Circulating Matrix Gla-protein

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**Circulating Matrix Gla-protein:
a biomarker for vascular disease**

Ellen C.M. Cranenburg

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Circulating Matrix Gla-protein: a biomarker for vascular disease

PROEFSCHRIFT

ter verkrijging van de graad van doctor
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Shoot for the moon.

Even if you miss, you will land among the stars.

- Les Brown -

Aan mijn ouders

Voor Arthur

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CHAPTER 1

General Introduction

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1. Matrix Gla-protein

1.1. Background

Matrix γ -carboxyglutamate (Gla)-protein (MGP) is a small secretory protein that can undergo two post-translational modifications: γ -glutamate carboxylation and serine phosphorylation. The protein was first described by Price and co-workers, who purified it from the bovine bone matrix [1]. The authors concluded that this approximately 15 kD protein contained five unusual amino acids, which were designated as γ -carboxyglutamate(abbreviated as Gla) residues. This matrix-associated, Gla-containing protein was named matrix Gla-protein (Figure 1). After its discovery in bone, MGP was found to be expressed in several other tissues, including cartilage, lung, heart, kidney, and in vascular smooth muscle cells (VSMC) of the arterial wall [1-3].

Figure 1: Matrix Gla-protein

Matrix Gla-protein (MGP) is a small 84 amino-acids vitamin K-dependent protein. It can undergo two post-translational modifications: at positions 2, 37, 41, 48, and 52 the glutamic acid residues (E) can be γ -carboxylated (indicated with γ) and at at position 3, 6, and 9 the serine residues (S) can be phosphorylated (indicated with P above). One disulfide bond is present, linking two cysteines (C).

Mature MGP consists of 84 amino-acids and has a theoretical isoelectric point of 9.7. 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 presents putative binding sites for transcription

factors AP-1 and AP-2, and for cAMP-dependent transcription factors. The fat soluble vitamins A and D can modulate MGP expression, their effect depending on the cell type.

1.2. Post-translational modifications

MGP can undergo two post-translational modifications:

1. γ -Glutamate carboxylation

Of the nine glutamic acid (Glu) residues present in MGP, only five can be γ -carboxylated in a vitamin K-dependent reaction, resulting in the formation of Gla residues. These residues are located at position 2, and at positions 37, 41, 48, and 52 within the so-called Gla-domain (amino-acid sequence 35–54).

2. Serine phosphorylation

MGP contains five serine (Ser) residues, of which three can be phosphorylated. This results in the formation of phosphoserine (Pser) residues, which are located within the amino-acid sequence 3–15 (positions 3, 6, and 9).

The best studied post-translational modification of MGP is γ -glutamate carboxylation, for which the energy is provided by vitamin K (Figure 2). The Gla residues formed are negatively charged and possess calcium-binding properties. They are required for protein activity of the vitamin K-dependent proteins (also called Gla-proteins) [4]. In all Gla-proteins the affinity for γ -glutamyl carboxylase (GGCX), the enzyme necessary for carboxylation, 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. This may contribute to the unique properties of MGP.

Phosphorylation is thought to be carried out by the Golgi casein kinase [5, 6]. Price *et al.* showed that the motif in MGP recognized for serine phosphorylation is the tandemly repeated Ser-X-Glu sequence [5]. Wajih *et al.* showed that phosphorylated MGP exits VSMC via the secretory pathway, whereas non-phosphorylated MGP appears in the cytosol and is thus not secreted in the extracellular matrix [6]. Although the precise function of serine phosphorylation is not known, it may regulate MGP secretion into the extracellular environment.

Figure 2: γ -Glutamate carboxylation and vitamin K cycle

Vitamin K serves as a co-factor for the conversion of specific glutamic (Glu) acid residues into γ -carboxyglutamic acid (Gla) residues. This reaction is linked to the enzymatic vitamin K cycle, in which vitamin K is salvaged for reuse in the carboxylation reaction. Vitamin K-quinone (K, dietary form) is first reduced to vitamin K-hydroquinone (KH_2). The active form KH_2 is needed in the oxidation step, in which Glu residues are reduced to Gla residues. As a consequence of this reaction, KH_2 is oxidized to vitamin K epoxide (KO). KO can subsequently be reduced to K, and eventually again reduced to KH_2 . Many studies favour the assumption that the latter two steps are carried out by the same enzyme, vitamin K epoxide reductase (VKOR). Coumarin derivatives inhibit the enzyme VKOR, thereby blocking the vitamin K cycle and leading to a build up of KO in the cell. However, in the liver the NAD(P)H-dependent quinone reductase (known as DT-diaphorase) can serve as an alternative pathway. This pathway can convert only K into KH_2 , and not KO into KH_2 . 1=NAD (H)-dependent reductase, 2= γ -glutamylcarboxylase enzyme (GGCX), 3=extra introduced carboxy-group, 4=dithiol-dependent KO-reductase (VKOR), 5=dithiol-dependent K-reductase (VKOR)

MGP exists as various species according to their state of phosphorylation and/or carboxylation: phosphorylated (pMGP), non-phosphorylated (desphospho, dpMGP), carboxylated (cMGP), or uncarboxylated (ucMGP). Development of antibodies directed against specific MGP species (Table 1) and of MGP assays to measure circulating MGP species (Figure 3) has enabled the investigation of these species in tissue (paragraph 1.4) and in the circulation (paragraph 1.5).

Table 1: Species-specific MGP antibodies

Antibody	Directed against	Amino-acid	Specificity	Used
mAb-ucMGP	uncarboxylated MGP	35-49	IgG I a	W, I, S, E
mAb-cMGP	carboxylated MGP	35-54	IgG I a	W, I, S, E
mAb-dpMGP	desphosphorylated MGP	3-15	IgG I a	W, I, S, E
mAb-pMGP	phosphorylated MGP	3-15	IgG I a	W, I, S, E

Abbreviations used:

mAb, monoclonal antibody; W, Western blot; I, Immunoprecipitation; S, Section staining; E, ELISA.

Figure 3: MGP ELISA

The following ELISA are depicted:

Single-antibody (competitive) ELISA (lined areas)

Four single antibody ELISAs could be developed, measuring ucMGP, cMGP, dpMGP, and pMGP. The first MGP ELISA described in the literature measures total desphosphorylated (t-dpMGP) and is commercially available (Biomedica, Vienna, Austria). The second MGP ELISA described measures total uncarboxylated (t-ucMGP) and its characterization and application in several patient populations is described in this thesis (Chapters 2–4).

Dual-antibody (sandwich) ELISAs (dotted area)

Combinations of the conformation-specific antibodies can be used to develop several MGP sandwich-combinations (in dotted area). Two novel dual-antibody ELISAs are described in this thesis (Chapter 5). The MGP ELISA indicated in bold have been developed and used in the research described in this thesis.

1.3. Functions

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 indication that a Gla-protein was involved in the inhibition of tissue calcification came from rats treated with the vitamin K-antagonist warfarin [7]. These animals developed extensive cartilage calcification with growth plate closure of the tibia, resulting in a reduced overall tibial length. In later years, Howe and Webster reported large areas of calcification in the nasal septum in the same rat model, resulting in maxillonasal hypoplasia and reduction in the length of the nasal bones [8]. These features resembled warfarin embryopathy (the fetal warfarin syndrome) in humans, resulting from the use of warfarin during the first trimester of pregnancy (see paragraph 2.6). It was only after the identification of MGP in cartilage, that it was hypothesized that the cartilage calcification in the warfarin model resulted from loss of MGP function [9].

After its discovery in bone and cartilage, it was thought for many years that the activity of MGP was restricted to these tissues. By targeted deletion of the *MGP* gene in mice it became clear, however, that MGP also inhibits medial artery calcification. *MGP* null mice 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 aorta [10]. The arterial calcifications in the *MGP* null mice consisted of calcium-phosphate precipitation in a ratio similar to hydroxyapatite, thus mimicking bone mineralization. The warfarin rat model provided further evidence that impairment of MGP function results in vascular calcification [11]. Price *et al.* investigated the presence of vascular calcification in this model and reported that aortic calcification was detectable after only two weeks of warfarin treatment. The calcification was localized at the elastic fibres within the tunica media, similar as seen in *MGP* null mice.

A mechanism explaining the strong calcification inhibitory activity of MGP was first suggested by Price, which was that MGP binds tightly to crystal nuclei, thereby preventing further growth [11]. Vascular calcification is currently considered to be an active, cellular-mediated process, in which the differentiation of VSMC from a contractile phenotype into a synthetic phenotype with chondrocyte- and osteoblast-like cell features, plays an important role (see 3. Arterial calcification) [12]. A second mechanism of MGP action is thought to be direct inhibition of VSMC differentiation, which is supported by the findings in *MGP* null mice. VSMC of these mice were changed into chondrocyte-like cells at sites of calcification, with the appearance of cartilage nodules in the calcified aorta at the age of one month [10, 13]. In addition, a loss of smooth muscle markers and upregulated expression of the bone-specific transcription factor *cbf1a/Runx2* and the osteogenic protein osteopontin was found in *MGP* null mice [14].

The ability of MGP to keep VSMC in the contractile phenotype may be accomplished by its binding to bone morphogenetic protein-2 (BMP-2) [15, 16]. BMP-2 is a member of the transforming growth factor- β (TGF- β) superfamily, and is an osteogenic growth factor. BMP-2 has been shown to be expressed in human atherosclerotic lesions [17]. Wallin *et al.* demonstrated that only cMGP species bind to BMP-2 [15], thereby likely blocking the osteo-inductive properties of BMP-2 [18]. Via its C-terminal region, MGP can also bind to the extracellular glycoprotein vitronectin, which is present in the extracellular matrix of the arteries [19]. The C-terminal part of MGP is hydrophobic and does not contain Glu or Ser residues, which are all present in the more hydrophilic N-terminal and mid-section of the molecule. It may be hypothesized that the binding of MGP to vitronectin results in a concentration of calcification-inhibitory activity in the milieu surrounding the elastic fibers, thereby protecting them from mineralization.

The specific knock-in expression of MGP in VSMC of *MGP* null mice completely rescued the calcification phenotype [20]. Expression of MGP in the liver of *MGP* null mice resulted in high levels of circulating MGP. However, the high systemic levels of MGP had no effect on inhibition of arterial calcification. This implies that MGP inhibits calcification by acting within its tissue of synthesis, and not by acting systemically. Evidence for the pivotal role of Gla residues for MGP function was provided by Murshed *et al.* who generated transgenic mice that produced MGP which could not be carboxylated, since the Glu residues in the Gla-domain were mutated into aspartate residues. Using these mice, it was demonstrated that only cMGP exhibits anti-mineralization properties [20].

In humans, loss-of-function mutations result in the Keutel syndrome [21], a rare autosomal recessive disorder characterized by abnormal cartilage calcification and peripheral pulmonary artery stenoses [22]. Keutel *et al.* first described the syndrome in two siblings presenting abnormal cartilage calcification, brachytelephalangism, neural hearing loss, and peripheral pulmonary stenosis [23]. At the age of 38, one of the two siblings died of acute right heart failure after several years of obstructive pulmonary disease; post-mortem examination revealed calcification of arteries throughout the body resulting in pulmonary artery stenoses and multiple brain infarctions [24].

1.4. MGP in vascular tissue

Immunohistochemical studies have shown that in healthy vessels MGP is synthesized at relatively low rate [2, 25, 26], 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 [27]. High MGP levels have been detected in arteries with calcification [2, 25, 26]. This may originate from increased MGP synthesis, which has been reported in both medial and intimal arterial calcification [2, 25, 26], or from accumulation of MGP due to binding to calcium salt crystals.

With the development of species-specific antibodies, enabling the detection of cMGP and ucMGP, it became clear that specifically ucMGP species accumulate in atherosclerotic and calcified arteries [25, 28, 29]. cMGP species were nearly absent in these arteries. Sweatt *et al.* demonstrated that calcified arterial lesions in aging rats contained elevated levels of ucMGP species, which were not able to bind BMP-2 [28]. Our group demonstrated massive accumulation of ucMGP around calcified 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 [30]. The group of Shanahan showed that arteries from children with chronic kidney disease (before the initiation of dialysis), contained more ucMGP species in comparison with vessels from healthy children. This shift preceded the formation of vascular calcification.

1.5. MGP in the circulation

MGP is a local inhibitor of vascular calcification and it has been demonstrated that circulating MGP has no biological function [20]. However, circulating MGP may reflect calcification processes and inhibition of those processes in the vascular wall. Circulating levels of MGP will depend on the rate of MGP synthesis in tissue, its secretion from VSMC, and subsequent binding of MGP to calcified areas that may be present within the arterial wall. Mature MGP is highly insoluble and it is currently not known in which forms MGP circulates. It may circulate as a free protein, or in association with a carrier protein. Full length MGP has been purified from the plasma of rats as a complex including calcium, phosphate, carboxylated MGP, and fetuin-A [31]. Additionally, it is likely that also a substantial fraction of circulating MGP occurs as fragments.

Two assays to measure circulating MGP species were described in the literature before we started the work described in this thesis. The first assay is a radioimmunoassay (not commercially available), in which polyclonal antibodies directed against MGP purified from human bone are used [32]. It is therefore not possible to discriminate between the different MGP species. 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 [33]. Especially the traditional lipid-risk factors correlated significantly with serum MGP levels. However, no significant correlation was found between MGP levels and the extent of coronary artery calcification (CAC).

