



GLA PROTEINS – THE WARNING SIGN FOR THE BLOOD VESSELS

Yulian S. Snegarov, Bistra T. Galunska

Department of Biochemistry, Molecular medicine and Nutrigenomics, Pharmacy faculty, Medical University – Varna, Bulgaria.

SUMMARY

The aim of the present work was to perform a critical analysis of the literature related to the role of the extrahepatic matrix Gla-protein (MGP) as an inhibitor of vascular calcification.

Online databases PubMed, Google Scholar, Scopus, Science Direct, and Medline were thoroughly searched using key words such as Gla protein, Gla rich protein (GRP), matrix GLA protein (MGP), cardiovascular calcification, vitamin K, calcification inhibitor, aortic valve calcification. More than 1400 articles in the last 10 years were found.

The structure and the biologic functions of matrix-Gla-protein (MGP) were discussed. MGP is thought to be an inhibitor of vascular calcification, and evidence for this stems from its Ca²⁺-binding gamma-carboxyglutamic acid motif, inhibition of cartilage calcification, binding and antagonizing BMP-2. The introduction of Gla-residues is by vitamin K-dependent carboxylase. Vitamin K hydroquinone (KH₂) is used as the active cofactor in the carboxylation reaction. Upon oxidation of KH₂, the released energy is used to introduce an extra carboxyl group at the c-position of a glutamate residue.

Discovery of the relationship between the different isoforms of vitamin K and extrahepatic Gla-proteins can be a prerequisite for making an adequate solution for supplementing with vitamin K preparations. Since the methods of bioavailability testing of vitamin K are not applicable in routine practice, various functional forms of extrahepatic vitamin K-dependent Gla-protein MGP can serve as biomarkers for assessing the need for supplementation with vitamin K.

Keywords: matrix Gla-protein, vitamin K, vascular calcification,

INTRODUCTION

Cardiovascular diseases (CVD) accounts for more than half of the deaths in Europe and are one of the main causes of disability and reduced quality of life. In addition to traditional preventative measures such as blood pressure and cholesterol lowering, a healthy lifestyle in terms of nutrition, smoking and physical activity, particular attention has been paid in recent years to the establishment of molecular biomarkers for early detection and follow-up of CVD and their complications.

Coronary arterial calcification is one of the early manifestations in the pathogenesis of atherosclerosis and is an independent risk factor for the occurrence of cardiovascular complications. It is believed that vascular calcification is not a passive process. It is actively regulated multifactor process caused by an imbalance between the mechanisms stimulating calcium deposition in the vascular wall and those that suppress it [1]. In recent years, it has been proven that one of the mechanisms regulating vascular calcification is its active inhibition by the use of inhibitory molecules important for the prevention of pathological calcification. These are proteins rich in gamma-glutamyl residues (Gla-proteins), whose mechanism of action and role in regulatory processes affecting calcium deposition in the vascular wall is intensively studied in recent years [2].

The discovery of vitamin K-dependent matrix Gla-protein (MGP) as a vascular calcification inhibitor has considerably altered the mechanistic understanding of this process and has opened new opportunities for biomarker search for diagnosis and treatment. The importance of MGP for cardiovascular health is also because no other alternative mechanism for inhibiting vascular mineralization has been found so far [3].

The aim of the present work was to perform a critical analysis of the literature related to the role of the extrahepatic matrix Gla-protein (MGP) as inhibitor of vascular calcification.

