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Matrix Gla-protein: The calcification inhibitor in need of vitamin K

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Summary

Among the proteins involved in vascular calcium metabolism, the vitamin K-dependent matrix Gla-protein (MGP) plays a dominant role. Although on a molecular level its mechanism of action is not completely understood, it is generally accepted that MGP is a potent inhibitor of arterial calcification. Its pivotal importance for vascular health is demonstrated by the fact that there seems to be no effective alternative mechanism for calcification inhibition in the vasculature. An optimal vitamin K intake is therefore important to maintain the risk and rate of calcification as low as possible. With the aid of conformation-specific antibodies MGP species in both tissue and the circulation have been detected in the healthy population, and significant differences were found in patients with cardiovascular disease (CVD). Using ELISA-based assays, uncarboxylated MGP (ucMGP) was demonstrated to be a promising biomarker for cardiovascular calcification detection. These assays may have potential value for identifying patients as well as apparently healthy subjects at high risk for CVD and/or cardiovascular calcification and for monitoring the treatment of CVD and vascular calcification.

Keywords

Matrix Gla-protein, vitamin K, calcification, cardiovascular disease, oral anticoagulants

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Background

Matrix Gla-protein (MGP) is a small secretory protein that can undergo two types of posttranslational modification: γ-glutamate carboxylation and serine phosphorylation. The protein was first described in 1983 by Price et al. who purified it from the bovine bone matrix (1). The authors concluded that this approximately 14 kD protein contains five unusual amino-acids designated as γ-carboxyglutamate (abbreviated as Gla), and therefore the protein was designated as matrix Gla-protein (Fig. 1). Soon after its discovery in bone, MGP synthesis in cartilage, lung, heart, kidney, arteries and calcified atherosclerotic plaques was confirmed (1–3). 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 γ-carboxylated in a vitamin K-dependent reaction, and three of its five serine residues can be phosphorylated into phosphoserine (abbreviated as Pser). The MGP gene is located on chromosome 12 (p13.1-p12.3), 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 increased MGP transcription in VSMC whereas retinoic acid down regulates its expression (4).

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 (5). 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. A common characteristic of all known members of this protein family is that the Gla-residues are absolutely required for protein activity (6). 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; MGP is the exception in this respect, since 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 (Fig. 1). Price et al. showed that the motif in MGP recognized for serine phosphorylation is the tandemly repeated Ser-X-Glu sequence (4). Phosphorylation is carried out by the Golgi casein kinase (4, 7). 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 environ-

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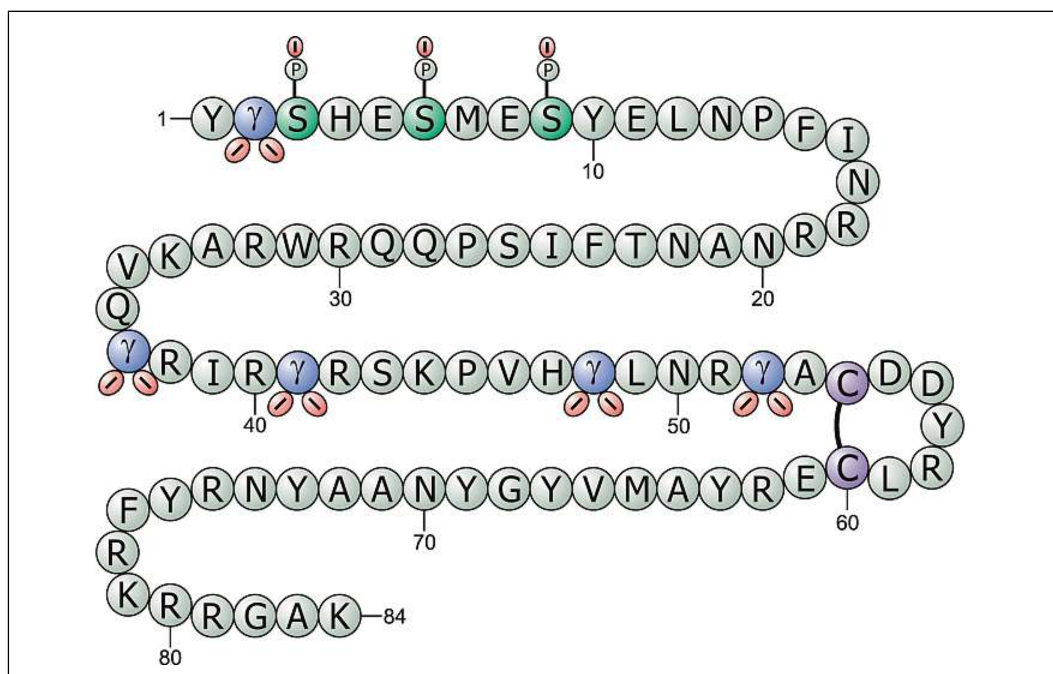


Figure 1: Matrix Gla-protein (MGP) is a small 84aa vitamin K-dependent protein. Although its 14 kD size, it can undergo two posttranslation modifications: at position 3, 6, and 9 the serine residues can be phosphorylated by a Golgi-casein kinase, and at positions 2, 37, 41, 47 and 52 the glutamate residues can be γ-carboxylated (117).

ment. 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 (7).

