preventing further calcification growth [39]. Animal studies show that a deficiency or impairment of MGP (blocking vitamin K action by coumarins) lead to rapid and extreme calcification of the vascular matrix [40, 41]. In the human 'Keutel syndrome', an autosomal recessive disorder in which patients lack mature MGP, excessive calcification of large arteries is seen [42]. Circulating uncarboxylated MGP levels are inversely proportional to coronary calcification and were significantly lower in patients who underwent PTCA *versus* a healthy control population [43, 44]. Several experimental studies suggest that MGP, produced in the vascular matrix, is transported to plasma in combination with fetuin-A, forming the fetuin-A-mineral complex [45, 46]. Whether uncarboxylated MGP levels are a reliable reflection of the calcification process in the vascular wall is not fully clear, although from the previously mentioned studies it seems that patients with high cardiovascular risk have lower serum MGP levels [47]. One advantage of MGP is that the protein's activity completely depends upon vitamin K, and thereby can be modulated by extra vitamin K intake. On the other hand, blocking carboxylation of MGP (*i.e.* with coumarines) or low vitamin K levels (*i.e.* deficient intake) results in excessive calcification [48–50].

### Bone morphogenetic protein

BMPs are members of the transforming growth factor (TGF)- $\beta$  superfamily, and play key signalling roles in the maintenance and repair of bone and other tissues in the adult. Their role in vascular

calcification is complex. When VSMCs change their phenotype from contractile to synthetic, they enter a state of proliferation in which the expression of smooth muscle markers is diminished. Additionally, they produce large amounts of extracellular matrix proteins and may become osteoblast-like cells. This reduction in smooth muscle marker expression is thought to be crucial in the pathogenesis of atherosclerosis and M<sup>u</sup>nckeberg's sclerosis. The loss of smooth muscle markers can be influenced by BMPs. Two BMPs, BMP-2 and BMP-7, have been extensively studied in relation to vascular calcification [51, 52]. Expression of BMP-2 is found in atherosclerotic lesions, in peri-adventitial myofibroblasts and tunica media cells. Induction of BMP-2 in the vasculature is related to oxidative stress, inflammation, oxidized lipids and hyperglycaemia [53–55]. Increased expression of BMP-2 stimulates the osteoregulatory gene MSX-2. Then core binding factor-1 (Cbfa-1 or RUNX2) and osterix, both transcription factors, stimulate differentiation of multipotent vascular mesenchymal cells into 'osteoblast-like' cells capable of bone formation and increased intramembranous bone formation in the artery wall [51, 56, 57]. The effect of BMP-2 on bone formation is suggested to be modulated by MGP [31, 58]. Diminished VSMC expression of MGP or inactive MGP may lead to unopposed BMP-2 action and hence vascular calcification. Another important inhibiting mechanism of BMP-2 is mediated by the Smad-6 gene [59]. Smad-6 gene expression is limited to the heart and the vasculature. Interruption of Smad-6 gene function leads to calcification, only in the areas where it is expressed, suggesting an important modulating role of BMP-2 function.

Strikingly, BMP-2 is associated with a decrease in smooth muscle cell markers whereas BMP-7 promotes the VSMC phenotype. The exact mechanism of this difference in action of these very similar proteins is not yet known, although it is mediated by induction of Smad-6 amongst others [60]. BMP-7 promotes increased bone formation and phosphate deposition in bone. High serum phosphate levels and vascular calcification are thus prevented. In chronic kidney disease (CKD) sufficient levels can reverse arterial calcification. However, in patients with CKD, BMP-7 levels (mainly produced by the kidney) are low [61]. A potential protective effect against vascular calcification in these patients might, therefore, be compromised resulting in excessive calcification.

### Osteopontin (OPN)

OPN is an acidic extracellular phospho-protein. Phosphoserines within OPN are negatively charged amino acids, and have a strong affinity for hydroxyapatite. It is normally present in mineralized tissues like bones and teeth. Mice lacking OPN are susceptible to vascular calcifications [32]. OPN regulates mineralization in two different ways. On the one hand it inhibits apatite crystal growth, on the other it promotes osteoclast function. In normal arteries OPN is absent, whereas in calcified plaques it is abundantly present. Research suggests that OPN is important in regulating calcification when the artery is injured [62].

