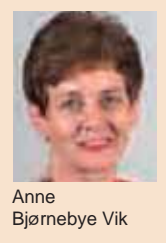


Vitamin K2 and arterial calcification



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ABSTRACT: Arterial calcification is strongly linked to poor cardiovascular health. Mechanisms for arterial calcification involve many factors and are highly regulated. Among participating factors strong focus is now on vitamin K dependent proteins, especially the Matrix Gla protein (MGP). This protein is synthesized in several cell types, among them vascular smooth muscle cells. MGP needs vitamin K-mediated activation for optimum biological activity, and when activated it functions as a strong inhibitor of arterial calcification. An optimum form of vitamin K is menaquinone-7 from natto. This article describes the background for understanding how MK-7 can influence arterial calcification through the inhibitory action of MGP.

VITAMIN K – A GROUP OF RELATED COMPOUNDS

Vitamin K is a group name for a family of related compounds, generally divided into the naturally occurring phyloquinone (vitamin K1) and menaquinones (MK-n; K2 vitamins- Figure 1).

Vitamin K activates the so-called vitamin K-dependent proteins by serving as a cofactor for the enzyme catalyzing the posttranscriptional carboxylation of specific glutamic acid (Glu) to γ -carboxyglutamate (Gla) residues (Figure 2). Dietary vitamin K is absorbed and transported in the circulation in its quinone form, and in tissue vitamin K is reduced to vitamin K hydroquinone (KH_2). Carboxylation requires intracellular recycling of the vitamin K product, vitamin K epoxide (KO), back to KH_2 . The conversion of Glu to Gla residues equips vitamin K-dependent proteins with structural integrity, metal-binding characteristics, and functionality. Beyond their central role in blood coagulation, Gla-containing proteins have a diversity of regulatory functions in important physiological processes. Examples are inhibition of soft tissue calcification (matrix-Gla protein, MGP), bone formation (osteocalcin), and cell growth and apoptosis (growth-arrest specific gene 6, Gas-6). Vitamin K-deficiency or insufficiency will lead to the production of undercarboxylated proteins, and accordingly to interference with the physiological processes mentioned above. These undercarboxylated proteins are being used as new markers of suboptimal vitamin K nutrition.

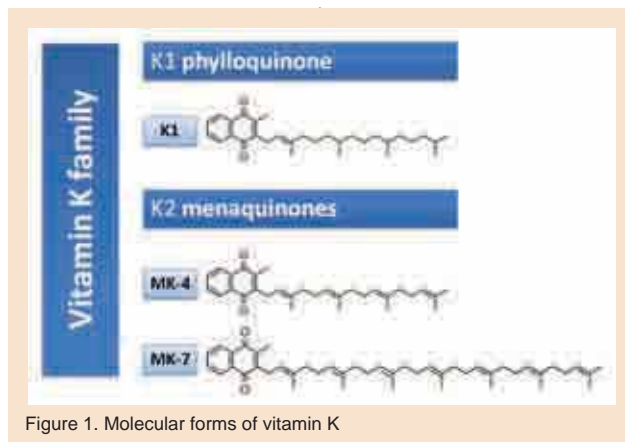


Figure 1. Molecular forms of vitamin K

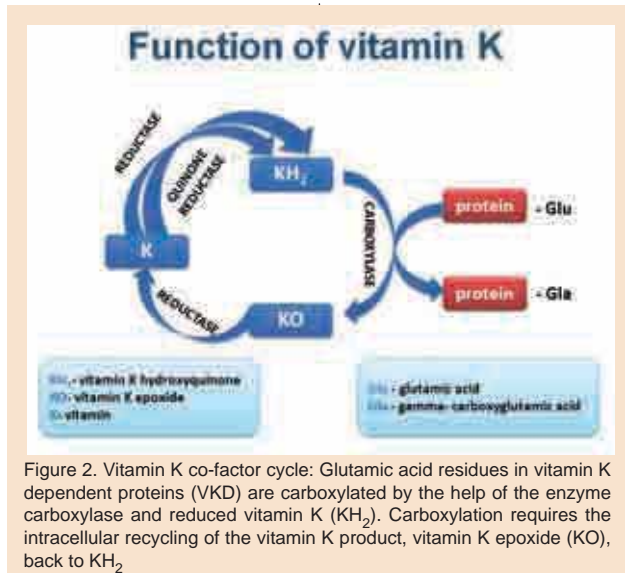


Figure 2. Vitamin K co-factor cycle: Glutamic acid residues in vitamin K dependent proteins (VKD) are carboxylated by the help of the enzyme carboxylase and reduced vitamin K (KH_2). Carboxylation requires the intracellular recycling of the vitamin K product, vitamin K epoxide (KO), back to KH_2 .