The fat-soluble vitamins A and D may modulate MGP expression. Retinoic acid is a regulator of chondrocyte maturation and mineralization (8). Its effect on mRNA expression levels of MGP is cell type-dependent: in fibroblasts, chondrocytes, osteoblasts, and type II pneumocytes, retinoic acid upregulates MGP mRNA expression (9, 10) whereas in kidney cells and VSMCs, retinoic acid downregulates MGP expression (11, 12). 1,25(OH)D_3 was shown to increase MGP expression *in vitro* in VSMCs (11). In models *in vitro* (13), animal models (14) and humans (15), extremely high vitamin D intakes may cause vascular calcification, most likely due to an effect on calcium-metabolism.

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 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 (16). 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 (17). It was only after the identification of MGP in cartilage that it was recognized that the cartilage calcification was brought about by loss of MGP function (18). 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 (19). The arterial calcification in the MGP null mice resulted from the precipitation of calcium-phosphate in a ratio similar to hydroxyapatite, thus mimicking bone mineralisation. Using histochemical techniques, the authors demonstrated that the arterial calcification was associated with the differentiation of VSMCs into chondrocyte-like cells. 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 (20). Inhibition of the differentiation of VSMCs into chondocyte- 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 (21). The ability of MGP to keep VSMCs in the contractile phenotype may be accomplished by binding to the bone morphogenetic protein-2 (BMP-2) (22, 23). 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 atherosclerotic lesions (24). Wallin et al. demonstrated that only the carboxylated form of MGP binds to BMP-2 (22); moreover, Bostrom et al. presented data suggesting that MGP blocks the osteo-inductive properties of BMP-2 (25). 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 (26). Via its C-terminal region, MGP can also bind to the extra-cellular glycoprotein vitronectin, which is present in the extra cellular matrix of the arteries (27). 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 (28). 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 (29, 30), arterial injury (31) and Mönckeberg's sclerosis (32, 33). 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. Reynolds et al. showed in cell culture systems that VSMC derived MV and AB both contained MGP which is thought to limit the rate of calcification (34).

The specific knock-in expression of MGP in VSMCs of MGP-deficient mice completely rescued the calcification phenotype (35). In the same article the authors also expressed MGP in the liver of MGP-deficient mice, resulting in high levels of circulating MGP. However, the elevated systemic levels of MGP had no effect on inhibition of arterial calcification 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 (36), a rare disorder characterized by abnormal cartilage calcification and peripheral pulmonary stenosis (37). Post mortem examination of a young Keutel patient also revealed extensive arterial calcification (38).

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 (39–41). Clinically, vascular calcification causes 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 (42, 43). Calcification is common in the elderly population, and in patients suffering from diseases such as chronic kidney disease (CKD), diabetes, aortic stenosis, and atherosclerosis (44). 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 (45–48), demonstrating that also the regression process is an actively regulated process. In humans, attempts to use lipid lowering drugs (statins) to stabilize or regress calcification have so far failed to show a significant effect (49, 50).

CKD patients have the highest incidence of arterial calcification, and cardiovascular mortality is 20-fold higher than in the apparently healthy population (51, 52). Moreover, moderate to severe vascular calcifications are found in 60–80% of patients on hemodialysis (53, 54). Recently, it was shown that vitamin K-status in CKD patients is low (55). Circulating vitamin K levels were measured and reported that some 30% of the haemodialysis 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 (55). 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 uncarboxylated MGP (ucMGP) present in calcified areas (47, 56, 57), these data are suggestive for high vitamin K intake as a novel treatment option for cardiovascular calcification (see also below). The first clinical studies in dialysis patients are in progress.

Factors affecting MGP activity

Vitamin K / warfarin

It has been known for a long time that women receiving anticoagulant therapy with vitamin K antagonists (coumarin derivatives) during the first trimester of pregnancy are at risk of delivering children with a syndrome characterized by nasal hypoplasia, depression of the nasal bridge and punctuate calcifications in the axial skeleton, proximal femurs and calcanei (58). This syndrome is known as warfarin embryopathy (fetal warfarin syndrome), and the abnormalities were first believed to be caused by haemorrhages in the developing fetal cartilages with subsequent calcification of these areas (58–60). However, it was soon recognized that this was unlikely, since clotting factors were known to be absent during the first trimester of pregnancy (58, 61). Similarities between the facial and skeletal abnormalities seen in warfarin embryopathy and the fetal phenytoin (hydantoin) syndrome suggested that prenatal vitamin K-deficiency may underlie these abnormalities (58, 62). This was confirmed by Pauli et al. who described a congenital deficiency of the enzyme vitamin K-epoxide reductase (VKOR, needed for recycling of vitamin K), causing prenatal vitamin K deficiency, and resulting in a similar phenotype (63, 64). In later years it was reported that also dietary vitamin K-deficiency results in comparable calcification abnormalities (65–69), which were remarkably similar to the bone and cartilage defects observed in warfarin-treated rats (16). The same animal model provided the first evidence that impairment of MGP function results in vascular calcification (20). It was found that within two weeks of warfarin treatment, the elastin fibres in the tunica media were significantly calcified. Further evidence for the pivotal role of Gla residues for MGP function was provided by Murshed et al. who used the MGP null mice in which MGP cannot be carboxylated, since the glutamate residues in the Gla-domain were mutated into aspartate; in this way it was demonstrated that only carboxylated MGP (cMGP) exhibits anti-mineralization properties (35). Vitamin K antagonists are frequently used to prevent thrombosis in