## RANKL

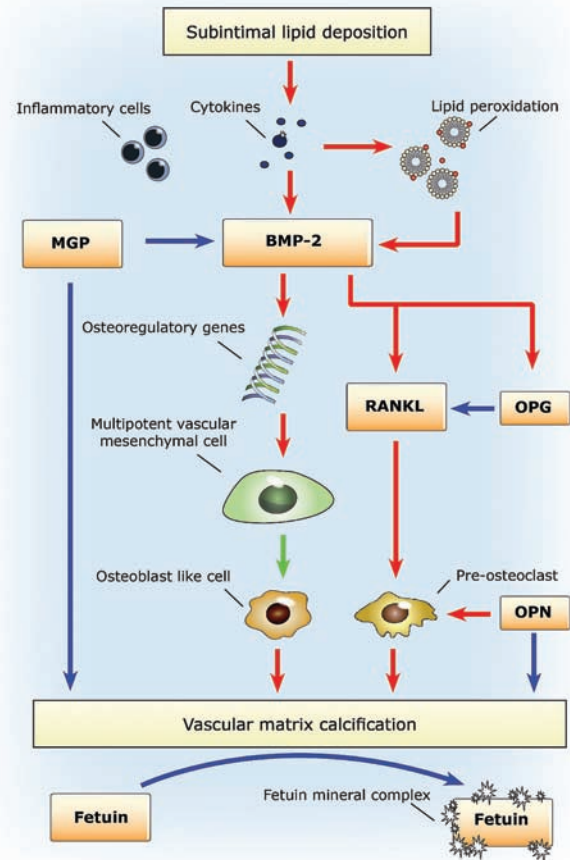
RANKL is a 316-amino acid transmembrane protein. It is highly expressed by T-cells in lymphoid tissue and by osteoblasts in trabecular bone. RANKL binds to RANK, a 616-amino acid transmembrane receptor which is present amongst others in osteoclasts and their precursors. This binding generates multiple intracellular signals that regulate cell differentiation, function and survival. RANKL action can be blocked by osteoprotegerin (OPG), thereby inhibiting vascular calcification. RANK/RANKL/OPG belong to the tumour necrosis factor (TNF)- $\alpha$  family. RANKL is up-regulated in osteoblasts by  $1\alpha$  25-dihydroxyvitamin D<sub>3</sub>, parathyroid hormone (PTH), glucocorticoids, prostaglandin E<sub>2</sub>, interleukin-1 $\alpha$ , TNF- $\alpha$ , interleukin-6, interleukin-11, interleukin-17, calcium or immunosuppressants like cyclosporin A. Down-regulation of RANKL is mediated through transforming growth factor  $\beta$ . In animal studies RANKL increases osteoclast size and function. Disruption of RANKL results in inhibition of osteoclast formation and function [63–66].

## Osteoprotegerin

OPG is a 380 amino acid acting as a soluble (decoy)-receptor for RANKL. It is produced by many tissues in the body, including the cardiovascular system. OPG expression is particularly high in VSMCs and vascular endothelial cells of the aorta and the renal arteries. It prevents the binding of RANKL to RANK [63, 65]. OPG is increased by some of the stimuli that also increase RANKL and by oestrogen, TGF- $\beta$  and BMP-2. Decreased levels are seen with increased PTH levels, glucocorticoids, prostaglandin E<sub>2</sub>, insulin-like growth factor-1 or immunosuppressants. A steady balance between RANKL and OPG prevents disorders in bone remodelling and vascular calcification. Low OPG expression leads to unopposed RANKL binding to RANK and thus osteoporosis and vascular calcification, whereas high OPG expression leads to osteopetrosis [67, 68]. Clinically, high serum levels of OPG are associated with atherosclerosis or risk factors for atherosclerotic disease indicating a compensatory increase in OPG levels in response to progressive atherosclerosis and thus OPG may lessen vascular calcification [68–70].

## Fetuin-A

Fetuin-A is a serum glycoprotein produced in the liver and present in high serum concentrations (0.4–1.0 g/l) [71]. In end-stage renal disease patients the role of fetuin-A has been extensively studied. With a molecular weight of 56 kD it is non-dialysable. It acts as a negative acute phase protein and is a powerful calcification inhibitor [71–73]. Together with MGP, fetuin-A is able to make up a complex with calcium and phosphate thereby transporting and clearing the insoluble calcium-phosphate salt, and preventing its extra skeletal deposition [74]. In transgenic fetuin-A deficient mice (fetuin-A<sup>-/-</sup> mice), extra skeletal calcification, including soft tissue and peri-vertebral arterial calcification develop [72]. Large arteries



**Fig. 1** Blue arrows indicate an inhibiting effect and red arrows indicate a stimulating effect.

are spared from calcifications, most likely because of up-regulation of other potent calcification inhibitors such as MGP and OPN [75]. In dialysis patients, serum fetuin-A negatively correlates with CAC [76]. It has been shown that low fetuin-A levels were associated with higher all-cause and cardiovascular mortality [77–79]. This effect was partially mediated by inflammation, according to its correlation with higher levels of C-reactive protein.