The second assay is an enzyme-linked immunosorbent assay (ELISA). With the development of species-specific antibodies by our group, it became possible to construct MGP ELISA for different MGP species (Figure 3). Two types of ELISA were developed: mono-antibody (competitive) and dual-antibody (sandwich) ELISA. The first MGP ELISA was described by our group in 2000, and is now commercially available as an ELISA kit produced by Biomedica (Austria, Vienna) [34, 35]. This is a mono-antibody ELISA, in which a monoclonal antibody against the non-phosphorylated N-terminal MGP amino-acid sequence 3–15 is used. This assay measures dpMGP species, and does not discriminate between cMGP and ucMGP. Therefore, the MGP measurements with this assay are referred to as measurements of total dpMGP (t-dpMGP) in this thesis (Figure 3). Increased levels of serum t-dpMGP were measured in patients with severe atherosclerosis and with type I diabetes mellitus [34]. The same assay was used in collaboration with Jono and colleagues, in which the severity of CAC was measured with electron beam computed tomography in subjects with suspected coronary artery disease [36]. The serum levels of t-dpMGP were significantly lower in subjects with CAC compared to those without CAC. Moreover, t-dpMGP levels inversely correlated with the severity of CAC.

Since 2000, we developed three new MGP ELISA: a mono-antibody assay measuring total uncarboxylated MGP species (t-ucMGP) and two dual-antibody assays measuring desphosphorylated-uncarboxylated MGP (dp-ucMGP) and desphosphorylated-carboxylated (dp-cMGP) species, respectively. The characteristics of these assays as well as their application in several patient populations are described in this thesis.

2. Vitamin K

2.1. Discovery

Vitamin K was discovered in the early 1930s by the Danish biochemist Henrik Dam. During his research on cholesterol metabolism in chickens, Dam observed that chicks reared on a diet free of sterols and poor in fat developed large subcutaneous and intramuscular hemorrhages [37]. Additionally, it was reported by McFarlane *et al.* that blood of chicks fed an ether-extracted fish or meat meal failed to coagulate overnight [38]. Initial experiments with additions of lemon juice, cholesterol, cod-liver oil, or vitamin C to the diet failed to prevent these haemorrhages [37]. Further testing of different foods, ranging from cereals and seeds to animal organs, revealed that “the antihemorrhagic factor” was fat-soluble, with one of its richest sources being hog-liver [39]. Hendrik Dam designated this factor as Koagulations vitamin (abbreviated as vitamin K), because of its requirement for normal haemostasis [39]. Subsequently, Almquist and Stokstad reported that the haemorrhagic syndrome could be prevented by concentrated extracts of alfalfa as well as by fish meals and bran preparations, which were subjected to the action of microorganisms [40].

In the mid 1930s, Schönheyder proposed that the prolonged clotting time seen in animals with the hemorrhagic syndrome was due to a decreased concentration of plasma prothrombin [41]; indeed, precipitates of prothrombin from the plasma of “K-avitaminous” chicks were found to be inactive [42]. Additionally, the prothrombin time of chicks and rabbits fed with spoiled sweet clover hay - resulting in hemorrhagic disease - was increased [43, 44]. In later years, it was established that sweet clover hay contained dicoumarin, a vitamin K antagonist produced by moulds acting on the hay [45].

In the late 1930s, several research groups were able to isolate vitamin K from alfalfa as a yellow oil [46-49]. The group at St. Louis University led by Edward A. Doisy could also isolate vitamin K from putrefied fish meals as a crystalline product and designated this product as vitamin K₂ [50, 51]. During this time, the first clinical studies with administration of vitamin K to patients with obstructive jaundice or biliary problems were conducted [52], since it had been recognized that the bleeding tendency seen in these cases was related to vitamin K deficiency [53]. Additionally, vitamin K was administered to newborns suffering from haemorrhagic disease or who were found to have a prolonged prothrombin time [54]. In later years, the prophylactic treatment of newborns (and/or their mothers) with vitamin K gained increasing attention [55]. Dam mentioned in his Nobel lecture that prophylactic treatment with vitamin K decreased the neonatal mortality rate among newborn infants from 4.6% to 1.8% [56, 57]. Dam and Doisy received the 1943 Nobel prize in Physiology/Medicine for the discovery of vitamin K and its chemical nature [56].

2.2. Function

Until 1974, various hypotheses about the mechanism of vitamin K action were proposed, including a role in mitochondrial electron transport and induction of protein synthesis. The discovery of the unusual amino acid γ -carboxyglutamate acid (Gla) in prothrombin as the product of vitamin K action in 1974, finally identified the unequivocal role of vitamin K as a cofactor for the post-translational carboxylation of glutamate (Glu) residues [58, 59] (Figure 2). These findings triggered the development of an *in vitro* carboxylase assay by the group of Suttie, in which $^{14}\text{CO}_2$ was incorporated into Glu-containing synthetic peptides [60]. Thus, it was demonstrated that the vitamin K-dependent step in prothrombin synthesis involved the γ -carboxylation of Glu residues in precursor prothrombin [60]. Fifteen years later, Stafford and co-workers succeeded in purifying the key enzyme GG CX [61], which carries out the carboxylation reaction. Only recently the purification and amino acid sequence of the second key enzyme in vitamin K metabolism, vitamin K epoxide reductase (VKOR), were reported by two independent groups [62, 63].

2.3. K vitamins

Vitamin K can be considered a group name for different chemical forms of vitamin K (K vitamins). All forms possess a common 2-methyl-1,4-naphthoquinone structure (menadione) and an aliphatic side-chain at the 3-position (Figure 4) [64]. The major dietary source of vitamin K is phyloquinone (vitamin K₁), originating from green leafy plants and green vegetables. The chemical structure of vitamin K₂ was elucidated in the 1960s and revealed that it consists of a family of related products now known as menaquinones [65]. Menaquinones are mainly of microbial origin and their nomenclature is based on the number of isoprenoid residues in their side-chain (Figure 4). Dietary relevant menaquinones range from menaquinone-4 (MK-4) through menaquinone-10 (MK-10). In the human diet the main sources of menaquinones are cheese, curd, and the Japanese food natto. MK-4 is the only menaquinone that can be produced in mammalian tissues, where it is formed by conversion of phyloquinone and possibly other menaquinones [64, 66]. Vitamin K₁, MK-4, and MK-7 are commercially available products.

Figure 4: K vitamins

Vitamin K₃ (menadione)

Vitamin K₁ (phylloquinone)

Vitamin K₂ (menaquinone)

Chemical structure of vitamin K₃ (menadione), K₁ (phylloquinone), and vitamin K₂ (menaquinone). The (n) stands for number of isoprenoid residues.

2.4. Vitamin K-dependent proteins (Gla-proteins)

After the discovery of Gla residues in prothrombin, the subsequently identified proteins containing these residues were those involved in blood coagulation: factors VII, IX, and X as well as the proteins C, S, and Z [67]. During this time, the presence of carboxylase was demonstrated in a wide variety of non-hepatic tissues, ranging from bone to skin [68, 69]. It was assumed that the endogenous substrate of carboxylase in these tissues (unidentified Gla-proteins) was not involved in blood coagulation.

The first extra-hepatic sites in which Gla-proteins have been identified and characterized are bone and cartilage. Bone Gla-protein, also known as osteocalcin, was identified in the mid 1970s [70, 71] and presently serves as a marker for osteoblastic activity. MGP was identified in the early 1980s [1]. Since then, several new Gla-proteins have been identified, including growth-arrest-specific gene 6 protein (Gas-6) [72]. Gas-6, which plays a role in cell growth and apoptosis, has gained increasing attention in the past years. The most recently identified Gla-protein is Gla-Rich protein (GRP), which is thought to serve as an inhibitor of soft tissue calcification [73]. An overview of all known Gla-proteins is given in Table 2.

Table 2: Gla-proteins

Gla-protein	Function	Site of action
Hepatic [74-76]		
FII (prothrombin), FVII, FIX, FX	Procoagulants	Plasma
Protein C, Z	Anticoagulants	Plasma
Protein S	Anticoagulant role as cofactor for protein C	Plasma and variety of other tissues (endothelium, bone)
Extra-hepatic		
Osteocalcin (OC, bone gla-protein) [70, 77]	Regulator of orderly bone crystallization	Bone, dentin
Matrix Gla protein (MGP) [1]	Inhibitor of ectopic calcification	Cartilage and soft tissues including vasculature
Growth arrest specific gene-6 protein (Gas-6) [78]	Regulator of cell growth	Soft tissues
Gla-rich protein (GRP) [73]	Regulator of calcium availability in the extra-cellular matrix	Cartilage, bone, and soft tissues
Membrane-bound		
Carboxylase [79]	Enzyme for carboxylation	Soft tissues
Proline rich Gla-protein (PRGP) [80]	Potentially promotes signal transduction	Soft tissues
Transmembrane Gla-proteins (TMG) [81]	Unknown	Soft tissues
Extra-cellular matrix		
Periostin (osteoblast specific factor-2) [82]	Promotes cell adhesion, motility, migration, and angiogenesis	Remodelling tissues, bone (periosteum)
TGF-β induced gene h3 (βig-h3) [82]	Promotes cell migration and cell-matrix interaction	Soft tissues, bone, cartilage

Abbreviations used: F, factor; TGF-β, transforming growth factor-beta.

2.5. Dietary intake

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

The association between dietary vitamin K₁ and K₂ intake and cardiovascular mortality and calcification has been investigated in several Dutch population-based studies. In the Rotterdam Study, dietary vitamin K₂ intake (and not vitamin K₁) was inversely correlated with cardiovascular calcification and cardiovascular death [83]. Elderly subjects in the highest tertile of vitamin K₂ intake had about 50% reduction in both aortic calcification and cardiovascular mortality, and 25% decreased all-cause mortality. Among post-menopausal women, participants of the PROSPECT study investigating cancer and nutrition, vitamin K₂ intake (and not vitamin K₁) was associated with decreased coronary calcification (RR=0.80) [84]. Vitamin K₂ intake was also inversely associated with CHD risk (HR=0.91 per 10 µg/day intake), mainly due to the menaquinones MK-7, MK-8, and MK-9 [85]. In a 3-year clinical intervention study in 78 women between 55 and 65 years of age, the effect of vitamin K₁ supplementation (1 mg/day) on vascular characteristics (elasticity and distensibility) was investigated [86]. Vascular elasticity decreased by 10–13% in the placebo group, but in the vitamin K group vascular characteristics remained unchanged. This suggested that the process of vascular aging can be retarded by increased vitamin K intake.

The concept of calcification inhibition by high vitamin K intake has also been investigated in experimental animals, including the warfarin rat model [87]. In this model, the efficacy of vitamin K₁ and MK-4 (vitamin K₂) in preventing arterial calcification was compared. It was found that MK-4 completely inhibited tissue calcification, whereas a similar or even an 8-fold higher dose of vitamin K₁ had no measurable effect. The same animal model was used to study the effect of high vitamin K intake on potential regression of arterial calcification [30]. In this experiment, rats receiving the standard chow (control) had very low aortic calcium during the entire experiment, whereas a 6-week warfarin treatment led to the accrual of calcium salts up to 12-fold above the baseline values. During the following 6-week period, warfarin treatment was stopped and the animals received either standard chow or standard food fortified with a high dose of vitamin K₁ or K₂ (MK-4).

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 K₁ or K₂, 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 6 weeks. This effect was found both in the aorta and in the coronary arteries. Using immunohistochemical staining with the species-specific MGP antibodies, it was demonstrated that parallel to the regression of aortic calcium content, cMGP had increased and ucMGP had decreased. This suggests a role of activated MGP in the regression of calcified plaques.

2.6. Vitamin K antagonists

Vitamin K antagonists (also known as oral anticoagulants, OAC) block the recycling of vitamin K by inhibition of the enzyme VKOR, resulting in vitamin K deficiency. Treatment with vitamin K antagonists will therefore result in the synthesis of uncarboxylated vitamin K-dependent proteins. VKOR is a crucial enzyme in vitamin K metabolism and ensures the reutilization of vitamin K after it has been oxidized in the carboxylase reaction. Because of this recycling, human vitamin K requirement is low [88]. On a molecular level, VKOR reduces vitamin K epoxide (KO) in two steps: first to vitamin K quinone (K), and subsequently to vitamin K hydroquinone (KH₂), the latter being the active cofactor for GGCX (Figure 2). The cytoplasmic enzyme DT-diaphorase serves as a rescue enzyme when coumarins are used; it is capable of reducing vitamin K quinones to their hydroquinone forms. However, Wallin *et al.* showed that the enzyme DT-diaphorase in VSMC is 100-fold less active than in the liver [89]. Coumarin treatment may therefore have a detrimental effect in the arterial vessel wall, by blocking vitamin K-metabolism, which may impair MGP activity

It has been known for many years, that women receiving the vitamin K antagonist warfarin 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 [90]. This syndrome is known as warfarin embryopathy (fetal warfarin syndrome), and the abnormalities were first believed to result from calcified haemorrhages in the developing fetal cartilages [90-92]. However, this was unlikely since fetal clotting factors are absent during the first trimester of pregnancy [90, 93]. Similarities between the facial and skeletal abnormalities seen in warfarin embryopathy and the fetal phenytoin (hydantoin) syndrome suggest that prenatal vitamin K-deficiency may underlie these abnormalities [90, 94]. This was confirmed by Pauli *et al.* who described a congenital deficiency of the VKOR enzyme, causing prenatal vitamin K deficiency, resulting in a similar phenotype [95, 96].

In later years, it was reported that also dietary vitamin K deficiency results in comparable calcification abnormalities [97-101], which were remarkably similar to the bone and cartilage defects observed in warfarin-treated rats [7].