ARTERIAL CALCIFICATION AND CARDIOVASCULAR DISEASE

Arterial calcification is associated with a number of diseases, such as diabetes, hypertension, atherosclerosis, and chronic kidney disease. Calcium-phosphate deposition is the hallmark of arterial calcification and can occur in the blood vessels, myocardium, and cardiac valves.

Many excellent articles have been published dealing with vascular calcification and risk for cardiovascular disease (1-4). In the St. Francis Heart Study (5) more than 4900 asymptomatic men and women age 50 to 70 years underwent electron beam computer tomographic scanning (EBCT) of the coronary arteries to determine coronary calcification. They were observed for nearly 20 000 person years and checked for the predictive value of EBCT and other established risk factors including C-reactive protein (CRP). In this study coronary calcium scores predicted coronary artery disease more accurately than standard risk

factors and CRP.

Shaw et al. (6) measured coronary calcium scores in more than 10300 asymptomatic individuals referred for EBCT and followed them for 5 years. They found that calcium-adjusted age was a better predictor of mortality than observed age alone. If a young person had a high calcium score, his biological age was much higher than his chronological age, and as much as 30 years could be added to his age. On the other hand a 70 year old person with very low calcium score could reduce his age with as much as 10 years.

CALCIFICATION – THE ROLE OF VITAMIN K DEPENDENT PROTEINS

The most interesting protein in relation to vascular calcification is the matrix Gla protein (MGP) - first isolated and characterized by Price in 1976 and further described by him and others the following years (7). MGP is an 84-amino acid protein synthesized in bone cells, chondrocytes and vascular smooth muscle cells (SMC).

Lou et al. (8) created a MGP knock-out mouse model and published some striking results in 1997. Mice deprived of the gene for MGP died very early and with reduced body weight compared to its control counterpart. Even if the mice were normal at birth, they died of massive calcification of elastic arteries which ruptured causing an early death. Mice deprived of MGP had obviously lost their capacity to inhibit vascular calcification while the control mice with intact MGP developed normally with no specific signs of calcification. Such a knock-out model is thus a powerful way of showing the importance of MGP in relation to vascular calcification.

It is now well established that MGP belongs to the group of vitamin K dependent proteins. As for osteocalcin- the bone protein synthesized in osteoblasts taking part in bone remodeling - MGP must be carboxylated to become active. The 5 glutamic residues in MGP need vitamin K as a cofactor for carboxylation. In addition MGP has serine residues which can be phosphorylated.

MGP can be found in the circulation as well as in tissues where it can be stained immunohistochemically (9). In healthy human arteries carboxylated MGP is mainly found around elastic fibres in the tunica media. No undercarboxylated - or inactive- MGP (ucMGP) - was found in these healthy arteries, while ucMGP was found in intimal and medial vascular calcified tissues. Atherosclerotic intimal calcification occurs in the context of apoptotic cells in a lipid-rich environment and, since atherosclerosis is predominantly an inflammatory process, the cells most commonly associated with this form of calcification are inflammatory cells, such as T cells and macrophages. In contrast to intimal calcification, medial calcification is not necessarily associated with atherosclerosis and is commonly seen in the aging population, and in patients with diabetes and chronic kidney disease. In its mildest form, medial calcification appears as linear deposits along elastic lamellae. At its most severe, it forms a dense circumferential sheet of calcification in the centre of the media, bound on both sides by smooth muscle cells, often containing bone trabeculae and osteocytes.

There are presently many studies showing the importance of MGP. Both cell culture (10), animal experiments and assays for circulating human MGP (11) show that ucMGP is a marker for vascular calcification (12, 13). Active MGP binds calcium and thus prevents calcium super-saturation and crystallization within vessel walls. By doing so, activated (carboxylated) MGP inhibits further crystalline growth and stiffening of arteries due to calcium deposits.