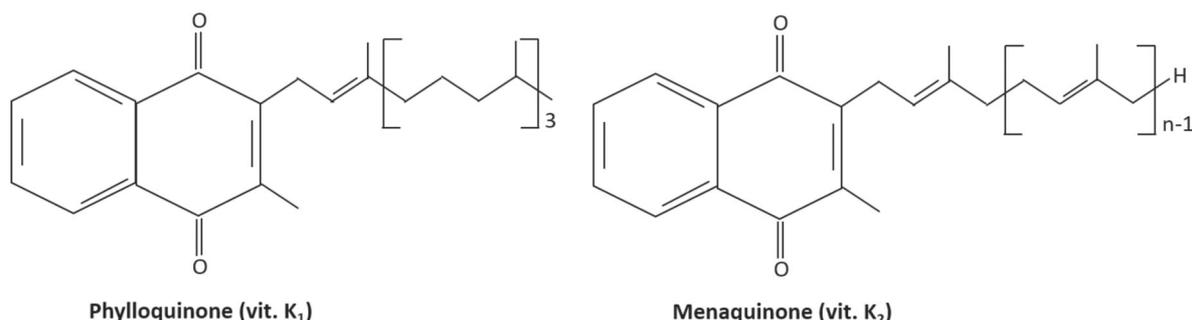
MATERIALS AND METHODS

Online databases PubMed, Google Scholar, Scopus, Science Direct, and Medline were thoroughly searched using key words such as Gla protein, Gla rich protein (GRP), matrix GLA protein (MGP), cardiovascular calcification, vitamin K, calcification inhibitor, aortic valve calcification. More than 1400 articles in the last 10 years were found.

Vitamin K related Gla-proteins

Vitamin K is a group name for a number of structurally related compounds including phyloquinone (vitamin K1) and menaquinones (vitamin K2). Menaquinones are classified according to the length of their aliphatic side chain and are designated as MK-n, where n stands for the number of isoprenoid residues in that chain (Fig. 1).

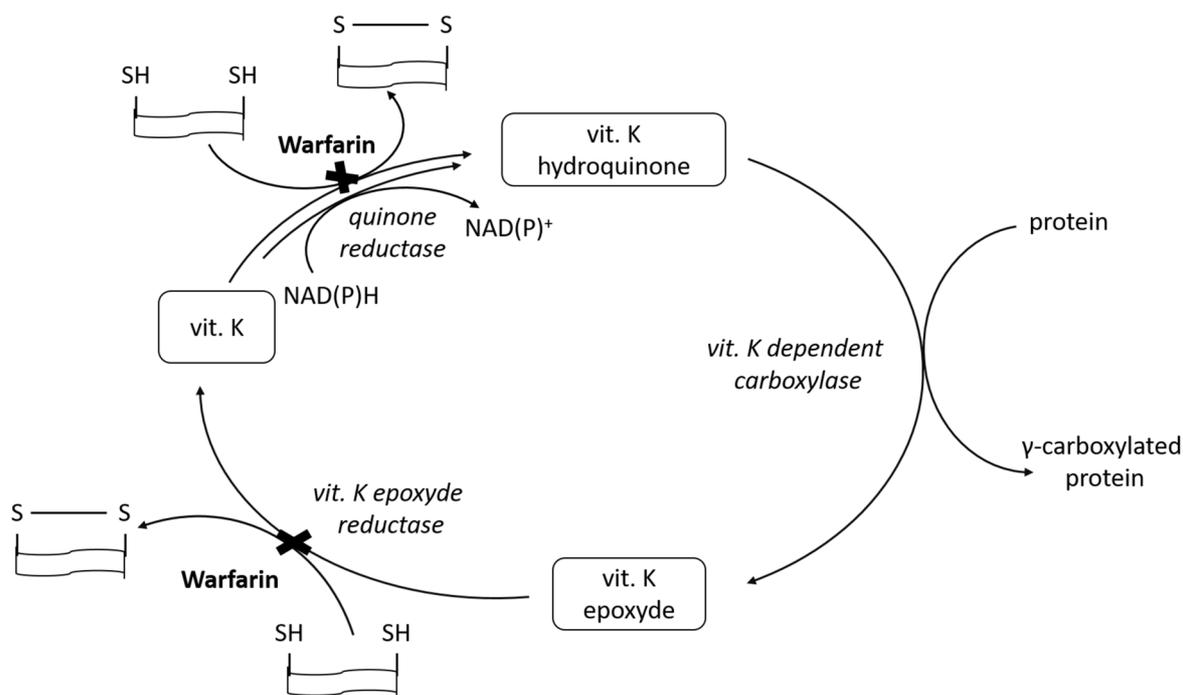
Fig. 1. Structures of phyloquinone and menaquinones