Vitamin K antagonists are frequently used to prevent thrombosis in high-risk patients [102]. Treatment periods range from several weeks to many years, often even life-long [103]. After demonstration in animal models that vitamin K antagonists induce vascular calcification, several independent studies showed an association between the use of coumarins and arterial and/or valvular calcification. Schurgers *et al.* compared the extent of aortic valve calcification in patients receiving marcoumar treatment (mean duration 25 months) with patients not on OAC before aortic valve replacement surgery [104]. The mean calcified area was 16% in the non-treated group and 37% in the marcoumar group. Histopathological evaluation showed partial or total valve destruction induced by amorphous calcified deposits in the latter group. These data were confirmed by Koos *et al.*, who showed that patients on long term OAC treatment had significantly higher aortic valve and coronary artery calcification scores compared patients without anticoagulation treatment, as assessed with MSCT scanning [105]. Rennenberg *et al.* compared 19 patients who used coumarins for more than 10 years with 18 matched healthy controls [106]. Vascular calcification was quantified using a plain X-ray of the thighs, from which femoral arterial calcifications could be assessed. The odds ratio for calcification in patients versus controls was 8.5. Coumarin use was independently associated with calcification. Since coumarin derivatives are widely used, physicians should be aware of this potential adverse effect on the vasculature.

3. Arterial calcification

Until a decade ago, vascular calcification was thought to be a passive, clinically irrelevant process, resulting from a high calcium-phosphate product, inflammation, lipid accumulation, and/or diabetes mellitus. However, during recent years it has become increasingly clear that vascular calcification is an active, cell-mediated process and an important, independent pathology that is strongly associated with increased risk of cardiovascular morbidity and mortality [107-109]. Four different types of cardiovascular calcification can be distinguished: atherosclerotic calcification, medial artery calcification, cardiac valve calcification, and calciphylaxis [110]. Atherosclerotic or intimal calcification is seen at sites of atherosclerotic plaques, in which cellular necrosis, inflammation, and cholesterol deposition occur. The presence of this type of calcification is associated with atherosclerotic burden. Medial artery calcification occurs in the elastic lamina of large- and medium- to small-sized arteries. It is commonly seen in CKD, diabetes, and aging. On radiographs, this type of calcification can be seen in distal arteries as a “railroad track” pattern. Whereas intimal calcification is mainly associated with arterial obstruction, medial calcification is associated with vascular stiffness. In cardiac valve calcification aspects of both medial and atherosclerotic calcification can be seen, with the primary stimulus being a combination of mechanical stress and inflammation [110]. Calciphylaxis (calcific uremic arteriolopathy) is characterized by medial calcification with intimal proliferation of small- to medium-sized arteries, resulting in tissue necrosis. The various types and localizations of vascular calcifications have an impact on cardiac morbidity mortality not only by increasing and complicating coronary atherosclerosis, but also by increasing the stiffness of the main arteries [111].

The phenotypic change of VSMC from a contractile phenotype into an osteo/chondrocytic phenotype plays a central role in the calcification process [12]. This transformation is characterized by the upregulation of bone-specific transcription factors and matrix proteins, resulting in an expression of osteogenic markers by VSMC [27]. In addition, a mineral imbalance, for instance hypercalcemia and/or hyperphosphatemia, can induce the formation of vesicles by the VSMC. These vesicles can be derived from apoptotic and viable VSMC and - if not phagocytosed properly - form a initial nidus for the deposition of hydroxyapatite crystals [112]. *In vitro* studies have shown that under physiological circumstances, these vesicles are loaded with mineralization inhibitors, including MGP [112], limiting their mineralization potential. A loss of inhibitors results in vesicles with mineralizing properties [112]. It is therefore thought that a loss of inhibitors, including MGP, is a major factor in inducing calcification [12].

Clinically, vascular calcification, particularly medial 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 [113, 114]. Calcification is common in the elderly population, and in patients suffering from diseases, such as chronic kidney disease (CKD), diabetes mellitus, aortic stenosis, and atherosclerosis [115]. 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 [30, 116-118], demonstrating that also the regression process is an actively regulated process.

Patients with CKD, especially those patients receiving hemodialysis or peritoneal dialysis therapy, have the highest incidence of arterial calcification, and cardiovascular mortality is 20-fold higher than in the apparently healthy population [119, 120]. Moreover, moderate to severe vascular calcifications are found in 60 to 80% of patients on hemodialysis [121, 122]. Booth and co-workers demonstrated that vitamin K status in CKD patients is low [123]. Some 30% of the hemodialysis patients had sub-clinical vitamin K deficiency, based on measurements of circulating vitamin K levels. It has been hypothesized that vitamin K may be a novel treatment option, or at least a modifiable risk factor for vascular calcification in dialysis patients [124].

Outline of this thesis

This thesis focuses on measurements of circulating MGP species and their potential diagnostic and clinical value. **Chapter 1** gives an introduction of MGP and vitamin K. In **Chapter 2**, the mono-antibody MGP ELISA measuring total uncarboxylated MGP (t-ucMGP) levels is presented and characterised, with application of t-ucMGP measurements in patient populations with cardiovascular disease (coronary angioplasty and aortic valve stenosis) and vascular calcification (hemodialysis and calciphylaxis). Further investigation of t-ucMGP as a biomarker in dialysis patients is described in Chapters 3 and 4. **Chapter 3** describes the correlation between t-ucMGP levels and the extent of coronary artery calcification in hemodialysis patients. In **Chapter 4**, the association between t-ucMGP levels and (cardiovascular) mortality in a large Dutch cohort of hemodialysis and peritoneal dialysis patients is investigated. In **Chapter 5**, we compared the four available MGP assays, including two novel dual-antibody MGP ELISA. All assays were applied in patients with cardiovascular disease, rheumatic diseases, and end-stage renal disease. To determine which assay would be suited to assess vascular vitamin K status, the effect of changes in vitamin K status on circulating MGP species was also investigated. For this, the assays were applied in two groups of healthy subjects with opposite vitamin K status: subjects receiving vitamin K supplementation and those receiving vitamin K antagonists. In **Chapter 6**, we investigated the functions of MGP in humans by phenotypic characterization of a newly-identified Dutch patient with Keutel syndrome. In order to further elucidate the difference in phenotype between MGP deficient mice and men, we characterized the circulating MGP species of this patient and his first-degree relatives. Additionally, MGP species were determined in tissues from the first described Keutel syndrome patient in the literature. **Chapter 7** describes the investigation of the vitamin K intake and the hepatic and extra-hepatic vitamin K status in a Dutch cohort of hemodialysis patients.

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CHAPTER 2

The circulating inactive form of Matrix Gla-protein (ucMGP) as a biomarker for cardiovascular calcification

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Abstract

Matrix γ -carboxyglutamate (Gla)-protein (MGP) is a vitamin K-dependent protein and a strong inhibitor of vascular calcification. Vitamin K deficiency leads to synthesis of inactive uncarboxylated MGP species (ucMGP), which accumulate at sites of arterial calcification. We hypothesized that as a result of ucMGP deposition around arterial calcification, the circulating fraction of ucMGP is decreased. Here we report on the development of an ucMGP assay and the potential diagnostic utility of monitoring serum ucMGP levels.

An enzyme-linked immunosorbent assay (ELISA) was developed with which circulating total ucMGP (t-ucMGP) can be determined. Serum t-ucMGP levels were measured in healthy subjects (n=165) and in four patient populations; patients who underwent coronary angioplasty (n=30), patients with aortic stenosis (n=25), hemodialysis (n=52), and calciphylaxis patients (n=10). All four patient populations had significantly lower t-ucMGP levels. In angioplasty patients and in those with aortic stenosis some overlap was observed with the control population. However, in the hemodialysis and calciphylaxis populations virtually all subjects had t-ucMGP levels below the normal adult range.

Serum t-ucMGP may be used as a biomarker to identify those at risk for developing vascular calcification. This assay may become an important tool in the diagnosis of cardiovascular calcification.

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Introduction

Vitamin K-dependent proteins are present in blood and in a wide variety of tissues throughout the body; they exert a broad range of functions, for example as blood coagulation factors in haemostasis [1]. In recent years one of these proteins has gained increasing attention: matrix γ -carboxyglutamate (Gla)-protein (MGP), a potent inhibitor of calcification present in cartilage and the vessel wall, where it is synthesized by chondrocytes and vascular smooth muscle cells (VSMC), respectively [2, 3]. MGP contains 9 glutamate residues, 5 of which may be γ -carboxylated in a posttranslational step catalyzed by the vitamin K dependent γ -glutamate carboxylase [1]. Deficiency of vitamin K, either due to nutritional deficiency or the use of coumarin-derivatives, results in uncarboxylation of MGP and impairment of its biological function [4].

With immunohistochemical techniques based on monoclonal antibodies specifically recognizing carboxylated and uncarboxylated MGP species (cMGP and ucMGP, respectively), we have recently demonstrated the massive accumulation of ucMGP in atherosclerotic lesions and areas of calcification [5]. This has been a consistent finding in several studies. Price *et al.* reported the accumulation of ucMGP at sites of arterial calcification in rats treated with vitamin D and warfarin [6] and Sweatt *et al.* showed that the aortic wall of aging rats contained elevated concentrations of ucMGP [7]. It was proposed that ucMGP could bind to apatite present in calcifying tissues irrespective of its Gla-residues, which was supported by the fact that thermal decarboxylation of MGP *in vitro* did not affect its affinity for hydroxyapatite [8].

The ability of MGP to inhibit calcification was demonstrated in transgenic MGP deficient mice. The MGP null mice were born normally, but all developed severe medial arterial calcifications and subsequent vascular ruptures leading to death within 6 weeks [9]. This vascular phenotype could only be rescued if MGP was restored locally in the VSMC and if MGP was γ -carboxylated [10], suggesting that MGP is a local inhibitor of vascular calcification and that its function depends on the presence of Gla residues, respectively. Glareidues are able to bind calcium both in solution and in calcium salt crystals, which is thought to be associated with the ability of MGP to inhibit crystal growth [8, 11]. Moreover, it has been suggested that its mechanism of action is based on the binding of MGP to BMP-2 [12, 13], a bone morphogenetic protein. MGP can also bind to vitronectin, a multifunctional protein present in plasma and extracellular matrix [14]. Nishimoto *et al.* proposed that this binding may assist cells in developing tissues to migrate and differentiate [14].

In humans, loss-of-function mutations in the *MGP* gene result in the Keutel syndrome (KS)[15]: a recessive disorder characterized by abnormal cartilage calcification, peripheral pulmonary artery stenoses, brachytelephalangism, and neural hearing loss [16]. Post-mortem examination of one of the first described KS patients has revealed calcification of arteries throughout the body [17]. The propensity to develop arterial calcification is present in a wide range of other patient populations including patients with diabetes, atherosclerosis, and renal dysfunction. Additionally, patients using oral anticoagulants (coumarins) have significantly increased aortic valve calcification and coronary artery calcification [18, 19]. Patients with moderate to severe arterial calcification (as assessed by multislice computed tomography [MSCT] scanning) have an unfavorable prognosis compared to patients with no or mild calcification [20–22]. Therefore, the measurement of biomarkers which can predict or reflect the disease becomes of substantial importance.

The first commercially available MGP assay does not discriminate between carboxylated and uncarboxylated MGP [23], but detects total desphosphorylated (dp) MGP species. Using this t-dpMGP assay, we have reported increased as well as decreased circulating MGP levels in patients suffering from various forms of atherosclerotic disease [5, 23, 24]. A correlation between t-dpMGP levels and coronary artery calcification (CAC) was found in subjects with suspected coronary artery disease [25]; however, no correlation with CAC could be established in patients with chronic kidney disease (CKD) [26]. Additionally, in CKD patients increased levels have been described [24], as well as no significant difference in MGP levels between these patients and control subjects [27]. Thus, although this assay performs well, results are sometimes conflicting and the strong overlap between serum t-dpMGP levels in patients and the normal range does not allow its use for individual diagnosis.

We have developed a competitive enzyme-linked immunosorbent assay (ELISA) which specifically recognizes ucMGP species. The accumulation of MGP in its uncarboxylated form at sites of arterial calcification and its probable high affinity for hydroxyapatite [5, 7, 8], suggest that ucMGP species are not set free into the circulation from calcified arteries. We hypothesized that in the presence of arterial calcification (arterial medial calcification or in atherosclerotic lesions), the levels of circulating ucMGP species are decreased. To test our hypothesis and to evaluate the potential diagnostic utility of the assay we have measured total ucMGP levels (t-ucMGP) in serum and plasma from different patient cohorts.

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Materials and methods

Subjects

Apparently healthy subjects, 91 males and 74 females, were recruited from the general Maastricht population for the assessment of the normal range and reference. Patient samples were acquired from a total of 117 subjects: 52 hemodialysis patients and 10 calciphylaxis patients from the department of Nephrology and Clinical Immunology of the University Hospital Aachen, 25 aortic stenosis patients from the department of Cardiology of the University Hospital Aachen, and 30 patients with a history of recent coronary angioplasty from the University Hospital Maastricht. An exclusion criterion for both the reference population and the patient groups was the use of coumarin derivatives. The study was approved by the local Medical Ethics Committee, and informed consent was obtained from all participants.

Serum handling

Blood was collected by venipuncture in serum tubes (10 ml; BD Vacutainer systems, Plymouth, UK) and in sodium citrate (10 ml; BD Vacutainer systems, Plymouth, UK) and stored for 20 minutes at room temperature before centrifugation (15 min 1580 *g*). Serum and plasma were sub-sampled in 250 μ l aliquots and frozen at -80 °C until testing.