patients at increased risk for thrombosis (70). Treatment periods range from several weeks to many years, even often life-long (71). After demonstration in animal models that vitamin K antagonists induce vascular calcification, studies in two independent populations revealed that indeed treatment with coumarin derivatives induces excessive calcification of the coronary arteries and the aortic heart valve (72, 73). Schurgers et al. compared valvular calcification in patients receiving oral anticoagulant treatment for a period of between 16 and 35 months with patients not on oral anticoagulation (72). Histopathological evaluation of the valves from the patients who had received oral anticoagulation showed partial or total valve destruction induced by amorphous calcified deposits. Quantification of the calcium contents of the aortic valves showed a statistically significant difference between valves from those who had never received oral anticoagulant treatment and those who had received this treatment. These data were confirmed by Koos et al., who used multislice spiral computed tomography (CT) to quantitate the extent of aortic calcification in patients on long term oral anticoagulant treatment (73). It was found that these patients had increased coronary calcification compared to patients without anticoagulation treatment (Agatston score 1,561 and 738, respectively). The present policy is opposite, however: large numbers of cardiovascular disease (CVD) patients receive anticoagulant therapy with vitamin K antagonists, which increases their calcification tendency. The data described above suggest that – if possible – other forms of anticoagulation (specific prothrombin or factor-X inhibitors) should be employed, preferably in combination with high vitamin K intake. This treatment could activate MGP, and the intriguing question remains whether it decreases CVD in parallel. Together, these data demonstrate a hitherto unrecognized adverse side-effect of coumarin derivatives which should be considered when designing optimal anti-thrombotic treatments for patients.

Vitamin K comprises a family including vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinones). The mechanism of vitamin K_1 is believed to be most important for activation of hepatic clotting factors whereas K_2 also is important for proteins synthesized in extra-hepatic tissues such as the vasculature. Moreover, there is now scientific evidence that K_2 vitamins have additional properties, including apoptosis and cell-cycle arrest and anticancer properties (74, 75), inhibition of the synthesis of prostaglandin E2 (PGE2) (76), osteoclast apoptosis (77), and binding to the SXR in the osteoblast, resulting in induction of osteoblastic markers (78). Recently, it was shown that vitamin K_2 could also down regulate osteoprotegrin and increase DT-diaphorase, implicating that vitamin K_2 is an anti-calcification component in the vessel wall (79). For a more extensive review on the gene regulatory functions of vitamin K_2, see Shearer article, this volume beginning on page 530.

The question of whether high vitamin K-intake is protective against arterial calcification was first addressed in a population-based study among participants of the Rotterdam Study. It was demonstrated that dietary vitamin K_2 intake (and not K_1) was inversely correlated with cardiovascular calcification and cardiovascular death (6). Elderly people in the highest tertile of vitamin K_2 intake had about 50% reduction in both aortic calcification and cardiovascular mortality and 25% decreased all-cause mortality. In a clinical intervention study in which 78 women between 55 and 65 years of age received either vitamin K (1 mg/day) or placebo for three years, vascular characteristics were assessed (elasticity and distensibility) (80). In subjects in the placebo group vascular elasticity had decreased by 10–13%, which is consistent with the normal decrease during the time-period of three years; in the vitamin K group, however, vascular characteristics had remained unchanged, suggesting that the process of vascular aging can be retarded by increased vitamin K intake.

The concept of calcification inhibition by high vitamin K intake was confirmed in experimental animals (81). In this model the efficacy of vitamins K_1 and K_2 in preventing arterial calcification was compared. It was found that K_2 completely inhibited tissue calcification, whereas a similar or even an eight-fold higher dose of K_1 had no measurable effect. To fully understand this model it is important to know that vitamin K_1 can be converted into MK-4 via MK4-O, but that warfarin blocks the conversion of MK4-O into reduced MK4, which is the active cofactor (82). Buitenhuis et al. showed that K_2 vitamins, especially the long chain K_2 vitamins such as menaquinone-7, have lower K_M values for the enzyme γ-glutamyl carboxylase, demonstrating that they are the preferred cofactor for vascular carboxylase (83). Additionally, recently Wallin et al. showed that specifically K_2 acts as an anti-calcification component in the vessel wall by increasing the gene expression with a 4.8-fold higher specific activity of DT-diaphorase, an enzyme of the vitamin K-cycle (79). The same animal model was used to study the effect of high vitamin K intake on potential regression of arterial calcification (47). Whereas rats receiving the standard chow (control) had very low aortic calcium during the entire experiment, a six-week warfarin treatment led to the accrual of calcium salts up to 12-fold above the baseline values. During the following six-week period warfarin treatment was stopped and the animals received either standard chow or standard food fortified with a high dose of vitamin K_1 or K_2. It was shown that once calcium deposits had formed, the accrual of calcium salts in the vasculature increased linearly after the warfarin diet had been replaced by the normal dose of vitamin K. At high doses of either K_1 or K_2, however, the process of calcification was not only stopped, but a significant fraction (some 40%) of the previously formed calcium salts had been removed within six weeks. This effect was found both in the aorta and in the coronary arteries. Using immunohistochemistry it was demonstrated that parallel to the regression of aortic calcium content, cMGP had increased and ucMGP had decreased, suggesting a role of activated MGP in the regression of calcified plaques. The fact that vitamins K_1 and K_2 had a similar effect in this experiment may be explained by the very high dosages used, and by the fact that in the absence of warfarin up to 25% of the vitamin K_1 may be converted into K_2 (84). Only by performing dose-response studies the efficacies of both vitamins may be compared in this model.

