Role of vitamin K-dependent proteins in the arterial vessel wall

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Summary
Vitamin K was discovered early last century at the same time as the vitamin K-antagonists. For many years the role of vitamin K was solely ascribed to coagulation and coagulation was thought to be involved only at the venous blood side. This view has dramatically changed with the discovery of vitamin K-dependent proteins outside the coagulation cascade and the role of coagulation factors at the arterial side. Vitamin K-dependent proteins are involved in the regulation of vascular smooth muscle cell migration, apoptosis, and calcification. Vascular calcification has become an important independent predictor of cardiovascular disease. Vitamin K-antagonists induce inactivity of inhibitors of vascular calcification, leading to accelerated calcification. The involvement of vitamin K-dependent proteins such as MGP in vascular calcification make that calcification is amendable for intervention with high intake of vitamin K. This review focuses on the effect of vitamin K-dependent proteins in vascular disease.

Vitamin K
Vitamin K is a fat-soluble vitamin and belongs to the family of vitamins A, D and E. For most people, vitamin K is the least known vitamin and even regarded as the Cinderella (1). After the discovery of vitamin K in the early days of last century clinicians and scientists believed that blood coagulation was the only physiological process in which vitamin K played a role. It was the Danish researcher Dam who discovered that chickens fed a fat-free diet suffered from serious bleedings. It took some time to isolate the micronutrient responsible for this action, and it is now known as vitamin K after the German word “Koagulation”.

In the 1970s it was discovered that vitamin K is a cofactor in the carboxylation reaction. Simultaneously Stenflo et al. (2) and Nehestuen et al. (3) reported the discovery of the unusual amino acid γ-carboxyglutamate acid (Gla) in prothrombin as the product of vitamin K action. These groups independently identified the unequivocal role of vitamin K as a cofactor for the post-translational carboxylation of glutamate (Glu) residues. This carboxylation-step is accomplished by an enzyme called gamma glutamyl-carboxylase (GGCX) (4), and requires a pro-peptide containing protein.

This process is driven by the oxidation of reduced vitamin K into vitamin K-epoxide. The vitamin K-epoxide must be recycled to vitamin K before it can be reused. This reaction is catalyzed by the enzyme vitamin K epoxide reductase (VKOR) (5, 6). In this way the efficiency of vitamin K is very high:

\[
\text{One molecule vitamin K can assure some 500 carboxylation reactions.}
\]

Recently, the group of Oldenburg added a new role for the VKOR enzyme in that the subunit VKORC1L1 is responsible for driving vitamin K-mediated intracellular antioxidation pathways critical to cell survival (7).

Dietary vitamin K
Vitamin K is an essential dietary micronutrient since man cannot synthesize it. Although in our gut flora some bacteria produce large amounts of vitamin K2 (8) their contribution to the vitamin K-status is

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questionable (9). The recommended daily intake for vitamin K1 is 1 μg/kg body weight and this is solely based on blood coagulation (10).

With the discovery of vitamin K-dependent proteins in extra-hepatic tissues – such as bone and vessel wall – this view needs to be revised. Nutritional vitamin K consists of two forms:
- vitamin K1 and
- vitamin K2.

Vitamin K1 (also called phylloquinone) is found in leafy green vegetables where it is tightly bound to the chloroplast membrane. This results in a poor absorption of vitamin K1 from vegetables (11, 12) and thus its contribution to the vitamin K-status is overestimated.

Vitamin K2 (group name for the menaquinones) is found in fermented foods such as cheese, sauerkraut and the Japanese natto (12) which is derived from the bacteria that are used for the fermentation process. The absorption of vitamin K2 is much better as compared to vitamin K1 (13). The difference between vitamin K1 and vitamin K2 is related to the aliphatic side chain.

After being absorbed in the intestine vitamin K is transported by lipoproteins, as it has no specific carrier protein. The different lipophilicity of K1 and K2 may result in substantial differences in plasma transport, half life and delivery to target tissues (14) (Tab. 1).

