

## Anniversary Issue Contribution

# Vitamin K: The coagulation vitamin that became omnipotent

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### Summary

Vitamin K, discovered in the 1930s, functions as cofactor for the posttranslational carboxylation of glutamate residues. Gamma-carboxy glutamic acid (Gla)-residues were first identified in prothrombin and coagulation factors in the 1970s; subsequently, extra-hepatic Gla proteins were described, including osteocalcin and matrix Gla protein (MGP). Impairment of the function of osteocalcin and MGP due to incomplete carboxylation results in an increased risk for developing osteoporosis and vascular calcification, respectively, and is an unexpected side effect of treatment with oral anticoagulants. It is conceivable that other side effects, possible involving growth-arrest-specific gene 6 (Gas6)

protein will be identified in forthcoming years. In healthy individuals, substantial fractions of osteocalcin and MGP circulate as incompletely carboxylated species, indicating that the majority of these individuals is subclinically vitamin K-deficient. Potential new application areas for vitamin K are therefore its use in dietary supplements and functional foods for healthy individuals to prevent bone and vascular disease, as well as for patients on oral anticoagulant treatment to offer them protection against coumarin-induced side effects and to reduce diet-induced fluctuations in their INR values.

### Keywords

Vitamin K, matrix Gla protein, osteocalcin, oral anticoagulants, diagnostics

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## Introduction

The dietary trace element vitamin K was discovered in the early 1930s by the Danish biochemist Henrik Dam. During his research on the cholesterol metabolism in chickens, Dam observed that chicks reared on a diet free of sterols and poor in fat developed large subcutaneous and intramuscular haemorrhages (1). Additionally, it was reported by McFarlane et al. that blood of chicks fed an ether-extracted fish or meat meal failed to coagulate on standing overnight in the laboratory (2). Initial experiments with additions of lemon juice, cholesterol, cod-liver oil or ascorbic acid to the diet failed to prevent haemorrhages (1). Further testing of different foods, ranging from cereals and seeds to animal organs, revealed that “the antihæmorrhagic factor” was fat-soluble with one of its richest sources being hog-liver (3). Hendrik Dam designated this factor as Koagulations vitamin (abbreviated as vitamin K), because of its requirement for normal haemostasis (3). Subsequently, Almquist and Stokstad reported that the hæmorrhagic syndrome could be prevented by concentrated extracts of alfalfa as well as by fish meals and bran preparations subjected to the action of microorganisms (4).

In the mid 1930s, Schönheyder proposed that the prolonged clotting time seen in animals with the hæmorrhagic syndrome

was due to a decreased concentration of plasma prothrombin (5); indeed, precipitates of prothrombin from the plasma of “K-avitaminous” chicks were found to be inactive (6) and the prothrombin time of chicks and rabbits fed with spoiled sweet clover hay – resulting in hæmorrhagic disease – to be increased (7, 8). In later years, it was established that sweet clover hay contained dicoumarin, a vitamin K antagonist produced by molds acting on the hay (9).

In the late 1930s several research groups were able to isolate vitamin K from alfalfa as a yellow oil (10–13); the group at St. Louis University led by Edward A. Doisy could also isolate vitamin K from putrefied fish meals as a crystalline product and designated this product as vitamin K<sub>2</sub> (14, 15). During this time, the first clinical studies with administration of vitamin K to patients with obstructive jaundice or biliary problems were conducted (16), since it had been recognized that the bleeding tendency seen in these cases was related to vitamin K deficiency (17). Additionally, vitamin K was administered to newborns suffering from hæmorrhagic disease or who were found to have a prolonged prothrombin time (18). In later years, the prophylactic treatment of newborns (or their mothers) with vitamin K gained increasing attention (19). In fact, Dam mentioned in his Nobel lecture that prophylactic treatment with vitamin K decreased the

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neonatal mortality rate among newborn infants from 4.6% to 1.8% (20, 21). Dam and Doisy received the 1943 Nobel prize in Physiology or Medicine for the discovery of vitamin K and its chemical nature (20).

Presently we know phyloquinone as vitamin K<sub>1</sub>; it originates from green leafy plants and green vegetables which are its most important dietary source for humans. The chemical structure of vitamin K<sub>2</sub> was elucidated in the 1960s (22) and revealed that it consists of a family of related products now known as menaquinones. Menaquinones are mainly of microbial origin and their nomenclature is based on the number of isoprenoid residues in their side chain. Dietary relevant menaquinones range from menaquinones-4 (MK-4) through menaquinones-10 (MK-10). In the human diet the main sources of menaquinones are cheese, curd, and the Japanese food natto. MK-4 is the only menaquinone that can be produced in mammalian tissues, where it is formed by conversion of vitamin K<sub>1</sub>, MK-7 and possibly other menaquinones.