The type of calcium crystals which accumulate in a typical calcified area of the blood vessel walls exist mostly in the form of calcium apatite - the same type of mineral found in bones (14). In addition one can also find small membrane-bound vesicles that bud off from vascular smooth muscle cells like the nuclei which can be seen during skeletal bone formation. The resemblance to bone formation has further been strengthened by findings of bone-related factors in the vasculature and in animal models using various gene-knock-out mice.

In a recent paper Danziger (15) gives an in depth overview over the vitamin K dependent proteins MGP and the less well known Growth Arrest Specific Gene 6 protein (Gas-6). Both proteins seem to protect vascular tissues from calcification and cell damage, however with different mechanisms. Even if a lot seems to be revealed, the exact mechanisms for the protective effects are still under investigation.

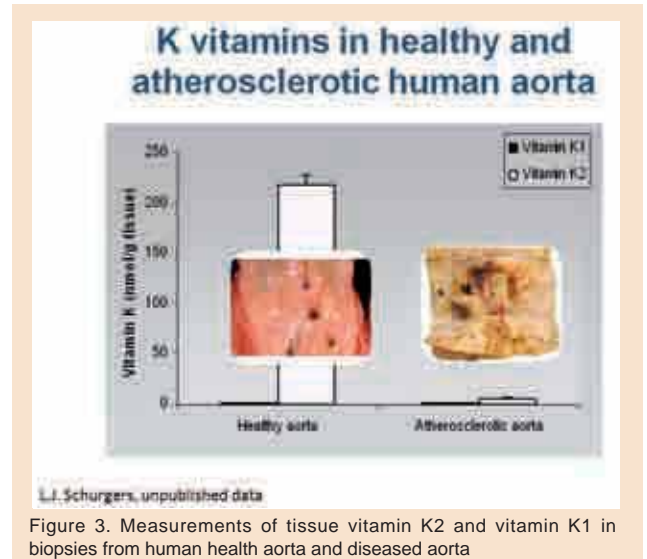


Figure 3. Measurements of tissue vitamin K2 and vitamin K1 in biopsies from human healthy aorta and diseased aorta

VITAMIN K IN TISSUES AND CALCIFICATION

An important issue is the relationship between availability of vitamin K in tissues and signs of calcification - or no calcification. As previously pointed out, MGP is found in tissues in different conformations. Carboxylated MGP - and hence enzymatically modified in the presence of vitamin K - was found in healthy arteries, while undercarboxylated MGP was associated with calcified vascular tissues. Experiments using samples of healthy and diseased biopsies from aorta show interestingly high tissue concentration of vitamin K2 in the healthy aorta but no vitamin K1 (16). In the diseased aorta neither any content of vitamin K2 nor K1 was found (Figure 3).

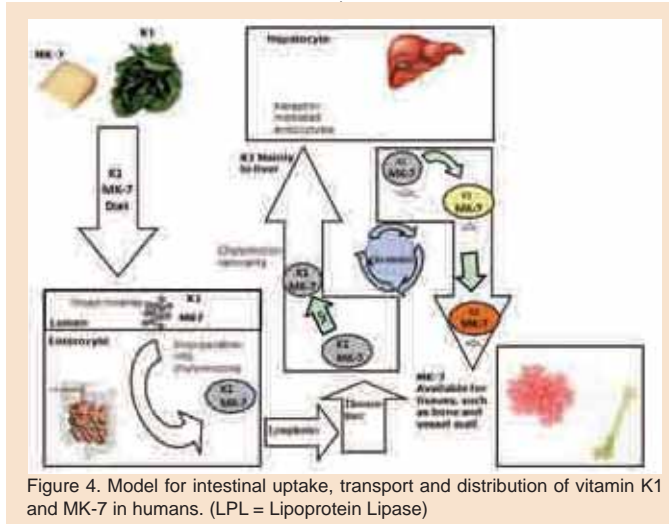
This indicates that vitamin K2 in these samples is present in healthy arteries, but not in calcified and diseased arteries - i.e. we see here an association between presence of vitamin K2, carboxylated MGP and a healthy aorta.

IS VASCULAR CALCIFICATION AN ACTIVE, REVERSIBLE AND WELL REGULATED PROCESS?