The function of all forms of vitamin K is that they serve as a cofactor for the posttranslational carboxylation of certain protein-bound glutamate residues, which are converted into gamma-carboxyglutamate (Gla). These Gla residues form calcium-binding sites that are essential for the activity of the proteins in which they are found. During

gamma-glutamate carboxylation, vitamin K is oxidized into its epoxide form (KO), which is reconverted to vitamin K quinone (K) by the enzyme vitamin K epoxide reductase (VKOR). Derivatives of 4-hydroxycoumarin (including warfarin and acenocoumarol) specifically inhibit VKOR, thus preventing the recycling of vitamin K (Fig. 2.)

Fig. 2. Vitamin K cycle (after [4])



Beside the well-known hepatic Gla-containing proteins such as the blood coagulation factors II, VII, IX, and X, the extrahepatic Gla-proteins are a group of vitamin K dependent proteins that play a crucial role as regulators of soft tissue calcium deposition, including the vascular and valvular calcification. Gla-proteins not related to blood clotting are osteocalcin (synthesized in bone), Gla-rich protein (GRP), and matrix Gla-protein (MGP) primarily synthesized in cartilage and in the vessel wall. Two of them, MGP and GRP, are powerful modulators of calcium availability and inhibitors of soft tissue calcification.

A common characteristic of all known members of this protein family is that the Gla-residues are absolutely required

for protein activity [5]. In all Gla-proteins the affinity for gamma-glutamate carboxylase is determined by a pro-sequence located immediately at the N-terminal site of the protein. In most Gla-proteins the pro-sequence is cleaved off during maturation [6]

Although in some cases conflicting data were obtained, accumulating evidence suggests that low vitamin K intake is associated with low bone mineral density, increased fracture risk, and increased risk of cardiovascular disease and mortality [7].

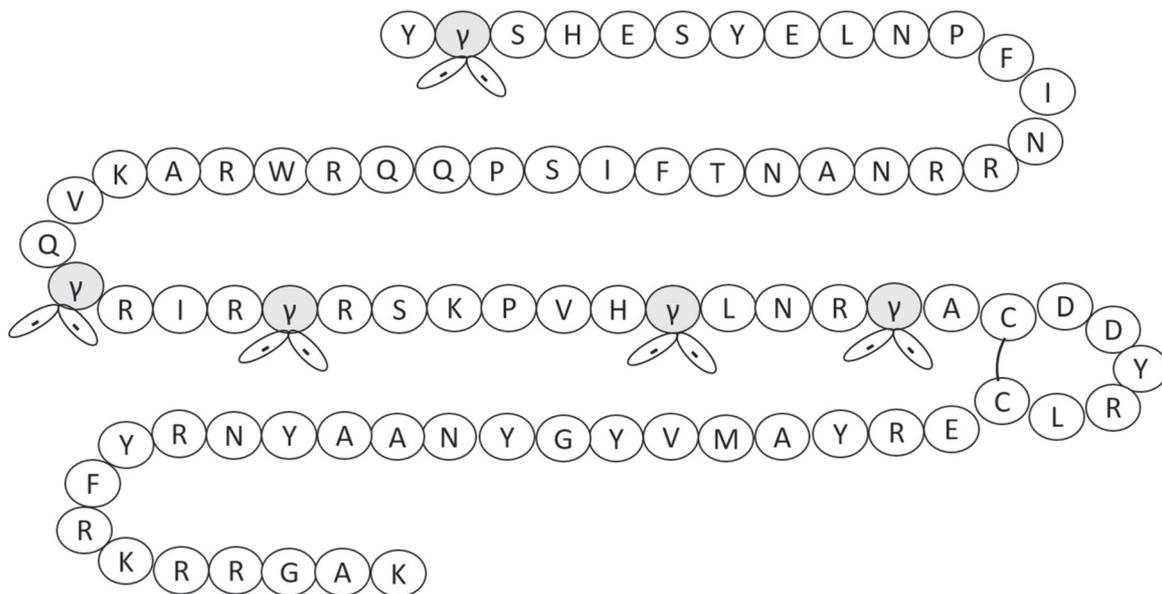
Matrix Gla protein (MGP)