Materials

All chemicals and reagents described below, were of analytical grade or better and obtained from commercial suppliers. Synthetic peptides homologous to the non-carboxylated MGP sequences 35–49 and 35–54 (designated as ucMGP^{35–49} and ucMGP^{35–54}, respectively) and carboxylated MGP sequences 35–49 and 35–54 (cMGP^{35–49} and cMGP^{35–54}) were made by Pepscan (Lelystad, The Netherlands). All peptides were purified and analyzed by LC/MS (ionspray, positive ion detection) and their purity level was >98% for all peptides. Synthetic full length MGP, cMGP or ucMGP [28] was used for antibody screening and to define assay specificity. The tracer for the assay was prepared by biotinylating the non-carboxylated 35–54 peptide (ucMGP^{35–54}) using the EZ-Link Maleimide PEO₂-Biotin kit according to the manufacturer's instructions (Pierce, Etten-Leur, the Netherlands).

t-ucMGP assay

The monoclonal antibody against ucMGP used for serum measurements was provided by VitaK BV (Maastricht, The Netherlands); the same antibody has been used previously for immunohistochemical staining. [5, 29] The monoclonal antibody (IgG1 isotype, designated as mAb-ucMGP) was raised against the human ucMGP Gla-domain (residues 35–49), and selected for its specificity towards ucMGP using standard techniques. [5] Polyclonal Rabbit-anti-Mouse IgG (Dako, Heverlee, Belgium) was diluted 2000 fold in carbonate buffer (0.1 mol/L sodium carbonate, pH 9.6) and used for the coating of the microtiter plate (100 μ L/well).

After incubation for 1.5 hours at 37 °C, remaining protein-binding sites were blocked with 200 µL of blocking buffer (2% HNBSA consisting of HEPES-NaCl buffer [175 mM NaCl, 25 mM HEPES, pH 7.7]) and bovine serum albumin (BSA; Sigma, St. Louis, MO) and incubated for 2 hours at room temperature. Next, the plate was washed 5 times with 300 µL of washing buffer (0.05% Tween-20 in HEPES-NaCl buffer) and incubated overnight at 4 °C with 100 µL mAb-ucMGP (0.69 µL/ml in 2% HNBSA; VitaK BV). Subsequently, the plate was washed 5 times with 300 µL of HEPES-Tween washing buffer. Five µL of either serum sample or standard were diluted in 70 µL of 2% HNBSA and 20 µL of this solution were supplemented with 100 µL of tracer (biotinylated ucMGP³⁵⁻⁵⁴, 0.7 µg/mL). After gentle mixing, 100 µL of this solution were transferred to the microtiter plate and incubated overnight at 4 °C. After 5 washing cycles with 300 µL of HEPES-Tween washing buffer, the plate was incubated with 100 µL Streptavidine-peroxidase (Zymed, Breda, The Netherlands) diluted 10,000-fold with 0.5% HNBSA. The plate was subsequently washed 5 times with 300 µL 0.05% HEPES-Tween washing buffer and stained with 100 µL of 3,3',5,5'-tetramethylbenzidine (KPL, Gennep, the Netherlands). The staining process was stopped by adding 50 µL of 1.0 mol/L H₂SO₄, and the plate was read at 450 nm. The ucMGP concentration was calculated with the aid of a calibration curve prepared with synthetic full length ucMGP.

Commercially available MGP assay

Serum t-dpMGP concentrations were quantified with the kit from Biomedica (Vienna, Austria). The kit is based on the competitive ELISA principle, with antibodies against dpMGP coated on the microtiter plate as has been described before. [24]

Immunoprecipitation

ucMGP species from plasma were purified by immunoprecipitation according to the method described by Michielsen *et al.* [30]. In brief, 200 µg of mAb-ucMGP were incubated for 1 hour at room temperature with 100 mg of protein A-sepharose (Pharmacia Biotech, Uppsala, Sweden). After incubation, antibodies were cross-linked with dimethyl pimelimidate (Sigma) and remaining reactive amino groups were quenched. The beads were added to a normal pool plasma sample (15 ml) and incubated overnight at 4 °C. After washing of the beads with PBS, the bound fraction was collected by adding 0.1 M Glycine, pH 2.7. The immunoprecipitated proteins were analyzed on SDS-polyacrylamide gel electrophoresis (-PAGE). One SDS-PAGE gel was used to transfer proteins to Immobilon-P membrane for Western blot analysis using either a polyclonal antibody raised against the MGP C-terminus (designated as pAb-MGP⁶¹⁻⁷⁹) or a monoclonal antibody raised against the MGP N-terminal peptide 3-15 (designated as mAb-MGP³⁻¹⁵; VitaK BV).

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Mass Spectrometry analysis

A second SDS-PAGE gel was used to collect the protein fraction for analysis by mass spectrometry. The MGP protein band was excised from the SDS-PAGE gel and processed on a MassPREP digestion robot (Waters). Generation of tryptic digest from the protein by in-gel digestion, MALDI-TOF analysis and subsequent database searching were performed as described previously. [31] For MS/MS analysis, 1.0 μ L of the peptide mixture and 1.0 μ L matrix solution (2.5 mg/mL CHCA in 50% acetonitrile/0.1% trifluoroacetic acid (TFA)) were spotted on a 384 wells target plate of a MALDI-TOF/TOF (4800 MALDI TOF/TOF analyzer, Applied Biosystems). Typically, 500 shots were combined for obtaining a spectrum. The generated peak list was searched with the MASCOT search engine v2.0 against the Swiss-Prot protein database for protein identification. One miss-cleavage was tolerated; carbamidomethylation was set as a fixed modification and oxidation of methionine as an optional modification. The protein charge was set at 1+. The peptide mass tolerance was set to 1 D and the MS/MS tolerance was set at 0.2 D. No restrictions were made on the protein mass. A protein was regarded identified when it had a significant Mascot probability score ($p < 0.05$).

Statistical analysis

Since t-ucMGP concentrations were not normally distributed, non-parametric testing was used. Non-parametric correlations (Spearman's correlation) were used to test for differences between plasma and serum ucMGP levels, and to determine the correlation with age. Healthy subjects were divided into 3 groups: subjects aged <40 years, between 40 and 60 years, and ≥ 60 years. The Kruskal-Wallis test was used to test for differences between the 3 groups of healthy subjects. Mann-Whitney U tests were used to test for differences between the reference population and the different patient populations. Unless stated otherwise, all data are means of duplicate measurements and presented as means $\pm$ SD. Data processing and statistical analysis was performed using SPSS for Windows (SPSS Corp, Chicago, IL, USA). Significance levels were based on two-tailed tests, with alpha level set at 0.05.

Results

Characteristics of the ucMGP assay

The intra-assay variability was tested for two different serum samples (t-ucMGP levels: 697 and 303 nmol/L) with 21 replicates per sample in the same plate. The variation coefficients were found to be 7.2% and 10.6%, respectively. The inter-assay variability was determined using pooled serum from 9 healthy subjects which was measured in duplicate on 21 different plates from different lot numbers on subsequent working days. The inter-assay variation coefficient was found to be 11.4%.

The lower detection limit of the assay was calculated as 3×SD of the zero standard obtained from 21 replicates in one plate, and was found to be 21 nmol/L. We compared the difference between citrated plasma and serum ucMGP measurements in paired samples from healthy subjects and patients, and found a good correlation ($r=0.897$; $p<0.001$).

Figure 1: Recovery after dilution

Recovery of t-ucMGP after dilution of serum. Serum from a healthy subject (t-ucMGP 869 nmol/L) was diluted with 2% HNBSA. The theoretical (—) as well as the observed ucMGP concentration (•) is shown. The points represent means of duplicate measurements.

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The recovery after dilution was assessed by serially diluting serum from a healthy subject (mean t-ucMGP 869 nmol/L) with dilution buffer (2% HNBSA) as depicted in Figure 1. Further, we assessed spiking recovery and spiked serum samples depleted from MGP by immunoprecipitation (t-ucMGP 1.7 nmol/L) with either synthetic full length ucMGP or cMGP. The ratio between the expected and observed t-ucMGP values was 64% for full length ucMGP and <2.5% for full length cMGP, as measured with the t-ucMGP assay.

Figure 2: Reactivity of mAb-ucMGP with synthetic MGP-derived peptides

Reactivity of the mAb-ucMGP with synthetic MGP-derived peptides (A) and synthetic full length MGP (B). Serum was mixed with either uncarboxylated (•) or carboxylated species (○) before testing. Points represent mean±SD (n=12).

To assess the assay specificity, mAb-ucMGP was tested for its cross reaction with synthetic MGP peptides (cMGP³⁵⁻⁵⁴ and ucMGP³⁵⁻⁵⁴; Figure 2A) and synthetic full length MGP (either cMGP or ucMGP; Figure 2B). These synthetic polypeptides were used in competitive inhibition experiments with the biotinylated ucMGP tracer (ucMGP³⁵⁻⁵⁴). As shown in Figure 2, both ucMGP³⁵⁻⁵⁴ and full length ucMGP were competitive inhibitors in the assay, whereas cMGP³⁵⁻⁵⁴ and full length cMGP were not. Moreover, we tested the synthetic peptides cMGP³⁵⁻⁴⁹ and ucMGP³⁵⁻⁴⁹, in which 3 glutamate (Glu) residues are present. Both peptides did not bind to the mAb-ucMGP (data not shown). Therefore, the mAb-ucMGP recognizes only ucMGP with at least 4 Glu residues. It was concluded that only ucMGP is recognized by the mAb-ucMGP used in our conformation-specific assay.

Identification of the product immunoprecipitated with the mAb-ucMGP was performed on Western blot analysis and showed a single band at ~11 kD, which reacted positively with a monoclonal antibody against the N-terminus and with a polyclonal antibody against the C-terminus of MGP (Figure 3). Analysis of the 11 kD-band of the second SDS-PAGE gel on MALDI-TOF/TOF after in-gel digestion revealed ucMGP as protein identity in the immunoprecipitated product (Table 1).

Figure 3: Western blot analysis

Western blot analysis of the immunoprecipitated product shows a single band at ~11kD as detected with both a polyclonal antibody raised against the MGP C-terminus (pAb-MGP⁶¹⁻⁷⁹) and a monoclonal antibody raised against the MGP N-terminus (mAb-MGP³⁻¹⁵).

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Table 1: Identification of ucMGP by MALDI TOF/TOF

Protein ID		MS		MS/MS		
Accession number	Protein name	MASCOT score	Sequence coverage	Matched peptides	Peptide sequence	MASCOT score
P08493	Matrix Gla-protein	62	32%	3	SKPVHELNR NANTFISPPQR	47

After excision of the 11 kD-protein band from the second SDS-PAGE gel followed by in-gel digestion, the obtained mixture of peptides was analyzed by mass-spectrometry (MS); the resulting collection of masses (peptide fingerprint) was searched with the MASCOT search engine against the Swiss-Prot protein database. Matrix Gla-protein (MGP) was identified with a significant MASCOT probability score of 62 and sequence coverage of 32%. To verify the identification of MGP, tandem mass spectrometry sequencing (MS/MS analysis) was performed; two peptide sequences of the three matched peptides could be determined with a significant MASCOT probability score of 47, confirming that ucMGP is indeed the protein identity in the immunoprecipitated product.

Sample preparation

To further characterize our assay, the influence of variations in the sample preparation procedure was tested using pooled serum from 20 healthy subjects (mean t-ucMGP 543±23 nmol/L). There was no measurable effect of centrifugation speed (1580 and 8000 g) during serum preparation (mean t-ucMGP 560±10 nmol/L and 526±22 nmol/L, respectively). Freeze-thawing up to 10 cycles did not affect the observed ucMGP concentration (mean t-ucMGP after 10 cycles 554±38 nmol/L).

Normal range, within-day variations, and day-to-day variations

The healthy subjects were divided into 3 groups: subjects under the age of 40 (mean age 30±6 years), those aged 40 to 60 years (mean age 51±6 years) and those aged 60 years or more (mean age 68±11 years). Baseline characteristics of the healthy subjects are shown in Table 2. The normal range for t-ucMGP was established in the group of 54 healthy males and females under the age of 40 (reference population), with a mean serum t-ucMGP level of 504±98 nmol/L. The normal range of t-ucMGP levels, defined as the mean±2×SD, was calculated to be between 308 and 699 nmol/L; no difference was observed between males and females. The mean level of serum t-ucMGP in the group of subjects aged 40 to 60 years was 481±77 nmol/L; in the group of subjects aged 60 years or more it was 441±97 nmol/L. The mean values of these two groups were within the normal range, with tailing towards lower values (Figure 4). There was a significant difference in t-ucMGP levels between the three groups of healthy subjects (p=0.02); additionally, an inverse correlation with age in the total population of healthy subjects was found (r =-0.81; p=0.02).

Figure 4: t-ucMGP levels in reference subjects

Serum t-ucMGP levels in the reference subjects, divided in 3 groups: subjects aged less than 40 years, aged 40 to 60 years and aged 60 or more. Mean±SD ucMGP levels were 504±98, 481±77, and 441±97 nmol/L respectively (depicted as horizontal bars). The difference between the 3 subpopulations was significant at p=0.02. The normal range, defined as the mean±2×SD, was established in the group of subjects aged less than 40 years (reference population) and was calculated to be between 308 and 699 nmol/L.

Table 2: Demographic and medical characteristics of healthy subjects

Reference population	Total	Aged < 40 years
N	165	54
Demographic characteristics		
Age (years)	49±16	30±6
BMI (kg/m ²)	23±4	22±3
Gender (m/f)	91/74	29/25
Clinical characteristics		
Diabetes (number [%])	0 (0)	0 (0)
Hypertension (number [%])	0 (0)	0 (0)
History of heart disease (number [%])	0 (0)	0 (0)
Medication use (number [%])	0 (0)	0 (0)

Data are given either as absolute values±SD or as numbers, with percentage of total between parentheses. Abbreviation used: BMI, body mass index.