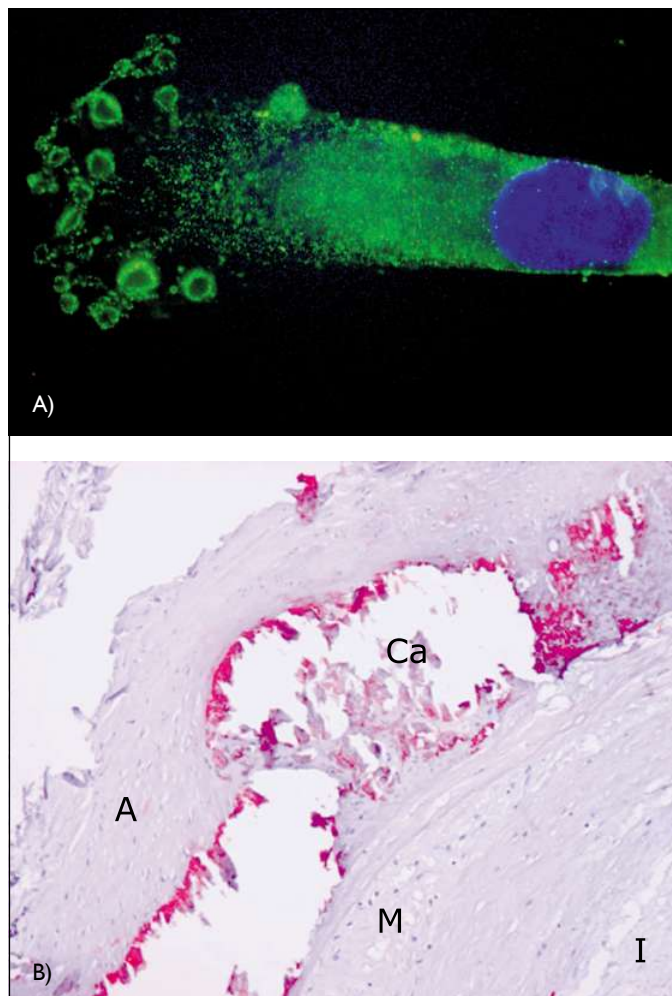
Mammals have developed an efficient system for recycling vitamin K, which is the reason why minute amounts are sufficient to cover the daily requirement. Therefore, circulating vitamin K levels are low, and accurate detection techniques for vitamin K in blood, tissues and food are only available in some specialized laboratories. Modern vitamin K assessment requires extraction, pre-purification, HPLC analysis and post-column reduction followed by fluorescence detection. Although several scientists have contributed in optimizing the procedure, Martin Shearer is generally regarded as the founder of modern vitamin K analysis (23). An alternative to fluorescence detection was recently provided by the group of Okano, who used tandem mass spectrometry as a detection system (24). The group of Shearer also initiated a worldwide Quality Assurance / Quality Control scheme together with our group, enabling participating researchers to compare their data and to check the reliability of their system on a regular basis (25).

## Function of vitamin K

Until 1974, various hypotheses about the mechanism of vitamin K action were proposed, including a role in mitochondrial electron transport and an inducer of protein synthesis; the discovery of the unusual amino acid  $\gamma$ -carboxy glutamic acid (Gla) in prothrombin as the product of vitamin K action in 1974 finally identified the unequivocal role of vitamin K as a cofactor for the posttranslational carboxylation of glutamate residues (26, 27). These findings triggered the development by the group of Suttie of an in-vitro carboxylase assay in which <sup>14</sup>CO<sub>2</sub> was incorporated into glutamate-containing synthetic peptides (28). More than 15 years ago Stafford and coworkers succeeded in purifying the key enzyme  $\gamma$ -glutamate carboxylase (GGCX) (29), only recently the purification and amino acid sequence of the second key enzyme in vitamin K metabolism, vitamin K epoxide reductase (VKOR) were reported by two independent groups (30, 31). These data have resulted in cloning the cDNA coding for both proteins in insect expression vectors, which has boosted the molecular research on the mechanism of vitamin K action (32, 33).