General characteristics and structure of MGP

The Matrix Gla protein was first described in 1983 by Price et al. who purified it from the bovine bone matrix. The authors concluded that this approximately 14 kD pro-

tein contains five unusual amino-acids designated as gamma-carboxyglutamate, and therefore the protein was designated as matrix Gla-protein (MGP) (Fig. 3).

Fig. 3. Structure of MGP



Soon after its discovery in bone, MGP synthesis in cartilage, lung, heart, kidney, arteries and calcified atherosclerotic plaques was confirmed. The mature protein consists of 84 amino-acids and has a theoretical PI of 9.7. Of the nine glutamate residues, only five can be gamma-carboxylated in a vitamin K-dependent reaction, and three of its five serine residues can be phosphorylated into phosphoserine (Pser). The MGP gene is located on chromosome 12, consists of four exons and three large introns and has a length of 3.9 kb. It contains metal responsive elements and presents putative binding sites for AP1 and AP2 and cAMP-dependent transcription factors. At physiological levels, vitamin D₃ increases MGP transcription in vascular smooth muscle cells (VSMC) whereas retinoic acid down regulates its expression.

MGP can undergo two types of posttranslational modification: γ -glutamate carboxylation and serine phosphorylation. The best studied posttranslational modulation of MGP is gamma-glutamate carboxylation. Gla-residues are formed in a unique posttranslational modification carried out by the enzyme gamma-glutamate carboxylase [8]. The only unequivocal role of vitamin K is to provide the energy to drive the carboxylase reaction. The Gla-residues formed are negatively charged, and proteins in which they are found are denominated as Gla-proteins. The mature protein contains an internal pro-peptide which may contribute to its unique properties. Phosphorylation, the other posttranslational modification in MGP, may take place at serine residues in positions 3, 6 and 9.

Price et al. showed that the motif in MGP recognized for serine phosphorylation is the tandemly repeated Ser-X-Glu sequence. Phosphorylation is carried out by the Golgi casein kinase [9]. The function of serine phosphorylation is

not precisely known, but recent data suggest that it plays a role in regulating the secretion of proteins into the extracellular environment. Wajih et al. showed that phosphorylated MGP exits vascular smooth muscle cells (VSMCs) via the secretory pathway, whereas the non-phosphorylated MGP appears in the cytosol, and is thus not secreted [9]. The fat-soluble vitamins A and D may modulate MGP expression, while retinoic acid regulates chondrocyte maturation and mineralization [10]. The effect of retinoic acid on MGP mRNA expression levels is cell type-dependent: in fibroblasts, chondrocytes, osteoblasts, and type II pneumocytes, retinoic acid upregulates MGP mRNA expression whereas in kidney cells and VSMCs, it downregulates MGP expression. The active form of vitamin D, 1,25(OH)₂D₃, was shown to increase MGP expression *in vitro* in VSMCs. It was shown that in cell cultures and in animal models, as well as in humans, extremely high vitamin D intake may cause vascular calcification, most likely due to a stimulatory effect on calcium-metabolism.