**Vitamin K-antagonists (VKA)**

Vitamin K-antagonists are 4-hydroxycoumarin derivatives. VKA were discovered in the early 1920s as a malady of cattle involving fatal bleeding showing up almost simultaneously in the area of Wisconsin (15). It turned out that if cattle ate spoiled hay a loss in the clotting power of the blood and as a result an internal haemorrhage occurred, which usually became fatal. It was Campbell who isolated the crystalline dicoumarol in 1939 (16).

Dicoumarol was launched as rat poison in the early 1940s. After an unsuccessful suicide attempt of an US soldier it became a drug to lower the coagulation tendency of blood. VKA have been given to patients for more than six decades and besides an increased bleeding tendency they are relatively safe. By the year 2000 VKA were the 11th most prescribed drug in the United States (17).

In the USA, warfarin (named after the Wisconsin Alumni Research Federation) is the most used VKA whereas in Europe also phenprocoumon and acenocoumarol are used (Tab. 1).

The action of VKA is to block the VKOR enzyme thereby rapidly exhausting vitamin K tissue stores (18). VKA are used to prevent thrombosis in patients at increased risk for thrombosis. When patients are over-anticoagulated with VKA this can be reversed by a high amount of supplemental vitamin K.

The liver has a very active antidotal pathway for VKA, called the DT-diaphorase (19, 20). This enzyme is some 100-fold less active in bone and vessel wall. Therefore, VKA cause a pronounced vitamin K-deficiency in extra-hepatic tissues (21, 22). With the knowledge of today – 16 vitamin K-dependent proteins are now known, half of them synthesized by tissues other than the liver – the use of VKA may also induce unwanted side effects (Tab. 2).

**Vitamin K-dependent proteins**

It is now known that vitamin K-dependent proteins constitute a family of 16 known proteins with diverse functions, not only involved in the haemostatic pathway (Tab. 2). We will summarize the vitamin K-dependent proteins, in particular those involved in vascular disease.

**Coagulation proteins**

The clotting factors II, VII, IX and X are essential for the coagulation cascade and are γ-carboxylated in the liver to be functionally active. They are well balanced by the anticoagulant factors protein C, protein S and protein Z. These vitamin K-dependent proteins are mainly synthesized and γ-glutamylcarboxylated in the liver, with the exception of proteins S which is synthesized some 45% by endothelial cells (23). Only recently it has been realized that coagulation factors also play an important role in inflammation (24, 25). Minute amounts of the coagulation proteins prothrombin and FVII are synthesized de novo in the vessel wall (24). The inhibition of thrombin by melagatran (direct FIIa inhibitor) reduced atherosclerotic plaque size and features of plaque vulnerability (26).

Protein S acts in the coagulation cascade as a cofactor of activated protein C (APC) in the degradation of FVa and FVIIa.

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**Tab. 1** Vitamin K and vitamin K-antagonists

<table>
<thead>
<tr>
<th>name (trivial name)</th>
<th>primary source</th>
<th>half-life (t₁/₂) in hours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>vitamin K</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>phylloquinone</td>
<td>leafy green vegetables</td>
<td>∼3</td>
</tr>
<tr>
<td>menaquinone</td>
<td>meat, eggs</td>
<td>∼1.5</td>
</tr>
<tr>
<td>-4</td>
<td>natto, cheese</td>
<td>&gt; 70</td>
</tr>
<tr>
<td>-7</td>
<td>cheese, curd, sauerkraut</td>
<td>&gt; 70</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>vitamin K antagonists</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>acenocoumarol (Sintrom)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>dicoumarol (Warfarin)</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>phenprocoumon (Marcumar)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>brodifacum (super coumarin)</td>
<td>&gt; 1500</td>
<td></td>
</tr>
</tbody>
</table>

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Phagocytosis of apoptotic cells is thought to limit the inflammatory response (28). Protein S has been identified as a factor responsible for stimulation phagocytosis of apoptotic cells by macrophages (27). Additionally, protein S regulates the expression and function of scavenger receptor A (SR-A) on macrophages resulting in diminished uptake of acetylated low density lipoprotein (AcLDL) (29, 30).