After the discovery of Gla-residues in prothrombin, the subsequently identified Gla-proteins were those involved in blood

coagulation: factors VII, IX and X as well as the proteins C, S, and Z (34). During this time, the presence of carboxylase was demonstrated in a wide variety of non-hepatic tissues, ranging from bone to skin (35, 36). It was assumed that the endogenous substrate of carboxylase in these tissues (unidentified Gla-proteins), was not involved in blood coagulation. This could imply



**Figure 1: Matrix Gla-protein is synthesized by vascular smooth muscle cells (A) and accumulates at areas of calcification in the arterial wall (B).** Matrix Gla-protein (MGP) is a potent local inhibitor of soft tissue calcification; it contains nine glutamate residues, five of which can be carboxylated in a vitamin K-dependent way, resulting in carboxylated MGP (cMGP). Poor vitamin K status therefore results in synthesis of uncarboxylated MGP (ucMGP). A major source of MGP is the arterial wall, where it is synthesized by vascular smooth muscle cells (VSMC). A) A single human VSMC in which cMGP is stained with conformation-specific antibodies. The cell was grown in medium containing a high concentration of calcium (3.6 mM); its nucleus was visualized by Dapi-staining (blue) and MGP was stained with FITC-labeled anti-cMGP-antibody (green). MGP is carboxylated in the endoplasmic reticulum (dense green staining) and secreted in vesicular structures. Magnification 1,000x. B) The immunohistochemical localization of ucMGP in a calcified artery. A section from a peripheral artery of a diabetic patient was stained with anti-ucMGP-antibodies (red) and counterstained with haematoxylin. Calcification is present in the medial layer of the arterial wall and it can be seen that the inactive MGP has accumulated at the area of calcification. A, adventitia; Ca, calcification; I, intima; M, media. Magnification 40x.

that treatment with oral anticoagulants, known to influence the carboxylation of coagulation factors in the liver, would also impair the carboxylation of these unidentified Gla-proteins in extra-hepatic tissues. Treatment with coumarins could therefore have unwanted, and yet unknown, side-effects in these tissues. Indeed our group, which contributed to the identification of carboxylase in several tissues, urged medical doctors already in 1982 to report possible side-effects from treatment with coumarin-derivatives (37).

The first extra-hepatic sites in which Gla-proteins have been identified and characterized are bone and cartilage. Bone Gla-protein, also known as osteocalcin, was identified in the mid 1970s (38, 39) and presently serves as a marker for osteoblastic activity. Matrix Gla-protein (MGP), first described in 1983 (40), is the strongest inhibitor of tissue calcification presently known (Fig. 1). Its importance for vascular health was demonstrated in MGP-deficient animals, which all died of massive arterial calcification within 6–8 weeks after birth (41). In humans, loss-of-function mutations of MGP result in the Keutel syndrome (42), characterized by abnormal cartilage calcification (43). In 1971, Keutel et al. first described the syndrome in two siblings presenting with abnormal cartilage calcification, brachytelephalangism, neural hearing loss and peripheral pulmonary stenosis (44). At the age of 38, one of the two siblings died of acute right heart failure after several years of obstructive pulmonary disease; post-mortem examination revealed calcification of arteries throughout the body resulting in pulmonary artery stenosis and multiple brain infarctions (45).

It is remarkable that it took about half a century after the discovery of the relation between vitamin K and blood coagulation before the vital importance of vitamin K for bone and vascular health was recognized. It is even more remarkable that it is still not commonly known among medical doctors that vitamin K-dependent proteins not only regulate blood coagulation, but also other physiological processes including bone metabolism (osteocalcin), vascular calcification (MGP), cell growth, and apoptosis (growth-arrest-specific gene 6 protein, Gas6) (46) and that, as a consequence, oral anticoagulants may have undesired effects in extra-hepatic tissues.

## Unexpected side effects of oral anticoagulants

Oral anticoagulant therapy is a widely used treatment of subjects with increased thrombosis risk; it is based on the daily intake of 4-hydroxycoumarins (warfarin, acenocoumarol, phenprocoumon) which bind to the VKOR enzyme and thus inhibit recycling of vitamin K. Consequently, the carboxylation of coagulation factors is inhibited resulting in the formation of inactive, non-carboxylated species also known as PIVKAs (proteins induced by vitamin K absence or antagonists). It was generally assumed that this was the only effect of oral anticoagulants. The discovery of new Gla-proteins not involved in blood coagulation initiated the search for side effects of oral anticoagulant treatment.