Biological functions of MGP

Although the precise molecular mechanisms of MGP function are not known, accumulating data demonstrate its major role in the inhibition of soft-tissue and vascular calcification. The first clues for a Gla-protein being involved in the inhibition of tissue calcification came from rats treated with the vitamin K-antagonist warfarin. These animals developed massive cartilage calcification, notably in the epiphyses and facial bones, leading to impaired growth, maxillonasal hypoplasia and reduction in the length of the nasal bones. It was only after the identification of MGP in cartilage that it was recognized that the cartilage calcifica-

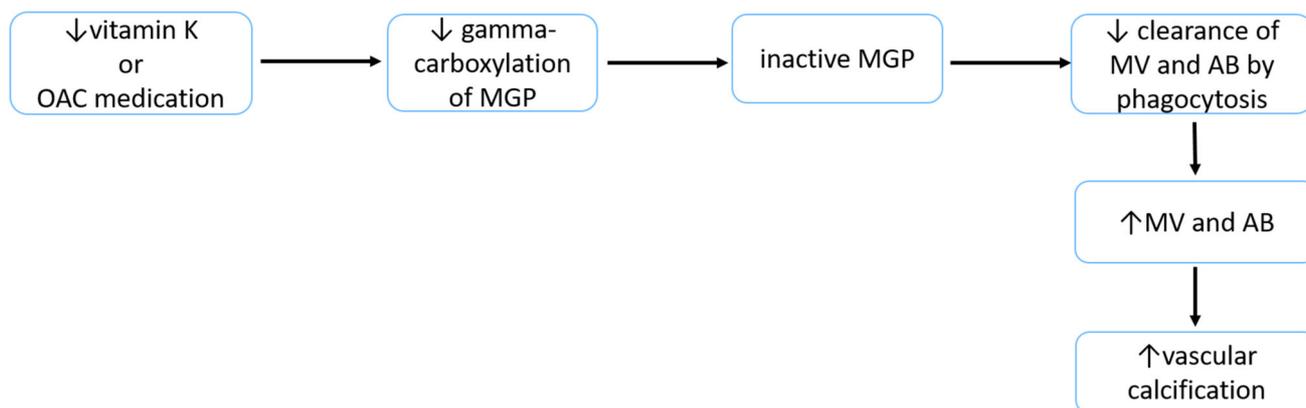
tion was brought about by loss of MGP function. After its discovery, it was thought for many years that the importance of MGP was restricted to bone and cartilage metabolism. By targeted deletion of the MGP gene in mice, it became clear, however, that its main function is the inhibition of medial calcification of the arteries: MGP-deficient animals all died within six to eight weeks after birth due to calcification of the elastic lamellae in the tunica media, resulting in rupture of the large arteries.

The arterial calcification in the MGP null mice resulted from the precipitation of calcium-phosphate in a ratio similar to hydroxyapatite, thus mimicking bone mineralization. Using histochemical techniques, it was demonstrated that the arterial calcification was associated with the differentiation of VSMCs into chondrocyte-like cells [11]. A mechanism explaining the strong calcification inhibitory activity of MGP was put forward by Price, who suggested that MGP binds tightly to the crystal nuclei thus preventing further growth. Inhibition of the differentiation of VSMCs into chondrocyte - and osteoblast-like cells may be a second function of MGP for which further support was provided in MGP-deficient mice by demonstrating a loss of smooth muscle markers and upregulated expression of the bone-specific transcription factor *cbf1a/Runx2* and the osteogenic protein osteopontin. The ability of MGP to keep VSMCs in the contractile phenotype may be accomplished by binding to the bone morphogenetic protein-2 (BMP-2). BMP-2 is a member of the transforming growth factor-beta (TGF-beta) superfamily, and is an osteogenic growth factor. BMP-2 has been shown to be expressed in human athero-