If there is an imbalance between the calcification protective and calcification inducing factors, progressive vascular calcification can be the result. Figure 1 depicts the different proteins and their role in the calcification process. First the conventional vascular risk factors result in intimal damage and sub-intimal lipid deposition. Subsequently, the inflammatory response induced leads to increased BMP-2. If not balanced by active (carboxylated) MGP, multipotent mesenchymal vascular cells are stimulated to differentiation into 'osteoblast-like' cells. At this point the presence of sufficient active MGP is important both for blocking the action of BMP-2 and for binding directly to calcium crystals in the vascular matrix [31, 39, 80, 81]. The use of vitamin K antagonists and/or a low vitamin K diet results in an increase of



dysfunctional MGP and thus favouring calcification [41, 82]. In addition, the interactions among RANKL, OPN and OPG influence the rate of calcification. (Fig. 1)

## Arterial calcification in specific patient populations at high risk for cardiovascular disease

### Chronic kidney disease

Elevated circulating levels of phosphate (P), calcium (Ca) and calcium phosphate product ( $P \times Ca$ ) are frequently encountered in dialysis patients and are associated with increased vascular calcifications [83–85]. The calcification process is not exclusively influenced by P and Ca. Many traditional risk factors, such as hypertension and hyperlipidaemia, and non-traditional atherosclerotic risk factors, including lipid oxidation, the presence of advanced glycation end-products, calcitriol and inflammation may affect VSCM-associated calcification, but their precise roles await clarification [53, 86–88]. Although the role of PTH in bone formation is eminent, the importance of PTH for vascular calcification in dialysis patients is less clear. Some studies show an association between PTH levels and vascular calcification, whereas others do not [84, 89, 90]. In addition, associations may differ between groups using calcium-containing or calcium-free phosphate binding therapy [91]. It has been suggested that vascular calcification under uremic conditions may act as a natural 'stent' by 'stabilizing' plaques [92, 93]. Arterial disease would then lead to less acute ischemic events, but more chronic ischemia and fibrosis through progressive luminal obliteration [92]. Arterial media calcification is mostly localized in muscle-type conduit arteries such as femoral and tibial arteries. These lesions in their most pure form do not obstruct the arterial lumen. This form of calcification has been associated with increased arterial stiffness and mortality [94, 95].

### Diabetes mellitus

Type 2 diabetes mellitus (DM2) patients suffer from increased rates of cardiovascular mortality and morbidity, especially in women, due to their extra unfavourable risk profile compared to men with DM2 [96, 97]. Medial artery calcification, *i.e.* Mönckeberg's sclerosis, is often seen and is associated with age and the severity of hyperglycaemia [98, 99]. The arteries are stiffened but not occluded [100]. OPG is increased in the tunica media but not in the intima of the vasculature of patients with diabetes [101]. Increased serum levels of OPG are associated with higher HbA1c levels and hypertension in DM2 patients and might be a marker of progressive atherosclerosis with accompanying calcification [102]. The latter might be related to overwhelming risk factors

(high glucose levels, high blood pressure or unfavourable lipid profile) for calcification or compromised function of other protective proteins and mechanisms [98, 103]. Clinically, patients with diabetes with medial calcifications have a significant excess risk for total mortality, stroke mortality and cardiovascular mortality than patients without. They also had a significantly higher incidence of coronary heart disease events, stroke events and lower extremity amputations [27, 28].

### Treatment and prevention of calcifications

Currently there is no evidence-based treatment regimen that can reduce calcification of large arteries. Experiments using calcium channel blockers or statins have not been convincing [104, 105]. Although the effect of treating cardiovascular risk factors on the progression of large artery calcification has not yet been evaluated, it seems reasonable to treat patients according to current guidelines because of their increased cardiovascular risk. Some current medical treatments, however, may be associated with an increased risk of calcification. For example, treatment of patients for recurrent thrombosis with coumarins results in accelerated calcification [49, 50]. Similarly, in end stage renal disease, treatment of hyperphosphatemia with phosphate binders that contain calcium have been associated with more CAC compared to treatments with non calcium-based phosphate binders [106].

## Summary

Arterial calcification is an actively regulated process, which involves different triggers and proteins. Patients with calcification of large arteries have an increased cardiovascular risk when compared to similar patients without calcification. A possible mechanism is that large artery calcification leads to increased arterial stiffness and reflects atherosclerotic burden. Therefore the presence of large artery calcification can be seen as an additional risk factor for cardiovascular events. Although there are no trials that prove risk reduction from aggressive risk management in patients with arterial calcifications, such patients probably benefit when cardiovascular risk factors like hypertension and dyslipidaemia are identified and treated according to current guidelines. Moreover, in patient groups with a strongly elevated risk for arterial calcification, patient-specific measures could possibly prevent arterial calcifications. Whether this will also result in a reduction of cardiovascular risk remains to be proven.

## Conflict of interest

The authors confirm that there are no conflicts of interest. There was no commercial funding involved.

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