For many years calcification of the vasculature was thought to be an irreversible process - once you had started to become calcified the degenerative damage had begun. However, new knowledge based upon molecular experiments as well as genetic knock-out studies with MGP show that arterial calcification is an actively regulated process which may be preventable or even reversible. The potential reversibility is clearly shown in an animal model where rat vasculature is being experimentally calcified (17). Normal young rats are put on anticoagulant drug (warfarin) - a strong inhibitor for vitamin K activity. This drug prevents the activation of MGP - thus inhibiting its action. Strong arterial calcification occurred after 6 weeks on warfarin anticoagulation treatment, but not in the control group. To test if the calcification could be reversed, the rats taken off warfarin treatment were given different additions to their diet: standard feed, normal or high vitamin K. The striking results showed that the group receiving high dose of vitamin K - but not normal dose of K1- reversed the extent of calcification with about 40 percent. Parallel to the regression of aortic calcium content, cMGP increased and ucMGP decreased, suggesting that activated MGP (cMGP) may play a role in the regression of calcified plaques.

This study shows that arterial calcification is a reversible process in animals dependent on active MGP. It also shows that the carboxylation process is complex, and that there exists genetic variants of the components participating in this reaction. This might result in different phenotypes which partly may explain the variation in individual susceptibility to anticoagulant drugs like warfarin (18).

In humans we have seen that moderate to extensive arterial calcification is a strong risk factor for cardiovascular disease, and that MGP likewise is indicative for playing an important role in the calcification process. Two interesting aspects for human health follow from these findings: Does MGP have the potential to be a biomarker predicting high risk for cardiovascular disease in humans? And is vitamin K2- as the bioactive MK-7- beneficial for activation of MGP and thus contributes to prevention – or even regression – of arterial calcification in humans?



have not yet been published (20). However, both unpublished and published data (21) show that menaquinone-7 has a much longer serum-half life than phyloquinone (vitamin K1) and menaquinone-4 (MK-4). This indicates that MK-7 has a much better bioavailability potential for extra-hepatic tissues like the vasculature compared to K1 and MK-4. Recent findings therefore indicate that MK-7 may be more effective at doses that do not exceed the present recommendations for daily vitamin K intake. The explanation to this is that

BIOAVAILABILITY OF VITAMIN K AND CARBOXYLATION OF MGP

It is clear that MGP needs to be activated in order to inhibit arterial calcification, and that vitamin K2 strongly influences this activation process. However, due to the fact that specific assays for measuring several conformational forms of MGP (carboxylated, undercarboxylated and phosphorylated as well as non- phosphorylated MGP) have not been readily available (19), few studies have reported how human consumption of vitamin K2 directly influences MGP. Presently, most human studies investigating dose-response effects of vitamin K2 on carboxylation of MGP

all K vitamins initially are absorbed by enterocytes in the intestines and packed into chylomicrons. As shown in Figure 4 these chylomicrons are enzymatically degraded to chylomicron remnants prior to being taken up by the liver. As vitamin K1 is mainly transported by the triacylglycerol rich part of the chylomicrons, the liver has a preference for this lipid fraction and thus absorbs K1 for the post translational modification (carboxylation) of the coagulation factors. Data suggest that MK-7 will be redistributed to extra hepatic tissues as this structurally different molecule mainly is bound to other lipid fractions - rich in HDL and LDL - surpassing the liver. Since LDL has a long half-life time in the circulation, MK-7 has better bioavailability for extra-hepatic tissue uptake compared to K1. As MK-4 is structurally very similar to vitamin K1 these two latter vitamin K derivatives will closely follow the same route of

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absorption and show the same pharmaco-dynamic behaviour. At regular intake of nutritional doses (50-150 µg/day) of MK-7, its long half-life time may lead to accumulation in extra-hepatic tissues to levels that may only be reached with much higher doses of either K1 or MK-4. Likewise, this longer half-life time may result in more stable levels of vitamin K in the circulation and tissue. Our unpublished data show that MK-7 is a good activator for MGP even if the activation is somewhat slower than activation of osteocalcin at the same dose. This may indicate that serum MGP is slower in reflecting tissue MGP or that MGP needs a higher dose of MK-7 than osteocalcin to fully carboxylate. When studying young healthy volunteers we see that they have a substantial fraction of ucMGP in serum at baseline. This unpublished data indicates that the normal, presumably healthy population has an insufficient supply of vitamin K2. However, we also see that their ucMGP levels decrease when supplementing with MK-7. One may thus ask if our volunteers are special – or if insufficient supply of vitamin MK-7 throughout life is an important contributing factor to sustained ucMGP and calcification of arteries.