Carboxylase / VKOR

Mutations in the γ-glutamate carboxylase gene result in bleeding disorders due to the inability to activate sufficient vitamin K-dependent clotting proteins (85, 86). Since only one gene encodes for the enzyme, also Gla-proteins produced in extra-hepatic tis-

sues are affected by this mutation. More recently it was shown that patients with the γ-glutamate carboxylase mutation not only presented with haemostatic disorders, but also with soft tissue calcifications, as is seen in patients with a pseudoxanthoma elasticum (PXE)-like phenotype (87). Pseudoxanthoma elasticum is an autosomal recessive multi-system disorder characterized by dystrophic mineralization of soft tissues, including skin, eyes, and arterial blood vessels (88). Whereas classic PXE is caused by mutations in the ABCC6 gene (ATP-binding cassette subfamily C member 6), patients with the PXE-like syndrome harboured known γ-glutamate carboxylase mutations in six out of seven patients analyzed. The involvement of MGP in classic PXE was recently demonstrated by two groups, showing that fibroblasts from PXE patients almost exclusively produce the inactive ucMGP, which is not able to block or inhibit calcification (89, 90). The very low cMGP production in pathological fibroblasts compared to controls suggests these cells have a deficient vitamin K metabolism which may play an important role in the ectopic calcification in PXE.

The vitamin K-epoxide reductase (VKOR) enzyme is a crucial enzyme in vitamin K metabolism and ensures the re-utilization of vitamin K after it has been oxidized in the carboxylase reaction. Because of this recycling, human vitamin K requirement is extremely low (91). On a molecular level VKOR reduces vitamin K-epoxide in two steps: first to the quinone, and subsequently to vitamin K hydroquinone (KH_2), which is the active cofactor for γ-glutamate carboxylase. VKOR is also the target for warfarin and related coumarin derivatives, which block the recycling of vitamin K thereby decreasing the vitamin K-status. Both vitamin K-epoxide and vitamin K quinone need to bind to the VKOR before being reduced. Wallin et al. showed that the enzyme DT-diaphorase in VSMCs is 100-fold less active than in the liver. The cytoplasmic DT-diaphorase is capable of reducing vitamin K quinones to their hydroquinone cofactors, and serves as a rescue enzyme in case the VKOR is blocked by coumarin (92, 93). Therefore, coumarin treatment has a detrimental effect in the arterial vessel wall, by blocking vitamin K-metabolism leading to impaired MGP. Moreover, the vitamin K binding site in VKOR is thought to be close to the coumarin-binding site and recently it was shown that the presence of various VKORC1 haplotypes correlates with arterial vascular disease (94).

Besides being a cofactor in the vitamin K-dependent carboxylation, KH_2 also possesses antioxidant activity (95, 96). This is consistent with its high sensitivity to free radicals, which may oxidize (and thus inactivate) KH_2 before it can take part in the carboxylation reaction. Especially in the atherosclerotic plaque, high levels of oxidized LDL are found, which may thus contribute to a local vitamin K deficiency.

MGP as biomarker

As discussed above, MGP is one of the strongest inhibitors of arterial calcification, its function depending 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 (35). However, circulating MGP may reflect calcification processes and inhibition of those processes in the vascular wall. Below we will discuss the presence of MGP in vascular tissue and in the circulation, and the potential of circulating MGP as a biomarker for cardiovascular calcification.

Table 1: Matrix Gla-protein antibodies. moAb = monoclonal antibody; W = Western blot; I = immunoprecipitation; S = section; E = ELISA. Antibodies available at VitaK Products BV (www.vitak.org)

	Amino-acid	Specificity	Used	Directed against
moAb dpMGP	3–15	IgG1a	W, I, S, E	Desphosphorylated MGP
moAb pMGP	3–15	IgG1a	W, I, S, E	Phosphorylated MGP
moAb ucMGP	35–54	IgG1a	W, I, S, E	Uncarboxylated MGP
moAb cMGP	35–54	IgG1a	W, I, S, E	Carboxylated MGP

Table 2: Possibilities of MGP ELISA's. Four single antibody competitive assays can be developed, namely dpMGP, pMGP, ucMGP and cMGP conformation. Also, antibodies directed against the C-terminal part of MGP could be used to measure the total of MGP proteins. These monoclonal antibodies are not available yet. Combinations of the conformation specific antibodies could result in several MGP sandwich combinations. Here we hypothesise that dp-ucMGP is the inactive fraction whereas p-cMGP represents the active MGP fraction. More research is needed to find out the exact role of the different MGP combinations.

Phosphorylation / Carboxylation	dpMGP	pMGP
ucMGP	inactive MGP fraction	
cMGP		active MGP fraction

MGP in vascular tissue

Immunohistochemical studies have shown that in healthy vessels MGP is synthesized at relatively low rate (2, 57, 97), most likely because the need for calcification inhibition is low. However, Shanahan et al. showed that in arteries of diabetic patients lower levels of MGP protein were present than in normal vessels, suggesting that low MGP levels might predispose for calcification (26). High MGP levels have been detected in arteries with calcification (2, 57, 97). This may originate from increased MGP synthesis, which has been reported in both medial and intimal arterial calcification (2, 57, 97), or increased subsequent adsorption to the calcium salt crystals.