sclerotic lesions. Wallin et al. demonstrated that only the carboxylated form of MGP binds to BMP-2. Moreover, Bostrom et al. presented data suggesting that MGP blocks the osteoinductive properties of BMP-2. This inhibitory function is further supported by work of Shanahan et al., who showed that MGP expression is lower in the media of arteries from diabetic patients with Mönckeberg's sclerosis than in normal vessels. Via its C-terminal region, MGP can also bind to the extra-cellular glycoprotein vitronectin, which is present in the extracellular matrix of the arteries [12]. The C-terminal part of MGP is hydrophobic and does not contain Gla- or Pser-residues, which are all present in the more hydrophilic N-terminal and mid-section of the molecule. It may be hypothesized that MGP's binding to vitronectin results in a concentration of calcification-inhibitory activity in the milieu surrounding the elastic fibers, thereby protecting them from mineralization. Formation of matrix vesicles (MV) and apoptotic bodies (AB) is thought to precede and/or initiate arterial calcification. VCSMs undergoing apoptosis provide negatively charged membrane particles which – if not phagocytosed properly – play a role in the initiation of calcification. The physiological function of these extracellular membrane particles is to serve as the initial nidus of calcification in cartilage. Also in the vessel wall, both MV and AB are relatively common, notably in atherosclerotic plaques, arterial injury and Mönckeberg's sclerosis. When VSMCs are grown in culture they can form multicellular nodules, containing a high number of AB. MGP expression is highest in this phase, suggesting an association between MGP and apoptosis (Fig. 4.).

Fig. 4. Proposed mechanism for MGP inhibitory activity (after [13])

OAC – oral anticoagulants; MV – matrix vesicles; AB – apoptotic bodies; MGP – matrix Gla-protein; ↓ - decreased; ↑ - increased



Reynolds et al. showed in cell culture systems that VSMC derived MV and AB both contain MGP which is thought to limit the rate of calcification [14]. On the contrary, specific knock-in expression of MGP in VSMCs of MGP-deficient mice completely rescued the calcification phenotype [15]. The same study showed that expression of MGP in the liver of MGP-deficient mice results in high circulating levels of MGP. However, the elevated systemic levels of MGP had no effect on inhibition of arterial calcifica-

tion implying that MGP inhibits calcification by acting locally within its tissue of synthesis, not systemically. In humans, mutations in the gene encoding for MGP – predicting a non-functional protein – cause the Keutel syndrome, a rare disorder characterized by abnormal cartilage calcification and peripheral pulmonary stenosis [16]. Post mortem examination of a young Keutel patient also revealed extensive arterial calcification.

MGP and arterial calcification

Until a decade ago, calcification of arteries was thought to be a passive, clinically irrelevant process, resulting from a high calcium x phosphate product, inflammation, lipid accumulation or diabetes. However, during recent years it has become increasingly clear that vascular calcification is an active process and an important, independent pathology that is strongly associated with increased risk of cardiovascular morbidity and mortality [17]. Clinically, vascular calcification caused stiffening of the vascular wall, which may result in decreased arterial compliance, development of left ventricular hypertrophy and decreased coronary perfusion leading to an increased risk of fatal complications. Calcification is common in the elderly population, and in patients suffering from diseases such as chronic kidney disease (CKD), diabetes, aortic stenosis, and atherosclerosis. Therefore, a lot of efforts have been directed towards retarding or reversing the development of calcification in the vasculature. In animal models it has been shown that arterial calcification is reversible [18-20], demonstrating that also the regression process is actively regulated. In humans, attempts to use lipid lowering drugs (statins) to stabilize or regress calcification have so far failed to show a significant effect [21, 22]. CKD patients have the highest incidence of arterial calcification, and cardiovascular mortality is 20-fold higher than in the apparently healthy population. Moreover, moderate to severe vascular calcifications are found in 60–80% of patients on hemodialysis. Recently, it was shown that vitamin K-status in CKD patients is low [23]. Circulating vitamin K levels were measured and reported that some 30% of the hemodialysis patients had sub-clinical vitamin K-deficiency. The authors discussed the possibility of giving these patients extra vitamin K to reduce the risk for cardiovascular events [23]. Additionally, the need for vitamin K in patients might be much higher than in the general population. Anticoagulation therapy with vitamin K antagonists, which is regularly prescribed in these patients, will exacerbate the low vitamin K-status in these patients. Together with the additional immunohistochemical evidence of high levels of undercarboxylated MGP (ucMGP) present in calcified areas [19, 24, 25], these data are suggestive for high vitamin K intake as a novel treatment option for cardiovascular calcification. The first clinical studies in dialysis patients are in progress [26].