Recently, a new function of protein S was discovered in vessel wall development. Mice with protein S deficiency die in utero (31). Mutants in which protein S was deleted specifically in hepatocytes – thought to be the major source of circulating protein S – were viable as adults due to the endothelial synthesized protein S. Protein S deleted in endothelial cells revealed that endothelial cells synthesize some 45% of the blood-borne protein S (23). Interestingly, the deficiency in the vessel wall resulted in impaired angiogenesis, independent of protein C (23). Mice with heterozygous protein C deficiency not only exhibit severe coagulation response to endotoxin but also have significant differences in their inflammatory response (32). Additionally, APC has been found to inhibit endotoxin-induced production of TNF-α, IL-1β, IL-6, and IL-8 by cultured monocytes/macrophages (33).

### Extrahepatic vitamin K-dependent proteins

Within the arterial vessel wall vitamin K-dependent proteins are synthesized with functions not related to blood coagulation.

**Gas-6**

Growth arrest specific gene 6 protein (Gas-6) is a vitamin K-dependent protein produced by vascular smooth muscle cells (VSMCs) and involved in a pleiotropic of physiologically processes (34). Gas-6 is associated with binding to its receptor Axl, stimulating the anti-apoptotic protein bcl-2 and inhibiting the pro-apoptotic protein caspase-3. Son et al. (35) showed that Gas-6-Axl signaling protects VSMCs from calcification by inhibiting apoptosis. It has been shown that apoptotic bodies may form a nidus for calcification (36).

**MGP**

The vitamin K-dependent matrix Gla-protein (MGP) is regarded as the strongest inhibitor of vascular calcification (VC) and produced by many cells, including VSMCs. MGP

- promotes VSMC differentiation,
- antagonizes BMP (BMP2 and BMP4) signaling and
- prevents osteochondrogenic lineage reprogramming of VSMCs.

Both a high local and circulating inactive MGP was associated with significantly more VC and cardiovascular death (37, 38). The role of MGP was elucidated in MGP null mice (39). These mice were born normally, but all died within eight weeks after birth from ruptures of the large vessels due to their massive calcification and loss of elasticity. Rescue experiments in MGP null mice demonstrated that MGP acts locally in the vascular tissue as restoration of MGP expression in arteries completely rescued the arterial mineralization phenotype, whereas hepatic MGP expression, resulting in high systemic MGP levels, did not (40).

The crucial role of vitamin K in the inhibition of VC became clear from experiments in which VKA was administered to experimental animals (41). In this model – in which VKA was given in the presence of vitamin K to prevent bleedings – all extrahepatic vitamin K-dependent proteins, including MGP, were synthesized in their inactive uncarboxylated form, resulting in vascular calcifications within 2–4 weeks.

Recently, we found that the VKA treatment also caused increased apoptosis in the

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**Tab. 2** Vitamin K-dependent proteins