With the development of conformation-specific antibodies, enabling the detection of active, carboxylated and inactive, uncarboxylated MGP (cMGP and ucMGP, respectively), it became clear that specifically the ucMGP conformation accumulates in atherosclerotic and calcified arteries (56, 57, 90). The cMGP conformation was nearly absent in these arteries. These conformation-specific antibodies have proven their value for MGP detection and studying vitamin K-metabolism in several animal models. Wajih et al. demonstrated the processing and transport of the different MGP conformations in cultured VSMCs (7). In this article the complexity of MGP processing and excretion was clearly presented by using conformation specific antibodies against MGP. Sweatt et al. demonstrated that calcified arterial lesions in aging rats contained elevated MGP levels, which was uncarboxylated and not able to bind BMP-2 (56). Our group demonstrated massive accumulation of ucMGP around calcified

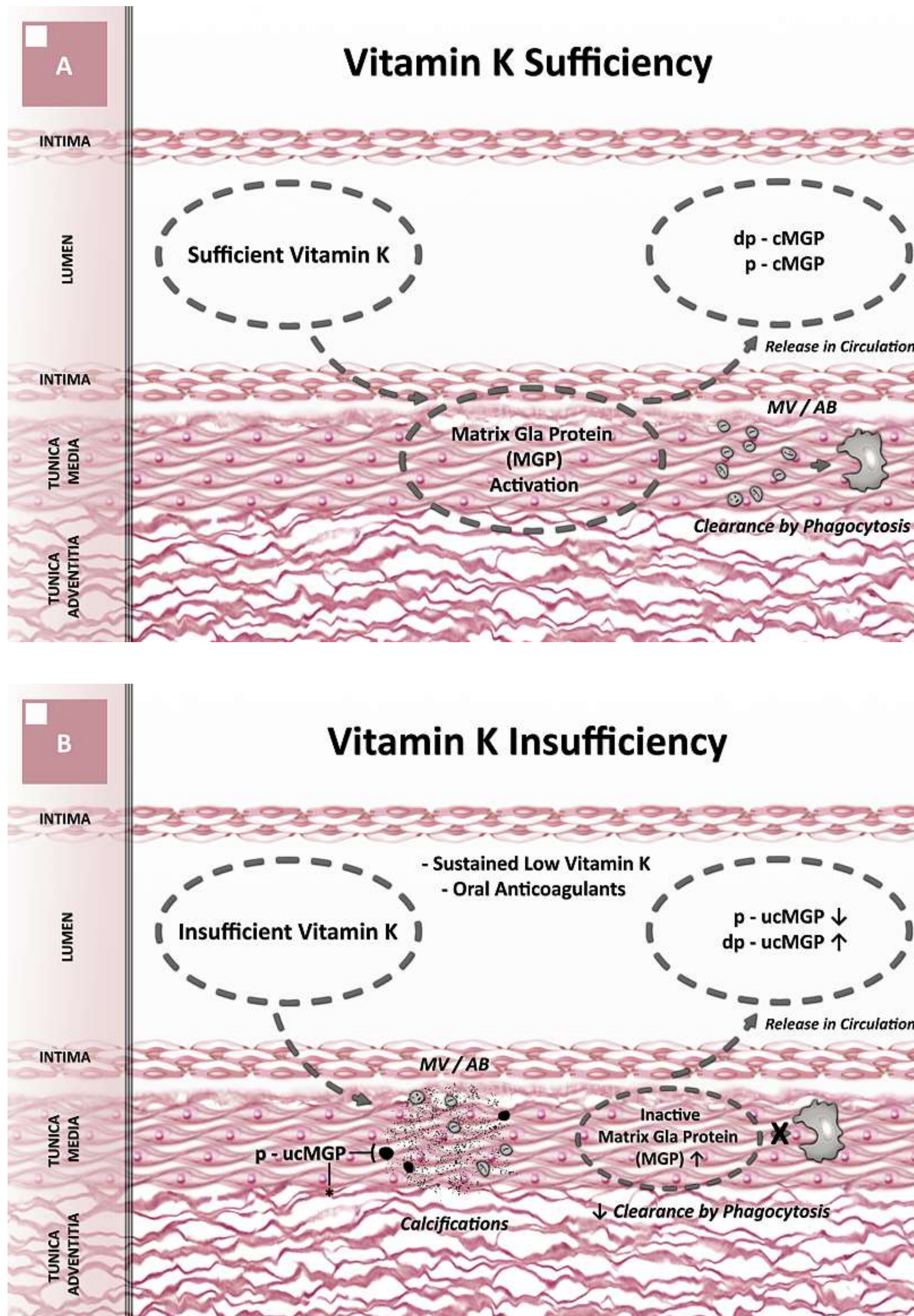
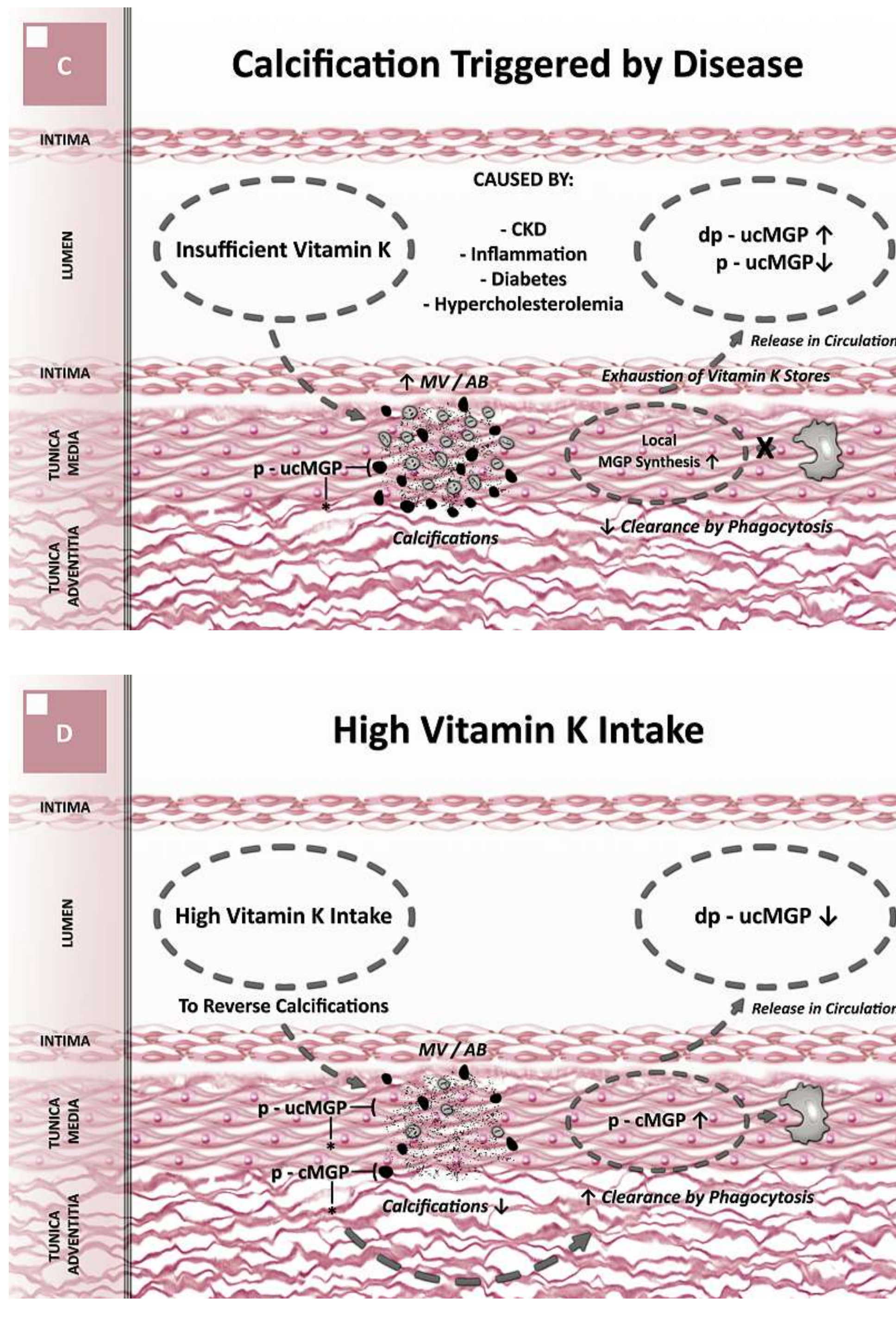