Pastoureau et al. showed that lambs receiving warfarin developed osteopenia within several months (47), which is probably due to the impaired function of osteocalcin. Careful analysis of bone mineral density in patients on long-term anticoagulation revealed that coumarin anticoagulants are associated with accel-

erated bone loss and low bone mass (48). Hence, long-term use of oral anticoagulants is considered as a risk factor for developing osteoporosis. Similarly, impairment of MGP must be regarded as a risk factor for arterial calcification. Indeed, two independent studies have demonstrated that subjects on long term anticoagulation have much more arterial and heart valve calcification than an age- and sex-matched control population (49, 50). It should be mentioned, however, that the effects in experimental animals were more pronounced than in humans, which may indicate more complex regulatory systems in man. Additionally, exposure to warfarin during the first trimester of pregnancy can cause warfarin embryopathy, characterized by punctuate calcifications mainly in the axial skeleton, proximal femurs and calcanei, nasal hypoplasia and depression of the nasal bridge (51). It has been suggested that impairment of MGP's ability to inhibit cartilage calcification, underlies the development of these abnormalities (52, 53).

These examples demonstrate that in forthcoming years more side effects of oral anticoagulants may be expected and highlight the importance of other strategies for anticoagulant treatment such as the well known platelet aggregation inhibitors (aspirin, clopidogrel, dipyridamole), as well as the direct thrombin and factor X inhibitors.

## Combination therapy

Another well recognized disadvantage of oral anticoagulant therapy is its instability requiring frequent monitoring. Since the level of anticoagulation is a balance between the anticoagulant and the dietary vitamin K, fluctuations of dietary vitamin K intake may lead to INR values outside the therapeutic window. One strategy to overcome this problem is to decrease the dietary fluctuations in vitamin K intake. This was not shown to be a practical approach. Alternatively, it has been proposed to increase the vitamin K intake in the form of dietary supplements, and to increase the anticoagulant concordantly (54, 55). In this way the fluctuations caused by diet will be small relative to the total vitamin K intake. Most studies which have addressed this point used vitamin K<sub>1</sub>. The potential danger of giving K<sub>1</sub> in combination with warfarin, however, is that K<sub>1</sub> is preferentially taken up by the liver, so that the increased warfarin used to compensate for the high vitamin K intake will exhaust the vitamin K supplies in the vessel wall, thus increasing the calcification risk. The correctness of this hypothesis was demonstrated in rats receiving a K<sub>1</sub> + warfarin treatment and which developed widespread arterial calcifications within 2–4 weeks (56). K<sub>2</sub> vitamins, on the other hand, are equally transported to extrahepatic tissues and would thus combine a maximal protection against unwanted side effects of oral anticoagulants and a hepatic vitamin K uptake which is sufficient to counteract the diet-induced fluctuations in INR. Consistently, K<sub>2</sub> and not K<sub>1</sub> was capable of preventing arterial calcification in the above animal model (57) (Fig. 2).

Recently, it was demonstrated in rats that high vitamin K intake was even capable of inducing regression of pre-formed arterial calcifications (58). The two K<sub>2</sub> vitamins that are commercially available are MK-4 and MK-7. To make a proper choice between both forms, it should be realized that MK-4 has a very short half-life time (about 1 hour), but that the half-life time of

MK-7 is much longer (about 3 days). If taken on a once daily basis, MK-4 will thus show substantial circulating and tissue fluctuations, whereas MK-7 will accumulate gradually and reach a steady state level after 2 weeks (59). Based on these considerations we have recently proposed that trials will be designed in which patients receive coumarin anticoagulants in combination with MK-7. These patients should be followed with respect to the stability of their INR value in time, as well as the development of bone mineral density and vascular characteristics and calcification (60).