<table>
<thead>
<tr>
<th>VKD protein in</th>
<th>tissue of γ-carboxylation (ref.)</th>
<th>function (ref.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>factor II (prothrombin)</td>
<td>liver, limited extra-hepatic (24)</td>
<td>coagulation, regulatory functions in inflammation (24, 25, 26)</td>
</tr>
<tr>
<td>factor VII</td>
<td></td>
<td>coagulation</td>
</tr>
<tr>
<td>factor IX</td>
<td>liver</td>
<td></td>
</tr>
<tr>
<td>factor X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein C</td>
<td>liver (80)</td>
<td>coagulation (25), anti-inflammatory (32, 33)</td>
</tr>
<tr>
<td>protein S</td>
<td>liver and endothelial cells (23)</td>
<td>coagulation (25), anti-inflammatory, phagocytosis and apoptosis (28–30)</td>
</tr>
<tr>
<td>protein Z</td>
<td>liver (81)</td>
<td>degradation of factor Xa (79)</td>
</tr>
<tr>
<td>MGP</td>
<td>bone, cartilage, vascular tissue and macrophages (38, 39, 57)</td>
<td>negative regulator of vascular calcification (37–39)</td>
</tr>
<tr>
<td>Gas-6</td>
<td>VSMCs and endothelial cells</td>
<td>TAM activating ligand (34)</td>
</tr>
<tr>
<td>osteocalcin</td>
<td>EH: primarily osteoblasts (82)</td>
<td>extra cellular matrix protein in bone (78, 82)</td>
</tr>
<tr>
<td>TGF-α inducible protein</td>
<td>most soft tissues</td>
<td>ECM protein</td>
</tr>
<tr>
<td>Gla-rich protein</td>
<td>regulator of mineralisation (83)</td>
<td></td>
</tr>
<tr>
<td>proline-rich Gla proteins</td>
<td>unknown (84)</td>
<td></td>
</tr>
<tr>
<td>transmembrane Gla proteins</td>
<td>most soft tissues</td>
<td>unknown (85)</td>
</tr>
<tr>
<td>periostin</td>
<td>bone marrow mesenchymal stromal cells, cardiomyocytes</td>
<td>bone (86), myocardial (87)</td>
</tr>
</tbody>
</table>
vascular media, which further supports the relation between apoptosis and calcification (36).

The role of vitamin K-dependent proteins has also been studied in patient populations and it confirmed that treatment with VKA induces excessive calcification of the vascular arteries and aortic heart valves (42–44).

Additionally, the duration of VKA treatment seems to correlate with an increase in vascular calcification.

**Vascular calcification**

VC is associated with increased cardiovascular mortality and morbidity, and is recognised as a strong and independent risk factor for cardiovascular death (45–47). The amount of VC, as measured and quantified by multidetector computed tomography is an important predictor of

- all-cause mortality,
- vascular complications and
- myocardial infarctions (45, 48, 49).

Patients with higher coronary artery calcification scores were approximately ten times more likely to have a cardiac event in the next 3–5 years (50). Not only the presence of coronary artery calcification is predictive for cardiovascular outcome, also its annual increase. It was shown that patients with a calcification-progression over 15% per year had a 17.2 fold increased risk of myocardial infarction compared to patients without significant progression (51). The amount of VC is even a stronger predictor than the Framingham risk score (FRS) (52), a well accepted 10-year risk predictor for coronary vascular disease. Clinically, VC causes stiffening of the vascular arteries via elastic fiber and VSMC calcification. The calcification may result in

- decreased arterial compliance,
- development of left ventricular hypertrophy and
- decreased coronary perfusion leading to an increased risk of fatal complications.

In spite of this, calcification of arteries has been neglected and considered to be clinically irrelevant. VC was regarded as an end-stage passive process not amenable to therapeutic intervention (53). However, recent reports demonstrate that punctated and spotty calcification in the atherosclerotic plaque influence stability negatively and render the plaque vulnerable to rupture (54, 55) (Fig. 1).

VC is now appreciated as a complex and actively regulated process involving cells and proteins acting as catalysts and inhibitors (56, 57).

Recruitment of macrophages in the atherosclerotic plaque and consequently their secretion of inflammatory cytokines may serve as a signal for intimal calcification. Indeed Nadra et al. showed that basic calcium phosphate crystals are taken up by macrophages in vitro (58). This was associated with the secretion of the pro-inflammatory cytokines TNF-α, IL-β and IL-8. Furthermore, Pazár et al. showed that basic calcium phosphate induced macrophage IL-1β secretion through activation of the NLRP3 inflammasome (59, 60). Also VSMCs can execute phagocytosis of calcium crystals. Ewence et al. showed that when VSMCs phagocytose calcium crystals it might destabilize atherosclerotic plaques by initiating inflammation and by causing VSMC death (61) (Fig. 2).