VITAMIN K2 INSUFFICIENCY AS BIOMARKER FOR CARDIOVASCULAR HEALTH

Although abnormal haemostasis is rarely detected in the normal population, undercarboxylation of extra-hepatic vitamin K-dependent proteins, such as MGP and osteocalcin is common (22). It has been demonstrated that, even with vitamin K intakes adequate for full carboxylation of all blood coagulation factors, 20-30 percent of the circulating osteocalcin remains undercarboxylated in the healthy population (23). As for osteocalcin, substantial undercarboxylation has also been reported for MGP. Consistent with findings in animal studies, massive accumulation of undercarboxylated MGP (ucMGP) has been shown in atherosclerotic lesions and areas of calcification. We have recently found that ucMGP may serve as a biomarker to identify subjects at risk for developing arterial calcifications (24). In line with these findings, an inverse correlation was found between vitamin K2 intake and aortic calcification, myocardial infarction, and cardiovascular death (Rotterdam Study). This study reveals that the diet is important for cardiovascular health, however, the diet needs to contain higher menaquinones like MK-7. It is thus important to know which food products that contain MK-7 and other higher menaquinones, and the availability of a test which can distinguish between active (carboxylated) and inactive (undercarboxylated) MGP. A test identifying persons with insufficient vitamin K2/ MK-7 level as well as responding to adequate supplementation of vitamin K2 as MK-7, could be of great interest. Presently we are investigating the new

conformation MGP specific assay in patients with cardiovascular disease to see if there is also a correlation between inactive MGP and the extent of disease status and/or cardiovascular calcification.

DIETARY MENAQUINONES – IMPORTANT FOR VASCULAR HEALTH

The best source of natural vitamin K2 is the Japanese food natto, which is a uniquely rich source of natural MK-7. Several studies have linked natto consumption in Japan to significant improvements in vitamin K status and bone health (25). The intensive odour and taste, however, makes this fermented product unappealing for the Western population. As a consequence, the consumption of MK-7 is very low. Higher menaquinones are found in dairy products like fermented cheeses and curd, however, in much less quantities (<40 µg/100 g).

This is shown in Figure 5. It is interesting to note that especially cheeses contain higher menaquinones and that cheese products probably contribute significantly to the total intake of vitamin K2 in the Netherlands. Traditional vitamin tables usually do not even distinguish between the vitamin K1 and K2 as assays for vitamin K usually have measured only vitamin K1. Green leafy vegetables (kale, spinach, lettuce, and broccoli) contain the highest content of K1 (150-800 µg/100 g food) and contribute to 40-50 percent of the total Western intake, followed by certain vegetable oils, such as soybean, cotton seed, canola, and olive oil (50-200 µg/100 g) (26). Meat, fish, dairy products and eggs contain both K1 (<10 µg/100 g) and MK-4 (<370 µg/100 g), with relatively high MK-4 concentrations in goose meat and liver, butter, and egg yolk. With our western dietary habits K1 comprises over 80 percent of total vitamin K intake. Despite this abundance of vitamin K1, most of us are vitamin K deficient- reflected in undercarboxylated biomarkers (osteocalcin, MGP). Biologically it is suggested that vitamin K2 is very much needed as it contributes to at least 50 percent of the total vitamin K absorbed. Supplementation of additional MK-7 would therefore be desirable in order to optimize ones vitamin K status.

POPULATION STUDIES – ARE THE CLINICAL FINDINGS RELATED TO VITAMIN K2?

The Rotterdam study (27) clearly links the reduction of risk for cardiovascular events and deaths to the consumption of higher menaquinones. The first indication that vitamin K could be associated with aortic atherosclerosis was however published already in 1995 by Jie et al. (28). They found a relationship between high dietary vitamin K intake and lower aortic calcification. At that time only vitamin K1 was analyzed and no data is

available in this study for vitamin K2. Knowing intakes of vitamin K2 would have been very interesting as the postmenopausal women included consumed both vegetables and dairy products, the latter known to contain higher menaquinones. In a new Dutch publication from Beulens et al. (29) it is clear that this study supports the conclusions of the Rotterdam study. This new study is a large population study of 564 postmenopausal women recruited from the Dutch PROSPECT study – one of two cohorts participating in the European Prospective Investigation into Cancer and Nutrition study. The findings in this study show that dietary intake of higher menaquinones – but probably not dietary K1- is associated with reduced