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The time-related variability of serum t-ucMGP was established in 4 healthy subjects (between 25 and 35 years old, 2 males and 2 females; mean t-ucMGP 578 ± 233 nmol/L) from whom blood was taken by venipuncture at 9 time points on one day and subsequently on 5 different days at 9 AM with 1-week intervals. The within-day variation was calculated for each subject separately by expressing the SD as a percentage of the mean of the 9 time points. Coefficients of variation for each subject were found to be 6.6% (mean t-ucMGP 476 ± 31), 15.2% (470 ± 71 nmol/L), 10.5% (1067 ± 112 nmol/L), and 10.9% (485 ± 53 nmol/L). There was no observation of a distinct circadian pattern. In a similar way, the day-to-day variation was calculated from the 6 samples obtained at weekly intervals and coefficients of variation were found to be 14.7% (513 ± 75 nmol/L), 9.2% (449 ± 41 nmol/L), 10.5% (1016 ± 107 nmol/L) and 10.7% (511 ± 55 nmol/L).

Patient samples

To evaluate the potential clinical utility of the t-ucMGP assay, we measured serum t-ucMGP in 4 different patient populations. The demographic and clinical characteristics of the patient populations are summarized in Table 3. Figure 5 shows the serum t-ucMGP distribution in the reference (mean t-ucMGP 504 ± 98 nmol/L) and coronary angioplasty (389 ± 182 nmol/L), aortic stenosis (312 ± 109 nmol/L), dialysis (172 ± 82 nmol/L), and calciphylaxis (140 ± 55 nmol/L) population. Obviously, all 4 patient populations had significantly lower circulating t-ucMGP levels in comparison with the reference population (healthy subjects under the age of 40). Both the angioplasty population and aortic stenosis population showed a broader range as compared to the reference population with a strong tailing towards lower values. The t-ucMGP levels in hemodialysis as well as in calciphylaxis patients showed virtually no overlap with the normal range (Figure 5).

Figure 5: t-ucMGP levels in the patient populations

Serum t-ucMGP levels in patients compared to the reference population (healthy subjects aged less than 40 years). Mean±SD t-ucMGP values of angioplasty, aortic stenosis, dialysis and calciphylaxis patients were 389±182, 312±109, 172±82, and 140±55 nmol/L, respectively (depicted as horizontal bars). Differences between the reference population and angioplasty, aortic stenosis, dialysis and calciphylaxis patients were statistically significant at $p=0.002$, $p<0.001$, $p<0.001$, and $p<0.001$, respectively.

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Table 3: Demographic and medical characteristics of patients

Patient populations	Coronary angioplasty	Aortic stenosis	Hemodialysis	Calciphylaxis
N	30	25	52	10
Demographic characteristics				
Age (years)	52±8	72±10	64±13	63±14
BMI (kg/m ²)	27±4	28±4	27±4	-
Gender (m/f)	18/12	14/11	28/24	5/5
Clinical characteristics				
Diabetes (number [%])	5 (15)	7 (28)	18 (35)	4 (40)
Hypertension (number [%])	6 (20)	16 (64)	46 (88)	9 (90)
CAD (number [%])	30 (100)	15 (60)	28 (54)	8 (80)
CKD (number [%])	0(0)	0 (0)	52 (100) ¹	10 (100) ²

Data are given either as absolute values±SD or as numbers, with percentage of total between parentheses. Abbreviations used: BMI, body mass index; CAD, coronary artery disease; CKD, chronic kidney disease.

¹ All 52 hemodialysis patients were by definition in CKD stage 5; ² seven patients in the calciphylaxis group were dialysis patients (CKD stage 5: 6 hemodialysis, 1 peritoneal dialysis), three patients were renal transplant recipients (CKD stage 3–4).

Additionally, t-ucMGP levels of the patient populations were compared with the group of healthy subjects of the same age. The t-ucMGP level of the angioplasty population was significantly lower in comparison with the group of healthy subjects aged 40 to 60 years ($p=0.003$). The t-ucMGP levels of the aortic stenosis, dialysis, and calciphylaxis populations were significantly lower as compared to the group of healthy subjects aged 60 years or more ($p<0.001$, $p<0.001$, and $p<0.001$, respectively). These results indicate that low ucMGP levels in the patient populations are not merely an effect of age.

Finally, t-dpMGP levels were measured with the commercially available MGP assay in the 4 patient populations. Only the group means of aortic stenosis and calciphylaxis patients were significantly different from the reference population (data not shown). Additionally, more than 70% of the t-dpMGP levels in the patients showed overlap with the normal range. These data do not allow individual risk assessment, since the majority of the patients cannot be discriminated from the healthy reference population.

Discussion

In this paper, we report on the development of an ELISA-based competitive assay for human total circulating ucMGP (t-ucMGP) species. To test the assay for its diagnostic utility, we analysed ucMGP in 4 different patient populations. Circulating t-ucMGP levels were significantly lower in both groups of cardiovascular patients as well as in dialysis and calciphylaxis patients. Moreover, t-ucMGP levels in the dialysis and calciphylaxis patients did not overlap with those in the reference population. Clearly, serum t-ucMGP is a marker capable of discriminating between healthy subjects and patients with end-stage renal disease.

Dialysis patients have a 60 to 80% prevalence of moderate to severe vascular calcifications [32–34]. Calciphylaxis is by definition characterized by extensive calcifications of cutaneous arterioles usually accompanied by large vessel calcifications [35, 36]. The angioplasty and aortic stenosis patient populations each represent a different vascular pathologic etiology. Aortic stenosis patients have a high incidence of calcification but can be free of atherosclerosis, whereas patients who underwent angioplasty suffer from intimal (atherosclerotic) vascular disease. Additionally, large inter-individual variation in the extent of vascular calcification can be expected in these patient groups and could explain the overlap with the reference population.

The specificity of the assay for ucMGP species was demonstrated by using synthetic MGP peptides; only the ucMGP peptides reacted with the mAb-ucMGP, whereas no affinity was observed for cMGP peptides. Recovery experiments using the same peptides to spike serum samples showed that only the recovery of ucMGP, and not of cMGP, was adequate. Full length MGP was identified by mass spectrometry as the major MGP species recognized by the mAb-ucMGP.

We hypothesized that in the presence of arterial calcification (arterial medial calcification or in atherosclerotic lesions), the circulating fraction of ucMGP may be decreased. The results obtained with our t-ucMGP assay show that in patient populations known for cardiovascular calcification serum t-ucMGP levels are significantly lower as compared to an apparently healthy reference population. The accumulation of ucMGP species in atherosclerotic lesions and areas of calcification has been reported in several studies [6, 7], including our recent study using immunohistochemical techniques [5]. The balance between tissue ucMGP and circulating ucMGP species in calcified arteries may be different from that in healthy ones with increased t-ucMGP levels in calcified tissues and consequently less ucMGP secretion in the circulation.

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However, the exact mechanisms – including the process of MGP secretion in the circulation – remain to be elucidated. Alternative explanations for the low ucMGP levels in the selected patient populations may be lower synthesis of MGP and increased degradation of MGP before carboxylation in these patients.

A limitation of our study was the inability to measure circulating levels of cMGP. At the moment there is no assay available to measure this conformation of MGP; in the near future we aim to develop a dual-antibody ELISA for cMGP as well as for ucMGP. Also, the reference population should be demonstrated to be free from disease, e.g. free from cardiovascular calcification; this would require MSCT or electron-beam computed tomography (EBCT) screening of all healthy volunteers. Since these imaging techniques are expensive, and also because they expose subjects to significant X-ray radiation, we have not characterized our reference population by MSCT or EBCT. However, it has been reported that the majority of subjects under the age of 40 have very low coronary artery calcium scores as measured with EBCT, with a mean Agatston score for males of 10 and for females of 9 [37]. Therefore we defined the normal range of circulating t-ucMGP in the group of healthy subjects under the age of 40.

Increasingly more patient populations at risk for vascular calcification are currently being identified, including young females with systemic lupus erythematosus [38], polycystic ovary syndrome [39], and patients with alkaptonuria [40]. The inactive ucMGP species could have potential to serve as a non-invasive biochemical marker for cardiovascular calcification and possibly contribute to the identification of patients requiring radiological assessment of cardiovascular calcification. Obviously, diagnostic applications of t-ucMGP measurements would require much further study. Seen from a therapeutic perspective, vitamin K therapy has the potential of increasing the activity of MGP, and possibly reducing the development of calcification. In an animal model system the efficacy of such therapy has recently been demonstrated [29, 41]. The effect of vitamin K therapy on circulating MGP levels remains to be determined.

The first commercially available MGP assay, measuring t-dpMGP levels, appears to be less suited for individual risk assessment. The cardiovascular patients as well as the patients prone for cardiovascular calcification could not be adequately separated from the reference population, since 73% of the t-dpMGP levels in these patients were within normal range. A principal difference with our newly developed t-ucMGP assay is that the commercial assay does not discriminate between carboxylated and uncarboxylated MGP species.

In conclusion, we have developed a robust assay for circulating t-ucMGP allowing the assessment of the circulating levels of uncarboxylated MGP species. It was found that different patient groups with cardiovascular disease or with a propensity to develop vascular calcification had significantly lower levels of circulating t-ucMGP and could be discriminated from the healthy reference population. Further studies are necessary to determine the exact correlation between t-ucMGP levels and the extent of vascular calcification as well as the predictive value of t-ucMGP measurements.

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CHAPTER 3

Total uncarboxylated Matrix Gla-protein (t-ucMGP) is associated with coronary artery calcification in hemodialysis patients

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Abstract

Matrix γ -carboxyglutamate (Gla)-protein (MGP) is a potent local inhibitor of cardiovascular calcification and accumulates at areas of calcification in its uncarboxylated form (ucMGP). We previously found significantly lower circulating total ucMGP (t-ucMGP) levels in patients with a high vascular calcification burden.

Here we report on the potential of circulating t-ucMGP to serve as a biomarker for vascular calcification in hemodialysis (HD) patients. Circulating t-ucMGP levels were measured with an enzyme-linked immunosorbent assay (ELISA) in 40 HD patients who underwent multislice computed tomography (MSCT) scanning to quantify the extent of coronary artery calcification (CAC). The mean t-ucMGP level in HD patients (193 ± 65 nmol/L) was significantly lower as compared to apparently healthy subjects of the same age (441 ± 97 nmol/L; $p < 0.001$) and patients with rheumatoid arthritis (RA) without CAC (560 ± 140 nmol/L; $p < 0.001$). Additionally, t-ucMGP levels correlated inversely with CAC scores ($r = -0.414$, $p = 0.009$), and this correlation persisted after adjustment for age, dialysis vintage, and high-sensitivity C-reactive protein (hs-CRP).

Since circulating t-ucMGP levels are significantly and inversely correlated with the extent of CAC in HD patients, t-ucMGP may become a tool for identifying HD patients with a high probability of cardiovascular calcification.

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Introduction

Cardiovascular disease (CVD) in patients on hemodialysis (HD) is a clinically outstanding problem: CVD accounts for approximately 50% of deaths among the dialysis population and cardiovascular mortality is about 10–20 times higher than in the general population [1, 2]. Arterial intimal calcification is associated with atherosclerotic lesions. Arterial medial calcification is found in diabetic and dialysis patients as well as in most elderly subjects [3, 4]. Arterial medial calcification was initially thought to be clinically irrelevant, but is now considered to be associated with all-cause and cardiovascular morbidity in dialysis patients, independent of traditional atherogenic risk factors [5, 6]. Clinically, arterial calcification causes stiffening of the vascular wall, which in turn may result in decreased arterial compliance, increased afterload with development of left ventricular hypertrophy, and decreased coronary perfusion with increased risk for death [5–7].

The exact pathophysiology of arterial calcification in chronic kidney disease (CKD) is not fully understood. Numerous factors have been correlated with arterial calcification in CKD including age, dialysis vintage, and calcium x phosphorus product [8–10]. Since concentrations of calcium and phosphate in extracellular fluid exceed their solubility product under physiological conditions, powerful calcification inhibitors are required to prevent spontaneous calcification of soft tissues throughout the body.

Matrix γ -carboxyglutamate (Gla)-protein (MGP) is the most potent inhibitor of soft tissue calcification presently known. Its main sources are cartilage and the vessel wall where it is synthesized by chondrocytes and vascular smooth muscle cells (VSMC), respectively [11–13]. MGP contains nine glutamate (Glu) residues, five of which can be carboxylated in a vitamin K-dependent reaction to form calcium-binding Gla residues [14]. Additionally, mature MGP contains three serine residues which may be phosphorylated. The function of these phosphoserine residues is presently unknown, but it seems highly probable that they significantly contribute to the calcium-binding properties of MGP [15].

A poor vitamin K status caused by poor nutritional intake or the use of vitamin K antagonists (oral anticoagulants), results in the synthesis of uncarboxylated MGP (ucMGP) [14, 16]. After the development of conformation-specific assays for MGP and osteocalcin, a vitamin K-dependent protein present in bone, it has been found that in healthy subjects uncarboxylated species of these proteins are present in the circulation [16]. Additionally, ucMGP was recently purified from plasma of healthy subjects as a mature 11 kD protein [17]. This suggests that the majority of healthy adults is subclinically vitamin K deficient, resulting in a sub-optimal activity of extra-hepatic vitamin K-dependent proteins.

Indeed, it was described that women with calcified atherosclerotic lesions had lower dietary vitamin K intake than women without calcifications [18]. In a large follow-up study it was found that vitamin K₂ intake was inversely associated with severe aortic calcification in asymptomatic subjects [19]. Additionally, the risk of coronary heart disease mortality was significantly reduced in the upper tertile of vitamin K₂ intake. Our recently developed competitive enzyme-linked immunosorbent assay (ELISA) measuring total ucMGP (t-ucMGP) provides a way to investigate the correlation between systemic t-ucMGP levels and vascular calcification, as described in this paper.

Impaired expression of MGP results in arterial calcification, as was demonstrated in transgenic MGP-deficient mice which developed extensive calcifications of the main arteries followed by rupture of the aorta a few weeks after birth [20]. The arterial mineralization phenotype of these mice has been rescued after restoration of MGP expression locally in arteries, however not after systemically raising circulating levels of MGP [21]. This emphasizes the function of MGP as a local inhibitor of vascular calcification, with circulating t-ucMGP levels possibly reflecting the status of calcification in the vessel wall [15].