## K-status in healthy adults

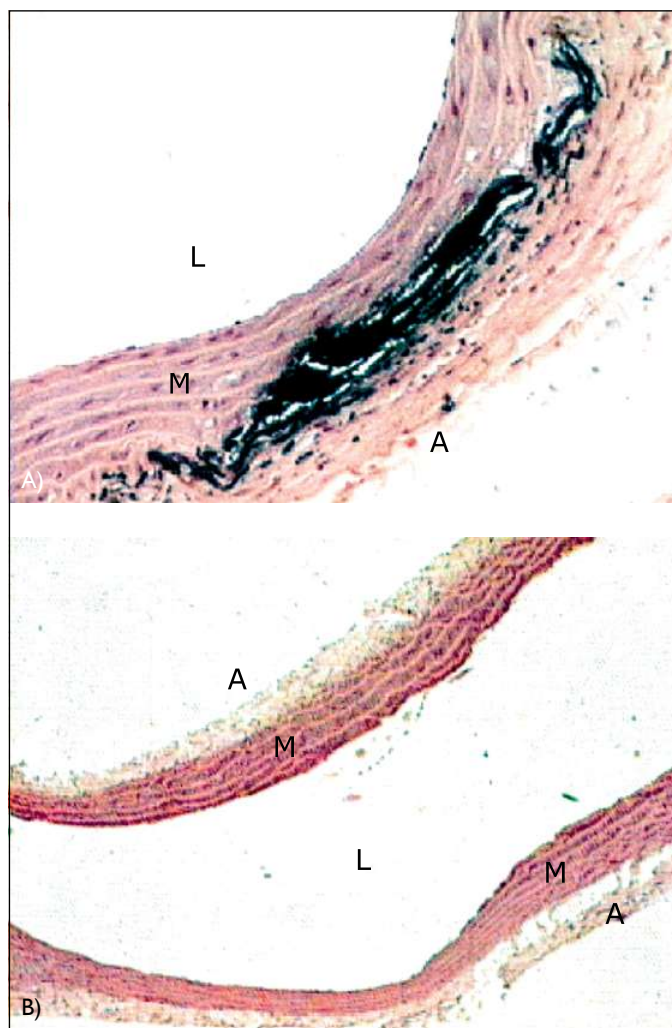
As compared with other vitamins, the dietary intake of vitamin K is very low. The recommended dietary allowance (RDA) is 1 µg/kg/day. Also, its biological half-life time is relatively short: upon deprivation experimental animals develop symptoms of vitamin K-deficiency within a few days. Yet, vitamin K intake is sufficient to ensure normal haemostasis in healthy adults. It should be kept in mind, however, that the liver – which is the place of clotting factor synthesis – is capable of extracting vitamin K from the circulation very efficiently. Therefore, it is questionable whether the present RDA for vitamin K intake is sufficient to cover the requirements of extrahepatic tissues.

Conformation-specific antibodies are now commercially available specifically recognizing under-carboxylated species of osteocalcin (Takara Shuzo, Shiga, Japan) and MGP (VitaK BV, Maastricht, the Netherlands) which are generated in subclinical vitamin K deficiency. Using these antibodies, ELISA-based assays were developed for testing circulating under-carboxylated species of osteocalcin and MGP. Surprisingly, these tests showed that in all apparently healthy subjects tested, a substantial fraction of both proteins occurs in under-carboxylated forms, which have no biological activity (61, 62). This raises the intriguing question of whether all (or: most) apparently healthy adults are subclinically vitamin K deficient. Increased circulating levels of undercarboxylated osteocalcin were shown to be associated with increased bone loss and osteoporosis in postmenopausal women (63), whereas under-carboxylated MGP has been associated with arterial calcification (64).

## New application areas for vitamin K

As mentioned above, extra-hepatic Gla-proteins are incompletely carboxylated in the majority of the healthy adults. Hence the biological activity of these proteins is sub-optimal. Only in vitamin K-supplemented subjects the plasma level of non-carboxylated osteocalcin (61, 65, 66) and MGP is below 5% of the total antigen (Cranenburg et al., unpublished observations). These findings suggest that, although the dietary vitamin K intake is adequate to maintain normal haemostasis, it may be insufficient for extrahepatic tissues. Indeed, the present dietary reference values are based on a proper functioning of the blood carboxylation factors, and not on the carboxylation of osteocalcin or MGP.

Poor vitamin K status must be regarded as a serious risk factor for increased postmenopausal bone loss and for artery calcification, notably in diabetes, endstage renal disease and aging. Population-based studies have demonstrated an inverse cor-



**Figure 2: Arterial calcification is induced by treatment with warfarin + vitamin K<sub>1</sub> (A) and prevented by vitamin K<sub>2</sub> (B).** Eight-week-old male rats received a diet containing 3 mg of warfarin and either 1.5 mg vitamin K<sub>1</sub> or 1.5 mg vitamin K<sub>2</sub> (menaquinone-4) per gram of food for four weeks. Rats were killed, and carotid arteries were fixed in 4% phosphate-buffered paraformaldehyde. Tissues were completely sectioned, and every fifth section was screened for calcification by Von Kossa staining. A) A typical section from an animal treated with warfarin + vitamin K<sub>1</sub> with calcification (black) clearly present in the medial layer of the arterial wall. Similar calcifications were found in animals receiving an 8-fold higher dose of vitamin K<sub>1</sub> (data not shown). In sections from animals treated with warfarin + vitamin K<sub>2</sub> (B), no calcification was observed. A, adventitia; L, lumen; M, media. Magnification 40x.