Figure 2: Vitamin K-dependent regulation of vascular calcification. A) Vitamin K-sufficiency: In the case of sufficient vitamin K supply (either via food or supplements) and in the absence of disease, all MGP synthesised in the VSMCs is activated to prevent calcification: the clearance of matrix vesicles (MV) and apoptotic bodies (AB) is supported by either macrophages and/or surrounding VSMCs. In this way, the nidus for calcification is absent. There is no hydroxyapatite matrix in the tunica media to bind to MGP. The fraction of MGP leaking into circulation is the dp-cMGP and p-cMGP. B) Vitamin K-insufficiency: Vitamin K insufficiency is present in subjects with sustained low vitamin K intake or patients on vitamin K-antagonist (coumarin derivatives). The expression of MGP is normal. The inactive MGP will lead to decreased clearance of MV and AB. The negatively charged phospholipid-remnants have the capacity to nucleate calcium and phosphate and subsequently calcify in the absence of the calcification inhibitory function of MGP. The phosphorylated ucMGP fraction will bind to the vascular calcification, and thus the p-ucMGP is lowered. The fraction easily released in the circulation is dp-ucMGP (this fraction has no or limited affinity for vascular calcium). Vitamin K-insufficiency (as deduced from inactive ucMGP species in the circulation) is present in the majority of the apparently healthy population. C) Calcification triggered by disease: In diseases leading to the shedding of high numbers of MV and AB, calcification is triggered and the need for



active MGP is high. Also in diseases with a balance in favour of calcification, e.g. high calcium-phosphate product (ESRD), inflammation (leading to apoptosis), a high synthesis of MGP is needed to counteract the nidus for calcification. In response, the demand for vitamin K increases. In the majority of the population the intake of vitamin K is insufficient to address this higher demand of vitamin K, required for the activation of all newly synthesised MGP. Therefore, a major part will occur in the ucMGP conformation, unable to stop or reverse calcification; MGP will bind to the local vascular calcifications via the p-ucMGP conformation which will be measured by the lower plasma p-ucMGP levels. The circulating MGP fraction which has no or limited affinity of calcium (dp-ucMGP) is high in this situation. D) High vitamin K-intake: Once calcifications are present, and dp-ucMGP is high, high intake of vitamin K could be used as treatment option. In this way, all newly synthesised MGP will be activated via the γ-carboxylation reaction. This will result in dp-cMGP and p-cMGP. As the active MGP fraction will support clearance / regression of calcification the amount of calcification will reduce. In the circulation, this will be reflected by a lowered dp-ucMGP level, demonstrating a beneficial shift in the tissue.

lesions which are rapidly formed in the arteries of rats during treatment with warfarin. Additionally, it appeared that high vitamin K intake resulted in improved MGP carboxylation, regression of pre-formed calcifications and subsequent increased vascular elasticity (47). The conformation-specific antibodies have recently become commercially available, which will facilitate the demonstration of their potential value as a diagnostic tool in specimens of patients with cardiovascular disease (Table 1). Together with expression levels of MGP, the activity-status of the protein may help to understand the precise function of MGP in the local inhibition of vascular calcification.