**Detection of vascular calcification**

VC can be visualized by various techniques. In the clinical setting, multidetector computed tomography is often used and generates a quantitative calcium score, which is used as a measure of atherosclerotic burden (62, 63). VC is therefore a potent predictor for cardiovascular events (64). Although some research has linked the amount of vascular calcium to a more stable plaque phenotype (65) most studies identified intimal calcification as predictor of a vulnerable plaque phenotype, in particular the punctuated “spotty calcification” (63, 66). Indeed, finite element analysis implied that macrocalcification in the plaque did not increase plaque stress or rupture (55, 67)
whereas small calcified spots in the atherosclerotic cap increased stress, sufficient for causing plaque rupture. This resulted in studies screening for regulating mechanisms of VC by multimodality imaging. Derlin et al. (68), used a combination of positron emitted tomography (PET) and CT to assess the cardiovascular risk in patients. The use of Na\(^{18}\)F for imaging calcified atherosclerotic plaques showed a more frequent uptake of Na\(^{18}\)F in patients with a high-risk profile. However, there was a weaker correlation with risk factors compared to the calcified plaque burden (68).

Imaging of VC in mice was done using bisphosphonate near-infrared conjugated probes. Bisphosphonates strongly bind to calcified structures in coronary arteries (69, 70). However, bisphosphonate also strongly accumulates in bone, which results in a high background signal. Therefore, these studies are only applied ex vivo. New specific probes imaging microcalcification can provide a platform to study the earliest events associated with VC at the molecular and cellular level.

The use of circulating biomarkers such as MGP for detecting or screening VC is an attractive possibility. Vitamin K-dependent proteins have been associated with the earliest calcification areas in the plaque (71). It was the uncarboxylated form of MGP that strongly correlated with both medial and intimal calcification (71,72). By measuring circulating MGP isoforms it was shown that the majority of the healthy population have sub-optimal levels of vascular vitamin K (73, 74). Preliminary data suggest that some MGP conformations are associated with aspects of cardiovascular disease (37, 38, 75, 76). Patients with high VC scores display high levels of inactive MGP, especially dialysis patients.

This creates possibilities for targeting VC with vitamin K. Indeed high intake of vitamin K has been shown to regress preformed medial calcifications in a rat model (77).

Recently, we conducted a first pilot study in dialysis patients showing that vitamin K supplementation markedly reduced the level in plasma (78) of

- uncarboxylated prothrombin (pivka-II),
- uncarboxylated osteocalcin (ucOC),
- inactive MGP (dp-ucMGP).

**Fig. 2** Mechanism of vascular calcification

1) Adaptation of VSMCs from a contractile to a synthetic phenotype as a result of multiple stress factors. VSMCs start loading calcium resulting in calcification of the media.

2, 3) Additionally, modified lipoprotein binding to macrophage scavenger receptors (2) are phagocytosed and accumulation results in foam cell formation (3).

4) Foam cells secrete pro-inflammatory cytokines that amplify the local inflammatory response (4) resulting in an accelerated calcium loading and vesicle release by VSMCs. These vesicles form a nidus for calcification. The increased inflammatory profile results in osteogenic differentiation of VSMCs. Additionally, VSMCs lose their calcifying inhibitors (such as MGP) resulting in an acceleration of calcification.

5) As a result, VSMCs undergo apoptosis releasing more apoptotic bodies, accelerating the calcification process.

6, 7) Macrophages phagocytose calcium crystals (7) which induces activation of the NLRP-3 inflammasome.

8) Subsequently cytokines (such as TNF-α, IL-β and IL-8) are released. Also VSMCs phagocytose calcium crystals, which leads to enhanced apoptosis of VSMCs.
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