The t-ucMGP assay specifically recognizes ucMGP, independent of its degree of phosphorylation. Using this assay we found that the serum t-ucMGP levels in HD and calciphylaxis patients as well as in patients with cardiovascular disease are lower than those in a healthy reference population [17]. We have previously demonstrated the massive accumulation of ucMGP at sites of arterial calcification [22], suggesting that – most probably through its negatively charged phosphoserine residues – MGP binds to calcified tissue independent of its Gla residues. This local tissue accumulation may be the reason for a diminished systemic release of ucMGP species into the circulation and may well explain the relatively low serum t-ucMGP levels in patients with pronounced vascular calcification.

A high coronary artery calcification (CAC) score as assessed with computed tomography is associated with the severity of coronary artery disease and the presence of significant, stenotic lesions [23] as well with increased mortality in HD patients [24]. To investigate whether circulating ucMGP may serve as a non-invasive biomarker for arterial calcification and – consequently – for cardiovascular disease in HD patients, we evaluated the relationship between serum t-ucMGP levels and the extent of CAC as measured with multislice computed tomography (MSCT) scanning.

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Methods

Subjects

We invited patients undergoing HD treatment at three dialysis centers to participate in this cross-sectional study. Patients with end-stage renal disease (CKD stage V) undergoing regular HD were eligible, irrespective of age, gender, the underlying primary renal disease, the presence of CVD, or traditional atherogenic risk factors. Exclusion criteria were a history of kidney transplantation, current bypass surgery, current treatment with oral anti-coagulants, and atrial fibrillation. Complete data and blood samples were acquired from 40 consecutive patients. Data were obtained on demographic, clinical, and dialysis-specific characteristics during interview, and physical examination as well as from review from patient's files. Additionally, all HD patients underwent MSCT scanning to measure the extent of CAC.

The reference population consists of two groups: 48 apparently healthy subjects aged 60 years or more, who were recruited from the general Maastricht population and 21 patients with rheumatoid arthritis (RA) from a tertiary RA referral center in Aachen. The subjects of the first reference group, 19 men and 29 women, had no overt history of cardiovascular disease and were described previously [17]; MSCT scanning was not available for these subjects. The second reference group consisted of 1 male and 20 female RA patients in whom MSCT scanning excluded the presence of CAC and aortic valve calcification (CAC score at both anatomical sites 0). Informed consent was obtained from all participants and the study was approved by the local Medical Ethics Committees.

Biochemical measurements

Venous blood samples from the HD patients were taken at the start of HD after a long dialysis interval. Serum from all participants was prepared by standard centrifugation and stored at -80 °C until testing.

The method for t-ucMGP measurement was a competitive enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody (mAb-ucMGP) which was raised against the uncarboxylated human MGP Gla-domain (MGP sequence 35–49; VitaK BV, Maastricht, The Netherlands) [17, 25]. The intra-assay and inter-assay coefficients of variation (CV) were 8.9% and 11.4% respectively. The specificity of the t-ucMGP assay has been demonstrated elsewhere by testing of mAb-ucMGP for its cross-reaction with synthetic peptides homologous to the uncarboxylated and carboxylated aminoacid sequences in human MGP (ucMGP^{35–54} and cMGP^{35–54}, respectively) and synthetic full length MGP (either ucMGP or cMGP) [17].

These polypeptides were used in competitive inhibition experiments with the biotinylated ucMGP tracer (ucMGP³⁵⁻⁵⁴). Both ucMGP³⁵⁻⁵⁴ and full length ucMGP were competitive inhibitors in the assay, whereas cMGP³⁵⁻⁵⁴ and full length cMGP were not. Additionally, immunoprecipitation was performed in plasma using the mAb-ucMGP. Identification of the immunoprecipitated product on Western blot analysis showed a single band at ~11 kD and analysis of the band on MALDI-TOF/TOF revealed ucMGP as protein identity in the immunoprecipitated product [17].

Calcium (Ca), phosphate (P), hemoglobin (Hb), and alkaline phosphatase (AP) were measured using standard laboratory techniques. Serum analysis for high-sensitivity C-reactive protein (hs-CRP) was performed by means of particle enhanced immunonephelometry using standard CardioPhase hsCRP for BNII (Dade Behring Holding GmbH, Liederbach, Germany), with a detection limit of 0.1 mg/L and precision of 0.1 mg/L. CRPI or CRPII assay protocols were used when appropriate. Interday precision controls revealed CV's below 9%; testing for precision with a 20-fold repetitive measurement of a single sample showed a CV of 7% [26]. Levels of intact parathyroid hormone (iPTH) were measured with the Roche's Elecsys Intact PTH immunochemiluminescent assay (Hoffmann-La Roche, Basel, Switzerland).

Imaging procedure

All MSCT examinations of the HD patients and RA patients were performed with a 16-slice MSCT scanner (SOMATOM, Sensation 16, Siemens, Forchheim, Germany). Scan parameters included a collimation of 12 x 0.75 mm, a rotation time of 420 ms, a table feed of 3.4 mm per rotation, a tube voltage of 120 kV and an effective tube current time product of 150 mAs_{eff}. For ECG-synchronization retrospective ECG-gating was applied. Axial images were reconstructed in mid-diastole at 60% of the RR-interval with an effective slice thickness of 3 mm and a reconstruction increment of 2 mm. A dedicated convolution kernel (B35f) [27], a field of view of 180 x 180 mm² and a matrix of 512x512 were applied. All MSCT images were assessed by two radiologists with several years of training in cardiac imaging. A consensus reading was performed with both readers being blinded to all patient data. Image analysis was performed on a separate computer workstation (Leonardo, Siemens, Forchheim, Germany) equipped with a dedicated software tool for calcium scoring (Calcium Scoring CT, Siemens, Forchheim, Germany). CAC scores were calculated according to the method originally described by Agatston [28].

Statistical analysis

Continuous variables are presented as median and range or mean±standard deviation (SD). The distribution of variables was evaluated with frequency histograms and normal plots.

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Differences between the patient population and the reference groups were analyzed by independent T-tests and Mann-Whitney tests (for normally and non-normally distributed continuous variables, respectively). The HD patient population was divided into tertiles of the CAC score which resulted in three groups with CAC scores of ≤ 103.0 , 103.1 to 600.0, and ≥ 600.1 . Differences in clinical and biochemical characteristics between the three HD patient groups were analyzed by Tukey post hoc testing and Kruskal-Wallis test (for normally and non-normally distributed continuous variables, respectively) and Fisher's exact test (for categorical variables).

Bivariate correlations between CAC scores, t-ucMGP, and other variables were performed using the Pearson correlation coefficient for normally distributed data and Spearman correlation coefficient for non-normally distributed data. Multivariate regression analysis was performed to investigate whether CAC was related to serum t-ucMGP levels in HD patients, simultaneously adjusting for all variables in the model; the dependent variable was log-transformed CAC score. Log-transformation was applied after adding a constant of 10, which resulted in normal distribution of CAC score, necessary for multivariate regression analysis. The independent variables were t-ucMGP, age, dialysis vintage, and hs-CRP. Significant colinearity was ruled out by calculating variance inflation factor for each independent variable. A pvalue of <0.05 was considered to be statistically significant; reported p values are based on two-tailed tests of statistical significance. All statistical analyses were conducted using SPSS version 12.0 for Windows (SPSS Corp, Chicago, IL, USA).

Results

Subject characteristics

The reference population consisted of two groups, apparently healthy subjects and RA patients in whom the presence of CAC or aortic valve calcification was excluded by MSCT. The healthy reference group was free from cardiovascular disease, hypertension, and diabetes and did not use medication, as described previously [17]. The median age of these subjects (67 years, range 60–90 years) was comparable with that of the HD patient population (67 years, range 36–87 years; $p=0.519$). The RA reference group consisted of 21 RA patients with a median age of 54 years (range 31 to 81 years), which was significantly lower than the median age of the HD patient population ($p<0.001$). The median duration of the RA was 16 years (range 7–32 years). A history of cardiovascular disease was present in 5 RA patients (23.8%), 1 patient had diabetes (4.8%), and 2 patients (9.5%) were current and 4 patients (19.0%) were former smokers, respectively. Median blood pressure in the RA patients was 125/80 mm Hg.

Demographic, clinical, and biochemical characteristics of the HD patient population are listed in Table 1, with HD patients stratified according to tertiles of the CAC score. Subjects in the intermediate CAC tertile was significantly older than the subjects in the lowest CAC tertile ($p=0.039$), however not as compared to the highest CAC tertile ($p=0.791$). There was no significant age difference between subjects in the lowest and the highest CAC tertiles ($p=0.163$). For the remaining demographic, clinical, and biochemical characteristics (Table 1), there were no significant differences between the tertiles.

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Table 1. Characteristics of HD patients stratified according to tertiles of the CAC score.

	CAC score ≤ 103.0	CAC score 103.1 - 600.0	CAC score ≥ 600.1	Total population
N	13	14	13	40
Demographic and clinical characteristics				
Age (years)	63 (36 – 87)	70 (57 – 83) *	68 (52 – 79)	67 (36 – 87)
Sex (male/female ratio)	3/10	6/8	8/5	17/23
Dialysis vintage (months)	44.9 (4.0 – 113.5)	30.4 (8.2 – 109.4)	33.8 (5.4 – 289.5)	34.3 (4.0 – 289.5)
Presumed cause of ESRD (number of patients)				
Glomerulonephritis, Vasculitis	3	4	2	9
Tubulointerstitial disease	2	1	1	4
Amyloid nephropathy	3	0	0	3
Vascular disease, Hypertension	1	5	2	8
Hereditary disease, Cystic disease	4	1	3	8
Diabetic kidney disease	0	0	3	3
Miscellaneous	0	3	2	5
Hypertension (number of patients)	12	13	1	38
Diabetes (number of patients)	1	2	3	6
Current smoking (number of patients)	1	2	4	7
BMI (kg/m ²)	25.6 (18.4 – 53.3)	24.1 (17.3 – 31.4)	23.9 (19.1 – 36.6)	24.4 (17.3 – 53.3)
History of cardiovascular disease (number of patients)				
CAD	2	6	7	15
CVD	2	5	5	12
CVD	1	1	3	4
PAD	1	2	3	6
History of parathyroidectomy (number of patients)	0	1	0	1
Vitamin D supplementation	7	12	8	27
Use of oral PB	13	12	11	31
Ca-containing PB	8	12	11	31
Aluminum-containing PB	2	6	3	11
Sevelamer	3	0	1	4
Biochemical characteristics				
Ca (mmol/L)	2.3 (1.4 – 2.7)	2.4 (2.1 – 3.0)	2.5 (2.1 – 2.8)	2.4 (1.4 – 3.0)
P (mmol/L)	1.8 (1.0 – 2.5)	1.4 (1.1 – 2.5)	1.8 (1.3 – 2.6)	1.7 (1.0 – 2.6)
Ca × P (nmol ² /L ²)	3.9 (2.3 – 6.6)	3.3 (2.7 – 5.7)	4.2 (2.9 – 7.3)	4.1 (2.3 – 7.3)
hs-CRP (mg/L)	2.5 (0.3 – 18.9)	5.6 (1.3 – 24.8)	7.2 (0.2 – 21.4)	4.8 (0.2 – 24.8)
Hemoglobin (mg/dL)	104 (88 – 124)	109 (95 – 144)	106 (95 – 156)	105 (88 – 156)
AP (U/L)	58 (41 – 232)	52 (40 – 252)	51 (34 – 217)	54 (34 – 252)
iPTH (pmol/L)	7.0 (1.1 – 276)	9.6 (1.2 – 116)	10.5 (0.7 – 164)	9.6 (0.7 – 276)

Demographic, clinical, and biochemical characteristics of patients stratified according to tertiles of the CAC score: CAC score ≤130.0, 103.1 to 600.0 and ≥600.1. Data are presented as median (range). *: The intermediate CAC tertile was significantly older as compared to the lowest CAC tertile (p=0.039). Otherwise, there were no significant differences between the CAC tertiles. Abbreviations used: CAC, coronary artery calcification; ESRD, end-stage renal disease; BMI, body mass index; CAD, coronary artery disease, defined as history of myocardial infarction, angina pectoris or evidence of obstructive disease by angiography; CVD, cerebrovascular disease, defined as a history of thrombotic stroke or transient ischemic attack; PAD, peripheral artery disease, defined as a history of claudication or lower extremity revascularization; PB, phosphate binders; Ca, calcium; P, phosphate; hs-CRP, high-sensitivity C-reactive protein; AP, alkaline phosphatase; iPTH, intact parathyroid hormone.

Circulating ucMGP

The mean t-ucMGP levels of the apparently healthy subjects and RA patients without CAC were 441±97 nmol/L and 560±140 nmol/L, respectively. The mean t-ucMGP level of the HD patient population was 193±65 nmol/L, with levels ranging from 60 to 352 nmol/L (Figure 1). The HD patient population had significantly lower circulating t-ucMGP levels in comparison with both reference groups (p<0.001). Moreover, the serum t-ucMGP levels of the healthy subjects were significantly lower compared than those in RA patients (p<0.001).

Figure 1: Serum t-ucMGP levels of the RA reference group (n=21), the healthy reference group (n=48), and the HD patient population (n=40).

Circulating t-ucMGP levels were significantly lower in HD patients than in RA patients (p<0.001) and apparently healthy subjects aged 60 years or more (p<0.001). One outlier, subject number 62 (depicted as ○ 62), was present in the healthy reference group. This subject (age 66 years) had a t-ucMGP level of 187 nmol/L. Box diagrams represent median (horizontal line), the 75th percentile (upper limit of the box) and the 25th percentile (lower limit of the box). Whiskers show the range of values, with exception of outliers and extremes. Per box the mean±SD ucMGP level is presented. Abbreviations used: RA, rheumatoid arthritis; HD, hemodialysis.