Circulating MGP

MGP can also be detected in the circulation with ELISA-based techniques. The use of a circulating biomarker for CVD and/or vascular calcification is an attractive possibility. Its value in the diagnostic area might be the pre-screening of patients before subjecting them to electron beam or multislice CT scanning. This technique is widely used to screen patients for coronary calcifications (98–100) but has the disadvantages of being expensive and posing an increased cancer risk due to the radiation load (101). The latter is especially important for regular follow-up during treatment. The crusade for finding biomarkers representing or predicting vascular disease has led to the development of ELISA's measuring proteins involved in the calcification process (20, 102–104). Of these proteins, only the function of MGP can be modulated, by either vitamin K or vitamin K-antagonists.

Circulating levels of MGP will depend on the rate of MGP synthesis in vascular tissue, its secretion from VSMCs and subsequent binding of MGP to calcified areas that may be present within the arterial wall. Currently it is not known in which forms MGP circulates. Mature MGP is highly insoluble and it is not fully understood how and whether it circulates as a free protein or associated with a carrier. Full-length MGP has been purified from the plasma of rats as a complex including calcium, phosphate, carboxylated MGP and fetuin (105). On the other hand, uncarboxylated MGP was purified from the plasma as mature 11 kD protein (106). Additionally, it is likely that also a substantial fraction of plasma MGP occurs as fragments.

MGP can undergo two posttranslational modifications: the aminoacid sequence 3–15 with three serine (ser) residues which can be phosphorylated, and aminoacid sequence 35–53 containing four glutamate residues (glu) which can be carboxylated into γ-carboxylglutamate (gla) (Fig. 1 and Table 1). These modifications result in several possible MGP conformations which can be set free in the circulation (Table 2). In the literature three assays for circulating MGP have been described. All three tests described are single antibody assays that do not discriminate between the full length molecule and fragments thereof (107–109).

The first published assay is a single antibody ELISA using a monoclonal antibody specific for the non-phosphorylated N-terminal MGP aminoacid sequence 3–15 (107, 108). This assay only measures non-phosphorylated MGP (dpMGP), and does not discriminate between cMGP and ucMGP. It has been reported the the dpMGP fraction is only a minority of the total MGP produced (4). Using a proteomics approach, Wajih et al. proposed that the phosphorylated fraction is secreted into the extra-cellular environment, whereas the non-phosphorylated form is only secreted as matrix vesicles or apopotic bodies (7). Thus, non-phosphorylated MGP may predict the VSMC stress locally. Increased levels of serum MGP were measured in patients with severe atherosclerosis and with type I diabetes mellitus using this assay (107). The same assay was used in collaboration with Jono et al. (110). In this study the severity of coronary artery calcification (CAC) was measured with electron beam CT (EBCT) in subjects with suspected coronary artery disease, and MGP was measured in serum samples from these subjects. The serum levels of MGP were significantly lower in subjects with CAC compared to those without CAC. Moreover, serum MGP levels were inversely correlated with the severity of CAC. This is consistent with data obtained in experimental animals in which significantly lower MGP levels were measured in animals with massive arterial calcifications (20, 47). An explanation for this can be the phenotypically change of VSMCs into osteoblast-like cells as response to the calcification, and subsequent down-regulation of MGP synthesis.

The second MGP assay, which is not commercially available, is a radioimmunoassay using polyclonal antibodies directed against MGP purified from human bone (109). With this assay it is not possible to discriminate between the different MGP conformations. Using this assay, O'Donnell et al. found a significant positive correlation between circulating MGP levels and coronary heart disease risk factors (Framingham CHD risk score) in both men and women. Especially the traditional lipid-risk factors correlated significantly with serum MGP levels. However, no significant correlation was found between MGP levels and CAC.

Recently, a competitive ELISA using monoclonal antibodies directed against the non-carboxylated MGP sequence 35–53 to measure circulating ucMGP levels was described (106, 111). Since this is also a single antibody assay, it does not discriminate between 1. full length MGP or fragments or 2. phosphorylated MGP (pMGP) and dpMGP. The assay was validated in a wide range of patient populations prone to develop arterial calcification, including patients with atherosclerosis and renal dysfunction (106). All patient groups had significantly lower ucMGP values than healthy subjects of comparable age. This assay was particularly successful in identifying patients with end-stage renal disease (ESRD) and calciphylaxis, a condition characterized by extensive calcification of cutaneous arterioles. MGP levels in these patients were almost without exceptions below the normal range (106). Additionally, it was demonstrated that circulating ucMGP levels were inversely associated with the aortic augmentation index (111). Moreover, in a well characterized cohort of ESRD patients we found an inverse correlation between circulating MGP levels and CAC scores measured by MSCT (E. C. M. Cranenburg et al., submitted for publication). These results could indicate that low MGP levels may be a marker of active calcification. The low MGP levels in these patient populations could be explained by the accumulation of ucMGP at sites of arterial calcification (14, 56, 57), suggesting that ucMGP is not set free into the circulation. An additional explanation for the low ucMGP levels could be that the majority of ucMGP is in the p-ucMGP form and that phosphorylation alone is sufficient for the binding of MGP to vascular calcifications (Fig. 2C). Indeed, previously we found no correlation between the dpMGP and ucMGP measurements in patients (R^2 0.008, p =

0.385) (106). To interpret these data, one could speculate that MGP is processed in a phosphorylated form, and that also the vitamin K-metabolism is impaired (as deduced from the ucMGP levels).

All MGP assays described above have in common that – if analysed on a group level – patients with CVD can be identified. However, further research is necessary to establish the value of this assay.