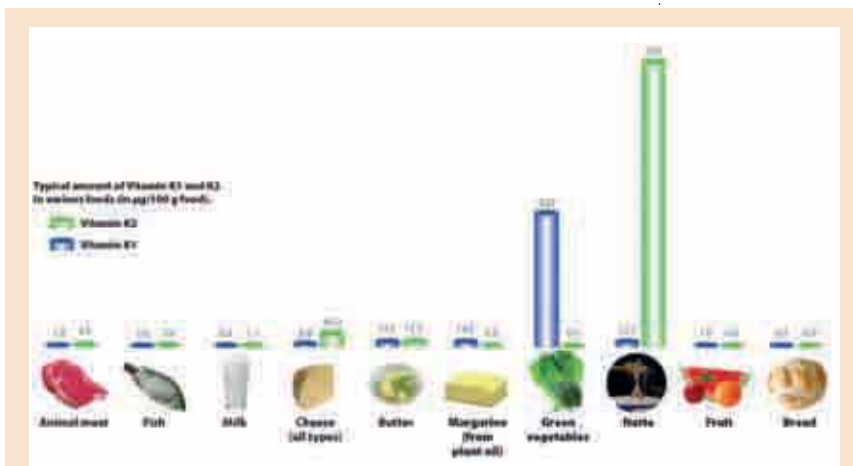


Figure 5. Content of vitamin K1 and K2 in different food products bought in The Netherlands

coronary calcification. An earlier American study correlating dietary intake of vitamin K1 in 807 consecutive active-duty US army personnel did also not find any significant correlation between inhibition of premature coronary calcification and vitamin K1 (30). Three studies examining large and independent populations have found the same, while a previous study (31) applying a high and pharmacological dose of K1 (1 mg/day, 3 years, and co-administered with minerals and vitamin D (referred to above)) has been shown to have beneficial effects on elastic properties of the arterial vessel wall. It is known that K1 is converted into K2 (as reviewed by Shearer and Newman (32)) which may provide a plausible explanation for these findings. Furthermore, it can be speculated that compared to the high dose of K1 used in this study a physiological dose of the higher menaquinones such as MK-7 will probably give similar results.

BONE HEALTH AND CALCIUM SUPPLEMENT- THE CALCIUM PARADOX

A 2008 published bone health study from New Zealand looked at 1471 postmenopausal women receiving 1 gram of calcium daily in a randomized controlled 5 year study (33). The women were followed up every six months during all these years, and any adverse events were recorded at each visit. The findings in this study show that many more adverse cardiovascular events (deaths, myocardial infarction, angina, other chest pains, stroke, transient ischemic attack and composite end point of myocardial infarction, stroke or sudden death) were recorded in the calcium supplemented group. The authors point to the fact that the results are surprising given the fact that calcium supplements acutely elevate serum calcium levels, possibly accelerating vascular calcification. As no data on daily dietary habits with respect to vitamin K containing food is given, we speculate that lack of substantial vitamin K2 can be one reason why it was recorded so many cardiovascular events. With insufficient vitamin K2 in the body, osteocalcin will not be fully carboxylated and thus less capable of binding calcium. This may partly be disguised by the very high calcium dose given and their vitamin D level (exclusion criteria: serum 25-hydroxyvitamin D level less than 25 nmol/l). However, with insufficient vitamin K2, MGP might not have been fully carboxylated – undercarboxylated MGP would probably have been found in serum as well as in the tissues. This inactive form of MGP will not bind calcium sufficiently with resulting calcification of the arteries.

CONCLUSIONS

MGP is a strong inhibitor of vascular calcification. However, this protein is dependent on post-translational modifications carried out by a carboxylase. This enzyme needs vitamin K as a co-factor, with a preference for the more bio available vitamin K2 in extra-hepatic tissues. Large population studies from different parts of the world suggest that vitamin K2 as higher menaquinones, like MK-7 from natto, are needed for efficient inhibition of vascular calcification. MGP is now under investigation for its relevance as a new biomarker for vitamin K2 insufficiency as well as a predictor of risk for cardiovascular disease.

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