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No significant correlations were observed between t-ucMGP levels and any demographic, clinical, or biochemical characteristic in the HD patient population. There was no difference in t-ucMGP levels between men and women; this was also found in both reference groups. Figure 2 shows the serum t-ucMGP levels of HD patients stratified according to tertiles of the CAC score; t-ucMGP levels were significantly lower in patients in the intermediate (174 ± 46 nmol/L) and highest CAC tertile (171 ± 66 nmol/L) in comparison with patients in the lowest CAC tertile (237 ± 66 nmol/L; $p=0.022$ and $p=0.021$, respectively).

Figure 2: Serum t-ucMGP levels of patients stratified according to tertiles of the CAC score (n = 40).

Circulating t-ucMGP levels in HD patients in the intermediate and highest CAC tertiles were significantly lower than in patients in the lowest CAC tertile ($p=0.022$ and $p=0.021$ respectively). One outlier, patient number 17 (depicted as $\circ 17$), was present in the intermediate CAC tertile. This HD patient (age 60 years, dialysis vintage 21 months) had a circulating ucMGP level of 300 nmol/L. Box diagrams represent median (horizontal line), the 75th percentile (upper limit of the box) and the 25th percentile (lower limit of the box). For further details, see the legend of Figure 1. Abbreviation used: CAC, coronary artery calcification.

t-ucMGP as a predictor for CAC in HD patients

A high prevalence of CAC was a major finding in our patient population, with 18 out of 40 patients (45%) having a CAC score of 400 or more, indicating extensive atherosclerotic plaque burden [29]. The median CAC score of the total HD patient population was 308.0 (range 0–2928). Serum t-ucMGP levels were inversely correlated with CAC scores ($r=-0.414$, $p=0.009$); the only other factor that correlated with CAC scores was hs-CRP ($r=0.404$, $p=0.011$; Table 2). In contrast, CAC scores did not correlate with any of the other clinical and biochemical characteristics (depicted in Table 2 for the main clinical and biochemical parameters). In a multivariate regression analysis t-ucMGP levels retained a strong and significant association with CAC scores ($\beta=-0.004$, $p=0.02$) after adjustment for age, dialysis vintage, and hs-CRP levels (Table 2).

Table 2. Coronary artery calcification correlations (n=40).

Bivariate	r	p	
Age	0.29	0.072	
Dialysis vintage	0.03	0.849	
BMI	-0.17	0.295	
Ca	0.27	0.098	
P	0.01	0.964	
Ca x P	0.06	0.713	
hs-CRP	0.40	0.011	
AP	-0.047	0.781	
iPTH	0.068	0.687	
ucMGP	-0.41	0.009	
Multivariate	β coefficient	p	95% CI
ucMGP	0.004	0.017	0.007 to -0.001
Age	0.016	0.056	0.001 to 0.032
Dialysis vintage	0.002	0.382	0.002 to 0.006
hs-CRP	0.035	0.031	0.003 to 0.067

For bivariate analysis (bivariate) the Spearman rank correlation coefficient was calculated. Circulating t-ucMGP levels and hs-CRP were significantly correlated with CAC scores; CAC scores did not correlate with any of the other clinical and biochemical parameters. In multivariate regression analysis (multivariate) t-ucMGP levels retained a strong association with (log-transformed) CAC scores while adjusting for age, dialysis vintage and hs-CRP-levels. The overall model p value was 0.002, the model R^2 was 0.377 (adjusted $R^2=0.306$); the β regression coefficient was calculated per 1 nmol/L t-ucMGP. Abbreviations used: BMI, body mass index; Ca, calcium; P, phosphate; AP, alkaline phosphatase; iPTH, intact parathyroid hormone; hs-CRP, high-sensitivity C-reactive protein.

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Discussion

The present study establishes serum levels of t-ucMGP as a possible novel biomarker for CAC in HD patients. HD patients had significantly lower levels of t-ucMGP than healthy subjects of the same age and patients without CAC. Additionally, HD patients in the intermediate and highest tertiles of the CAC score had significantly lower t-ucMGP levels in comparison with patients in the lowest CAC tertile. The inverse correlation between serum t-ucMGP and CAC scores was confirmed by multivariate regression analysis and persisted after adjustment for age, dialysis vintage, and hs-CRP – three well established predictors of CAC in HD patients [5, 6, 8–10, 30].

We previously found significantly lower t-ucMGP levels in several patient populations with a high vascular calcification burden, including HD patients and calciphylaxis patients [17]. The present study underlines and extends these findings and shows that t-ucMGP serum measurements may not only be used for intergroup comparisons of arterial calcification, but also help estimating the individual extent of CAC in a certain patient population. It is noteworthy that the mean $\pm$ SD t-ucMGP level of 140 $\pm$ 55 nmol/L previously found in patients with calciphylaxis – a condition characterized by widespread arteriolar calcification – is even lower than that of HD patients with the highest CAC scores in the present study [17].

Defining t-ucMGP as a sensitive indicator for the presence of vascular calcification might also be the explanation for the surprising finding of lower circulating t-ucMGP levels in the apparently healthy subjects compared to the RA patients. Only in the RA reference group, the presence of CAC was truly excluded, while clinically unapparent CAC might be present in the presumably healthy subjects who were aged 60 years or more. Indeed, several studies have described median CAC scores >100 in elderly subjects without overt signs of atherosclerosis, especially in elderly men [31–33]. We speculate that subclinical arterial disease might contribute to the lower t-ucMGP levels in the “healthy” reference group. At the present time it cannot be determined, whether RA itself, or a consecutive treatment influences t-ucMGP levels in the RA reference group.

The design of the present study is cross-sectional and therefore we cannot establish a cause-and-effect relation between low t-ucMGP levels and high CAC scores. However, it does allow for assessment of t-ucMGP as a biomarker for CAC. The number of HD patients is limited and therefore we are limited with respect to full adjustment for all possible confounders in our multivariate analysis. Nevertheless, the present patient population is well characterized and by including age, dialysis vintage, and hs-CRP levels we could adjust at least for three of the most important potential confounders regarding CAC development in ESRD patients.

ucMGP species have a high affinity to areas of calcification in the vessel wall. In rats treated with warfarin, Price *et al.* demonstrated that MGP accumulates at sites of calcification in its non-carboxylated form [34]. Sweatt *et al.* reported elevated concentrations of poorly carboxylated MGP in the aortic wall of aging rats [35]. Moreover, with immunohistochemical staining based on conformation-specific antibodies recognizing ucMGP and cMGP, we demonstrated that ucMGP rather than cMGP species are present at sites of arterial calcification [22]. Therefore, our working hypothesis is that the presence of Gla residues is not a requirement for the binding of MGP to sites of calcification. Indeed, thermal decarboxylation of MGP in vitro did not affect its ability to bind hydroxyapatite from serum [36].

Phosphorylation of MGP results in the presence of three phosphoserine residues in the N-terminal domain. These negatively-charged phosphate groups must be expected to contribute significantly to the calcium-binding capacity of MGP. We examined the role of phosphoserine-residues in a cell model of calcification [37]. This model consisted of VSMC exposed to high extracellular calcium concentrations. The synthetic MGP peptides pMGP³⁻¹⁵ and dpMGP³⁻¹⁵, which were used in the experiments, contain three serine residues that are either phosphorylated or non-phosphorylated, respectively. These peptides do not contain Gla residues. After addition of the (biotinylated) peptides to the calcifying VSMC, staining revealed that pMGP³⁻¹⁵ specifically adhered to vesicular structures derived from the (calcifying) VSMC. These vesicles were presumably matrix vesicles, which are thought to precede calcification [38]. In contrast, dpMGP³⁻¹⁵ did not bind to either the cell surface or the vesicles. pMGP³⁻¹⁵ was also able to inhibit the calcification process, whereas dpMGP³⁻¹⁵ had no measurable effect. These results indicate that MGP containing phosphoserine residues, but no Gla residues, is able to bind to (initial) sites of calcification.

Since ucMGP species in the vascular tissue have a strong association with areas of calcification, circulating t-ucMGP levels could be particularly suited to reflect this degree of this calcification. A low systemic t-ucMGP level in patients with a high vascular calcification burden has been a consistent finding [17, 25, 39]. The low t-ucMGP levels in these patients can be explained by the accumulation of ucMGP species in areas of calcification, which may cause a diminished release of ucMGP species into the circulation. This accumulation is most probably due to binding of MGP's negatively charged phosphoserine residues to vascular calcifications. It is therefore likely that the majority of circulating ucMGP species is phosphorylated. Indeed, we have found no correlation between circulating ucMGP levels and non-phosphorylated MGP levels in dialysis patients ($r=-0.144$, $p=0.38$; data not published).

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Since phosphorylation is often associated with cellular secretion, it is at least feasible that in the diseased vessel MGP phosphorylation is hampered, leading to decreased circulating levels. This could be an alternative explanation for the low circulating t-ucMGP levels in patients with vascular calcifications.

Recently, we developed a new sandwich MGP ELISA specifically measuring desphospho-uncarboxylated MGP (dp-ucMGP) [15]. Since strong calcium-binding groups such as phosphoserine- or Gla-residues are absent in this molecule, we expect that dp-ucMGP will be readily set free into the circulation independent of present vascular calcification. This assay would therefore be suited to assess and follow-up vascular vitamin K status (e.g. when vitamin K supplementation or vitamin K antagonists are used). Preliminary data showed that dp-ucMGP is present in the circulation of healthy subjects [15]. High dp-ucMGP levels were found in subjects receiving vitamin K antagonists and low levels in subjects receiving vitamin K supplementation. The data presently available strongly suggest that dp-ucMGP reflects vascular vitamin K status. In contrast, t-ucMGP levels as described in this paper seem to be relatively insensitive for vitamin K status (data not published) but are rather sensitive for the presence of arterial calcification. Full details of the dp-ucMGP assay will be published elsewhere.

The dialysis population exhibits a significant amount of vascular calcification that is associated with an increased risk of cardiovascular morbidity and mortality. According to the present data it can be hypothesized that measuring merely calcification promoters (e.g. serum calcium or serum phosphorus) does not give sufficient consideration to the role of calcification inhibitors in the process of vascular calcification. Based on the present data, circulating t-ucMGP could contribute substantially to the identification of those patients with high probability of cardiovascular calcification. Further studies need to be performed in order to clarify whether low t-ucMGP levels may eventually be used to identify those patients who should undergo a more thorough diagnostic cardiac work-up (e.g. non-invasive ischemia testing or coronary angiography).

In conclusion, the current study is the first analysis of circulating t-ucMGP levels in a well characterized population of adult HD patients and demonstrates that t-ucMGP is significantly and inversely correlated with CAC as assessed by MSCT.

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Chapter 4

Circulating total uncarboxylated Matrix Gla-protein (t-ucMGP) is not associated with mortality in dialysis patients

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Abstract

Matrix γ -carboxyglutamate (Gla)-protein (MGP), a vitamin K-dependent protein, is an important local inhibitor of vascular calcification. Patients undergoing dialysis treatment are at high risk to develop vascular calcification, which results in significant morbidity and mortality. Vitamin K deficiency leads to synthesis of uncarboxylated MGP (ucMGP), which accumulates at sites of calcification in the arterial wall. Previous studies showed that circulating total ucMGP levels (t-ucMGP) were low in dialysis patients, and were inversely correlated with vascular calcification parameters. In the present study, we have investigated the predictive value of t-ucMGP for overall and cardiovascular mortality in incident dialysis patients.

Serum t-ucMGP levels were measured with a competitive enzyme-linked immunosorbent assay in 952 patients from NECOSAD, a large prospective multicenter Dutch cohort of incident hemodialysis (HD) and peritoneal dialysis (PD) patients. Patients were divided into tertiles according to t-ucMGP levels (high, medium, and low) to investigate whether there was a difference in mortality risk between the tertiles.

Almost all patients had t-ucMGP levels far below the normal range (300–700 nmol/L), with HD patients having significantly lower levels (mean $\pm$ SD; 154 $\pm$ 59 nmol/L) compared to PD patients (189 $\pm$ 77 nmol/L; $p < 0.001$). In the total study population, t-ucMGP levels inversely correlated with pulse pressure ($r = -0.157$, $p < 0.001$). No significant differences were found between the high, low, and medium t-ucMGP tertiles in overall, cardiovascular, and non-cardiovascular mortality.

Our results indicate that t-ucMGP is less suited for predicting mortality in this patient population, probably due to very low t-ucMGP levels in dialysis patients.

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Introduction

Mortality among dialysis patients is high, with expected life times ranging from one-fifth to half of those in the general population [1]. The presence and extent of vascular calcification are predictors of mortality, independent of traditional atherogenic risk factors in dialysis patients [2]. Clinically, vascular calcification may result in left ventricular hypertrophy and decreased coronary perfusion with increased risk of death [2–4].

The process of vascular calcification is an active, cell-mediated process in which both promoters and inhibitors play a role. An important inhibitor of vascular calcification present in the arterial wall is matrix γ -carboxyglutamate (Gla)-protein (MGP), which is synthesized by vascular smooth muscle cells. MGP can undergo two post-translational modifications: γ -glutamate carboxylation and serine phosphorylation [5–7]. The oxidation of vitamin K-hydroquinone provides the energy for MGP carboxylation, and this results in the synthesis of carboxylated MGP (cMGP) [8]. cMGP is able to bind to calcium-crystals, and inhibit the osteogenic growth factor BMP-2 [9, 10]; both mechanisms underlie the calcification-inhibitory activity of MGP [11–13]. In case of vitamin K deficiency, uncarboxylated MGP (ucMGP) will be secreted, which has been consistently found to accumulate at sites of calcification in the arterial wall [14–16].