MGP as a biomarker

MGP is one of the strongest inhibitors of arterial calcification, which function depends on the presence of vitamin K. MGP is a local inhibitor of vascular calcification, and it has been demonstrated that circulating MGP has no biological function [15]. However, circulating MGP may reflect calcification processes and inhibition of those processes in the vascular wall.

Both cardiovascular calcification and MGP activity are directly correlated with vitamin K2 intake [5, 19, 27]. It is well documented that plasma dephosphorylated decarboxylated MGP (dp-ucMGP) is an established biomarker of poor vitamin K status [28]. High plasma inactive MGP levels, indicative of functional vitamin K insuffi-

ciency, were found to be associated with an increased risk for CVD [29] and mortality [30.] in patients with diabetes, chronic kidney disease (CKD), and CVD.

A recent study demonstrated that high plasma dp-ucMGP concentrations were associated with an increased risk for mortality in the general population [31]. A prospective study on 4275 subjects aged 53 ± 12 years showed that plasma dp-ucMGP concentrations were significantly higher among elderly and subjects with comorbidities like hypertension, type 2 diabetes, CKD, and CVD [32]. The authors demonstrated that these dp-ucMGP concentrations increased even further as the number of comorbidities increased. A J-shaped association was found between plasma dp-ucMGP concentrations and all-cause and cardiovascular mortality that remained significant after adjustment for potential confounders. It was shown that dp-ucMGP levels above 414 pmol/L were associated with an increased risk for all-cause mortality, and values above 557 pmol/L were related with increased risk for cardiovascular mortality.

The Flemish follow-up Study on Environment, Genes and Health Outcomes (FLEMENGHO) examined whether circulating dp-ucMGP could predict a decrease in estimated glomerular filtration rate (eGFR) in 1009 white Europeans, during a period from 1996 to 2015 year. They stated that circulating inactive dp-ucMGP, a biomarker of poor vitamin K status, predicts renal dysfunction exhibited by a decline in eGFR in all participants and microalbuminuria at follow-up. [33]. In a multiethnic cross-sectional study, including 66 participants with type 2 diabetes, an independent association between circulating dp-ucMGP and carotid-femoral pulse wave velocity was established. This suggests that deficient vitamin K-dependent activation of MGP may lead to large artery stiffening and vitamin K supplementation of the patients with diabetes could be beneficial [34].

Additionally, it appeared that high vitamin K intake resulted in improved MGP carboxylation, regression of preformed calcifications and subsequent increased vascular elasticity [35]. The development of commercially available conformation-specific antibodies will facilitate the usage of different functional forms of MGP as potential biomarkers for vascular calcification in patients with cardiovascular diseases.

CONCLUSION

Cardiovascular calcification is one of the earliest sign in the pathogenesis of cardiovascular disease. The discovery of the Vitamin K dependent matrix Gla protein (MGP) gives a new perspective on soft tissues calcification and future hope for the early detection and prevention of cardiovascular diseases. Discovery of the relationship between the different isoforms of vitamin K and extrahepatic Gla-proteins can be a prerequisite for making an adequate solution for supplementing with vitamin K preparations. Since the methods of bioavailability testing of vitamin K are not applicable in routine practice, the various functional forms of extrahepatic vitamin K-dependent Gla-protein MGP can serve as biomarkers for assessing the need for supplementation with vitamin K or its analogues or dietary changes with foods enriched with vitamin K.

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Address for correspondence:

Yulian Snezhanov Snegarov, MD
Department of Biochemistry, Molecular medicine and Nutrigenomics, Pharmacy faculty, Medical University – Varna, Varna,
84, Tzar Osvoboditel blvd., 9000 Varna, Bulgaria.
E-mail: yuliansnegarov@gmail.com