Ongoing research and future perspectives of MGP as biomarker

We aimed to develop an MGP ELISA to follow-up vitamin K-status after intervention (e.g. supplementation with vitamin K, or treatment with coumarin derivatives). Additionally, we intended to measure MGP species which are most readily set free in the circulation, independent of the presence of vascular calcification. It can be hypothesized that all forms of MGP containing the very negatively charged carboxylated or phosphorylated domains have a high affinity for precipitated calcium and will accumulate in and around calcified lesions in the vasculature (Fig. 2). As we found that in patients prone for vascular disease/calcification both the pMGP and ucMGP where decreased (see above), we hypothesise that only the non-phosphorylated, non-carboxylated MGP conformation will be easily set free in the circulation, independent of the vascular tissue calcium content, since this conformation has the lowest affinity for calcium (Fig. 2). Therefore, we developed a sandwich ELISA measuring non-phosphorylated, non-carboxylated MGP (dp-ucMGP).

We first tested the dp-ucMGP assay in a healthy control population, divided into groups aged 20 to 45 years and 50 years and older. The dp-ucMGP fraction was indeed measurable in plasma of these apparently healthy subjects (data not shown). The presence of inactive MGP in healthy subjects is consistent with data on osteocalcin, another vitamin K-dependent protein produced exclusively in bone (112, 113). It is generally accepted that the vitamin K-status is sufficient for normal haemostasis, but that extra-hepatic tissues such as bone and vascular are marginal in vitamin K (93, 112, 114). Secondly, we measured dp-ucMGP in two groups with extremes in vitamin K status: patients receiving vitamin K-antagonists (coumarin derivatives) as oral anticoagulant therapy and healthy volunteers receiving supplements with vitamin K (Fig. 3). Significantly increased circulating dp-ucMGP levels were found in patients receiving vitamin K-antagonists, whereas low levels of dp-ucMGP were present in healthy volunteers on vitamin K-supplementation. When MGP status was measured before and after a period of high vitamin K intake it was found that the dp-ucMGP level even decreased below the detection limit. These data indicate that this assay may become a marker for vitamin K-status of the arterial vessel wall. The dp-ucMGP assay is currently under validation, the details of which will be published elsewhere.

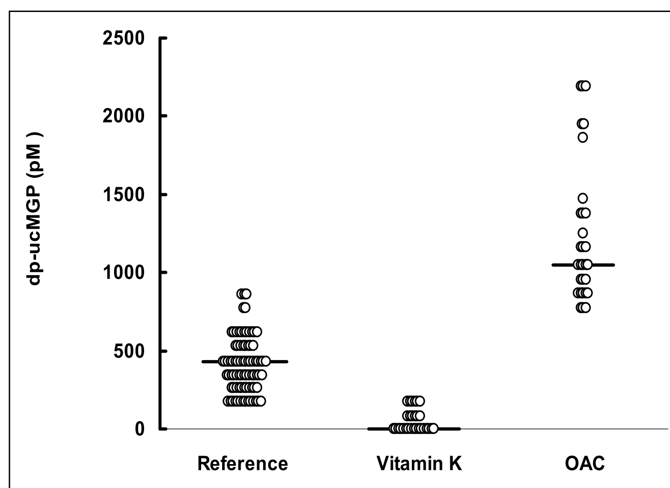


Figure 3: The dp-ucMGP assay is based on the sandwich ELISA-principle. In brief, monoclonal anti-dpMGP was coated to the microtiter plate. After blocking, either sample (citrated plasma or EDTA) or standard was incubated. The standard peptide was synthetic MGP, based on the non-phosphorylated 3–15aa sequence and the non-carboxylated 35–54aa sequence, linked with a hydrophilic spacer (Pepscan, Lelystad, the Netherlands). After incubation and washing, the standard or sample was detected using a biotinylated monoclonal ucMGP antibody. Plasma dp-ucMGP levels in the reference subjects, patients on oral anticoagulant treatment and subjects receiving vitamin K supplements are depicted. The reference group was divided into two groups: age less than 40 years, and age 50 or more. Mean ± SD ucMGP values of young healthy controls, elderly healthy controls, patients on coumarins and subjects receiving vitamin K-supplements were 389 ± 182, 312 ± 109, 172 ± 82, and 140 ± 55 nM, respectively (depicted as horizontal bars).

Conclusions

Vascular calcification is a major determinant of cardiovascular mortality and recent data demonstrate that one of the main calcification inhibitors in the vasculature is MGP. Although the vascular calcification is associated with poor cardiovascular outcome, Huang et al. showed in post-mortem coronary arteries that massive calcification is not related to plaque stress (115). Inspection of human atherosclerotic lesions revealed an association between plaque rupture and punctated intimal calcium deposition, possibly originated from small cell membrane fragments (116). Thus, the exact role of calcification in unstable plaque development is still unknown. The value of the calcification score (assessed by multislice CT scan) is therefore still under debate. Although calcification is regarded as an actively regulated process, it is likely that the massive arterial calcifications represent an end-stage process. The measurement of biomarkers, which can reflect the early signs of vascular disease could be of great importance. Both cardiovascular calcification and MGP activity are directly correlated with vitamin K_2 intake (6, 47, 81). Remarkably, most subjects in the healthy population are not optimally protected against calcification, since part of their MGP occurs in an uncarboxylated, inactive form (93, 106). The presence of MGP can now be detected accurately, and could be regarded as an independent risk factor for CVD; fortunately, this risk factor can be annihilated by increased vitamin K intake. Experiments in rats suggest this possibility by regression of calcification (47), but presently no data in humans are available to suggest that high vitamin K intake may contribute to regression of vascular calcification in CVD patients. Accumulating data suggest, however, that a high vitamin K_2 intake may be an effective interventional strategy to decrease the calcification risk in the general population.

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