relation between dietary vitamin K intake and bone fracture risk (67) as well as between dietary vitamin K intake and arterial calcification and cardiovascular mortality (68). Notably for vascular health, K<sub>2</sub> appeared to be superior to K<sub>1</sub>. Several clinical trials have demonstrated that supplementary vitamin K may result in decreased bone loss (69), as well as in maintenance of bone strength (61) and carotid artery elasticity (70). For K<sub>1</sub> and MK-4 these effects have been reported at pharmacological doses ranging between 1 and 45 milligrams per day. On the basis of its longer half-life time and extra-hepatic tissue distribution it is to be expected

that similar effects will be obtained with MK-7 at nutritional doses (i.e. below the RDA for vitamin K) (59). Therefore, MK-7 is the obvious choice for enrichment of dietary supplements and functional foods to be used for disease prevention in healthy subjects.

The third extrahepatic Gla-protein receiving increasing attention is Gas6. Gas6 exhibits a broad range of regulatory functions associated with cell growth regulation, migration and proliferation, cell survival, apoptosis, recognition of dying cells, phagocytosis and cell adhesion (71). It has a 43 % amino acid sequence identity (including the 11 Gla residues) with protein S, which is known as a negative regulator of blood coagulation. In contrast to Gas6, protein S also binds to the C4b-binding protein, which acts as a negative regulator of the complement system. Gas6 serves as a ligand for a subfamily of receptor tyrosine kinases, and it has been demonstrated that incomplete carboxylation results in loss of biological activity (72). Consequently, interfering with its Gla-content by using coumarin derivatives may influence the progress of a wide variety of pathologies including cancer, cardiovascular disease, neurological diseases, autoimmune disease and kidney disorders. Conformation-specific antibodies for Gas6 have not yet been described, and it is unknown whether also in this case partially carboxylated or uncarboxylated species occur in healthy adults. However, we would expect that the carboxylation status of Gas6 is substantially decreased by using oral anticoagulants; this may affect the course of diseases such as those mentioned above in an as yet unpredictable way. Once again, oral anticoagulants may surprise us with unexpected side effects although they have been widely used for more than 50 years.

## Circulating MGP as a diagnostic marker for cardiovascular disease

MGP is expressed in cartilage and the vasculature. In healthy tissues the expression is relatively low, but around ectopic calcifications in the arteries MGP expression is up-regulated several orders of magnitude, probably as a defence mechanism triggered

by the calcium crystals (73). Since mature MGP contains five Gla residues per molecule, hypothetically the local vitamin K requirement around calcified lesions is strongly increased, so that the vascular tissue is rapidly depleted of vitamin K. Hence, at these sites incompletely carboxylated MGP species are formed (Fig. 1B), which are not functional. Whether increased vitamin K intake may protect against further calcification is a major point of interest at this time. Several test kits for MGP in serum and plasma are presently available, and the first data suggest that circulating MGP total antigen may become a marker for cardiovascular disease (62, 64). Since MGP may circulate in at least four different conformations, it remains to be seen which test will prove to have the highest diagnostic utility.

Conformation-specific antibodies against carboxylated and non-carboxylated MGP are now available, and with ELISA-based techniques both species have been found in the circulation (ECM Cranenburg et al., submitted for publication). Whereas the absolute levels of either of these forms may depend on a number of factors including vascular smooth muscle cell activity, triggered up-regulation of MGP expression and cellular secretion of the different MGP fractions and local binding in and around the calcified tissue, the ratio between carboxylated and non-carboxylated MGP species will reflect vascular vitamin K status, and thus the extent to which an individual is protected against arterial calcification by the MGP-dependent pathway.

Obviously, the availability of tests with which the vascular vitamin K status can be tested in a small amount of blood is of major importance for the health food industry. Lateral flow tests are now being developed for carboxylated and non-carboxylated MGP; such tests may be used for point-of-care or even home diagnostics providing information to the consumer about his/her vitamin K status and the potential benefit of taking vitamin K supplements. By monitoring MGP carboxylation on a regularly basis, this system would provide a unique possibility to visualize short-term benefits of vitamin supplements. Without doubt, such a system should not be brought to the market before the health benefits of nutritional doses of supplemental vitamin K have been confirmed in long-term clinical trials.

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