Several enzyme-linked immunosorbent assays (ELISA) have been developed to measure circulating MGP levels. The first assay which was able to discriminate between cMGP and ucMGP, was the competitive ELISA detecting circulating total ucMGP (t-ucMGP), independent of phosphorylation state [17]. With this assay, low t-ucMGP levels have been found in patients with end-stage renal disease (ESRD) [17–19], which is consistent with the accumulation of ucMGP species in vascular tissue at sites of calcification [8]. In addition, an inverse correlation was found between circulating t-ucMGP levels and both the aortic augmentation index and the extent of coronary artery calcification in dialysis patients [18, 20].

In the present study, we evaluated the potential predictive value of circulating t-ucMGP for overall and cardiovascular (CV) mortality in dialysis patients, which has not been investigated previously. Serum t-ucMGP levels were therefore determined in a large, prospective, multicenter Dutch cohort study of incident hemodialysis (HD) and peritoneal dialysis (PD) patients.

Methods

Patients

The Netherlands Cooperative Study on the Adequacy of Dialysis (NECOSAD) is a large prospective multicenter cohort study, in which ESRD patients were followed from the initiation of dialysis treatment until transplantation or death. All incident ESRD patients in 38 dialysis units in The Netherlands were consecutively invited to participate in the study. Patients had to be 18 years or older, with dialysis as their first renal replacement therapy. The study was approved by all local medical ethics committees and all patients gave informed consent before inclusion. For the present analysis, we included incident HD and PD patients who started on long-term dialysis treatment between 1997 and 2005 and who survived the first 3 months of dialysis. Patients were followed until transplantation, mortality, or the end of the study period (26 May 2009).

Data collection

Data on demographics, primary kidney disease, and comorbidity were collected 0–4 weeks before start of dialysis treatment. Data on residual renal function, biochemistry and dialysis characteristics were collected at 3 months after the start of dialysis.

Primary kidney disease and causes of death were classified according to the codes of the European Renal Association-Dialysis and Transplantation Association (ERA-EDTA) [21]. CV mortality was defined as any death attributed to myocardial ischemia and infarction, cardiac failure, cardiac arrest (cause unknown), cerebrovascular accident, fluid overload, hyper- and hypokalemia, haemorrhage from a ruptured aneurysm or mesenteric infarction. If the cause of death was uncertain, unknown or could not be determined, mortality was classified as cardiovascular. All other causes of death were classified as non-cardiovascular.

Blood pressure measurements were performed at 3 months of dialysis. BP was measured before the start of a dialysis session and at the end of the same session in HD patients. In general, the measurements were performed with oscillometric devices incorporated in the dialysis machines.

Prevalent CV disease (CVD) was classified as the presence or history of ischemic heart disease, peripheral vascular disease, heart failure, and/or cerebrovascular disease. Comorbidity was defined according to the risk criteria of Khan *et al.* [22]. The Kahn index is a combination of age and comorbidity resulting in 3 risk groups: low, medium, and high.

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The nutritional status was scored on the standardized 7-point scale of the Subjective Global Assessment (SGA), which is based on the clinical judgement of the dialysis nurse. Malnourishment was defined as an SGA score of 5 or less.

Residual renal function was expressed as residual glomerular filtration rate (rGFR), calculated as the mean of creatinine and urea clearance adjusted for body surface area (in ml/min/1.73 m²). Dialysis dose, expressed as K_t/V urea per week, was calculated as dialysis urea clearance divided by urea distribution volume (V) according to Watson *et al.* [23]. For HD patients, dialysis urea clearance was calculated by using a second-generation Daugirdas formula [24], and for PD patients peritoneal K_t/V was calculated by using 24-h dialysate collection.

All laboratory analyses were performed in blood samples obtained 3 months after the initiation of dialysis treatment. In HD patients, blood samples were obtained just before the start of HD treatment after a short interval. Plasma calcium, phosphorus, and albumin were measured using standard laboratory techniques in the different centers. Calcium concentration (mmol/l) was corrected for albumin concentration. Measurements of intact parathyroid hormone (iPTH) were performed by various first-generation immunometric iPTH-assays depending on the different participating centers. Measurements of high-sensitivity C-reactive protein (hs-CRP) were performed by means of particle-enhanced immunonephelometry using a standard CardioPhase hsCRP for BNII (Dade Behring Holding GmbH, Liederbach, Germany). Serum t-ucMGP levels were measured with a competitive ELISA, using a monoclonal antibody directed against the uncarboxylated MGP sequence 35–49, as described previously [17]. The intra-assay and inter-assay coefficients of variation were 8.9% and 11.4%, respectively.

Statistical analysis

Continuous variables are presented as mean $\pm$ standard deviation (SD) or as median with interquartile range (IQR). Patients were divided into tertiles according to t-ucMGP concentrations: low (≤ 129 nmol/l), medium (130–188 nmol/l), and high (> 189 nmol/l). Differences in clinical and biochemical characteristics between the patients in the t-ucMGP tertiles were analyzed by one-way ANOVA or by Kruskal-Wallis tests (for normally and non-normally distributed continuous variables, respectively) and χ^2 tests (for categorical variables). Differences between HD and PD patients were analyzed by independent T-tests and Mann Whitney tests. Bivariate correlation analysis was performed to study the associations between t-ucMGP and clinical and biochemical variables. Multivariate regression analysis was used to adjust for potential confounders, which were age, rGFR, K_t/V, hsCRP, calcium, and phosphorus levels.

Cumulative mortality curves were calculated using Kaplan-Meier analysis for all-cause mortality. This method is known to profoundly overestimate the cumulative mortality when analyzing competing endpoints [25]. Separate analysis of CV mortality and non-CV mortality is a clear example of competing endpoints. For that reason, we calculated the cumulative mortality curves for CV mortality and non-CV mortality using competing risk analysis, taking account that patients dying for CV causes are no longer at risk to die for non-CV causes, and vice versa [26]. The survival time of patients was censored at the time of transplantation, withdrawal from the study, or at the end of the study period (May 2009) with a maximum follow-up of five years. We calculated hazard ratios (HRs) for the t-ucMGP tertiles by Cox proportional hazard analysis, using the high t-ucMGP tertile as reference category. The multivariate models contained the variables age, sex, primary kidney disease, CVD, diabetes mellitus (DM), smoking, calcium, phosphorus, CRP, rGFR, nutritional status (SGA), and BMI.

A p value of <0.05 was considered to be statistically significant; reported p values are based on two-tailed tests of statistical significance. All statistical analyses were conducted using SPSS version 12.0 for Windows (SPSS Corp, Chicago, IL, USA).

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Results

Serum samples for t-ucMGP measurements were available for 952 patients from a total of 996 patients included in the NECOSAD cohort. Three patients were excluded from the analysis because of missing data regarding dialysis modality and survival. The mean t-ucMGP level of the total patient population (n=949) was 165 ± 67 nmol/L, which is far below the normal range in healthy subjects (300–700 nmol/L; Figure 1). The baseline characteristics of the patient population, 3 months after the start of dialysis treatment, are presented in Table 1 according to t-ucMGP tertiles (low, medium, high). Patients in the low t-ucMGP tertile were significantly older, had a higher prevalence of renal vascular disease as the primary cause of renal disease, moderate co-morbidities, and malnourishment (Table 1). There was no difference in the prevalence of CVD and DM among the t-ucMGP tertiles. The use of vitamin K-antagonists (coumarin derivatives) did not differ between the tertiles.

Figure 1: t-ucMGP values for hemodialysis (HD; n=641), peritoneal dialysis (PD; n=308) patients, and a healthy reference population (RP; n=165)

Circulating t-ucMGP levels in HD and PD patients and a previously described reference population [17] consisting of apparently healthy subjects aged between 20 and 80 years. Ninety-six percent of the dialysis patients had t-ucMGP levels below the normal range (300–700 nmol/L). PD patients had significantly higher t-ucMGP levels compared to hemodialysis patients (** $p < 0.001$).

Table 1: Baseline characteristics of the study population stratified according to t-ucMGP tertiles

Tertile t-ucMGP (nmol/L)	High ≥189	Medium 130-188	Low ≤129	P-value
N	315	309	325	
Demographic and clinical characteristics				
Age (years)	57±16 ^a	59±14 ^a	62±15 ^b	<0.001
Sex (% men)	63	60	56	0.193
Dialysis modality (% HD)	55	70	77	<0.001
Primary kidney disease (%)				<0.001
Diabetes	16	15	15	
Glomerulonephritis	15	16	8	
Renal vascular disease	13	16	28	
Other	57	54	50	
Co-morbidity (%)				0.025
Low	44	36	32	
Moderate	32	36	42	
High	24	28	26	
Diabetes (%)	22	24	22	0.855
Cardiovascular disease (%)	36	35	37	0.882
Current smoking (%)	25	26	19	0.172
Biochemical characteristics				
rGFR (ml/min)*	4.1 (2.0-6.4) ^a	2.9 (1.1-4.9) ^b	3.2 (1.6-5.0) ^b	<0.001
Nutritional status (% malnourished)	21	29	33	0.002
BMI (kg/m ²)	25±4	25±5	24±4	0.081
hsCRP (mg/L)*	4.3 (1.7-10.2)	4.2 (1.7-13.3)	5.5 (1.7-15.5)	0.198
Albumin (g/L)	36±4.8	36±5.5	36±5.2	0.447
Hemoglobin (nmol/L)	7.1±0.9 ^a	6.8±1.0 ^b	6.8±1.1 ^b	<0.001
Corrected calcium (mmol/L)	2.5±0.2 ^a	2.4±0.3 ^{ab}	2.4±0.3 ^{bc}	0.003
Phosphorus (mmol/L)	1.8±0.5 ^a	1.9±0.5 ^{bc}	1.9±0.6 ^{ab}	0.039
iPTH (pmol/L)*	11.9 (4.2-28.0)	12.9 (5.5-29.3)	12.3 (4.2-29.0)	0.625
Pulse pressure (mmHg)	61±17 ^a	63±17 ^a	66±16 ^b	<0.001
Use of coumarin derivatives (%)	12	14	14	0.762

Demographic, clinical and biochemical characteristics of patients stratified according to t-ucMGP tertiles: high (≥189 nmol/L), medium (130–188 nmol/L), and low (≤129 nmol/L). Data are presented as number and percentage of the t-ucMGP tertile, mean ± standard deviation, or as median and IQR range (indicated with *). Abbreviations used: rGFR, residual glomerular filtration rate; BMI, body mass index; hsCRP, high-sensitivity C-reactive protein; iPTH, intact parathyroid hormone.

t-ucMGP and dialysis modality

The percentage of patients who underwent HD treatment was highest in the low t-ucMGP tertile (Table 1). Patients undergoing PD treatment had significantly higher t-ucMGP levels (189±77 nmol/L) compared to HD patients (154±59 nmol/L, $p<0.001$; Figure 1). The difference in t-ucMGP levels persisted after correction for age, sex, primary kidney disease, CVD, DM, rGFR, hs-CRP, calcium, phosphorus, and use of coumarin derivatives in multivariate linear regression analysis ($p=0.001$).

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t-ucMGP correlations with other relevant parameters

Circulating t-ucMGP levels significantly correlated with rGFR ($r=0.136$, $p<0.001$), K_t/V ($r=-0.073$, $p=0.029$), hsCRP ($r=-0.068$, $p=0.035$), calcium ($r=0.105$, $p=0.001$), and phosphorus levels ($r=-0.700$, $p=0.032$). There was no significant correlation between t-ucMGP and iPTH ($p=0.683$). Circulating t-ucMGP levels negatively correlated with pulse pressure ($r=-0.157$, $p<0.001$), which persisted after correction for age, rGFR, K_t/V , hsCRP, calcium, and phosphorus levels in multivariate regression analysis (B for t-ucMGP= -0.088 ; $p=0.010$).

t-ucMGP and survival

Overall, five-year patient survival was 61.3%. Median follow-up time was 862 days (IQR 410–1498). Censoring was observed in 488 patients. Of the total mortality (367 patients), approximately half (48.8%) was attributed to CV death and half (51.2%) to non-CV death. Common causes of non-CV death were infections ($n=41$, 11.2%), refusal of further treatment ($n=40$, 10.9%), cessation of therapy for any other reason ($n=31$, 8.5%), and malignant diseases ($n=29$, 7.9%). After 5 years, 278 HD patients (43.4%) had died and 89 PD patients (28.9%).

Figure 2 shows the crude survival curves for overall, CV and non-CV mortality of the total study population. Table 2 shows the adjusted hazard ratios (HR) for overall, CV and non-CV death for the low and medium t-ucMGP tertiles compared with the high t-ucMGP tertile (reference group) for HD and PD patients. PD patients in the low and medium t-ucMGP tertiles had significantly increased risks of non-cardiovascular mortality of 2.12 (95% CI 1.01–4.44) and 2.32 (95% CI 1.10–4.91), respectively. The non-CV mortality risk remained high for PD patients in the medium and low ucMGP tertiles when adjusting for age, sex, primary kidney disease, CVD, DM, smoking, calcium, phosphorus, CRP, rGFR, malnourishment, and BMI (2.60 [95% CI 1.02–6.16] and 2.50 [95% CI 1.02–6.16], respectively). For HD patients, neither the low nor the medium t-ucMGP tertile had an increased mortality risk in the unadjusted or adjusted Cox-regression analysis (Table 2). In the total study population, there were no significant differences between the t-ucMGP tertiles (data not shown).

Figure 2: Mortality curves.

Mortality curves showing all-cause (A), cardiovascular (B), and non-cardiovascular (C) mortality by tertile of t-ucMGP level (high ≥ 189 nmol/L, medium 130–188 nmol/L, and low ≤ 129 nmol/L) for the total study population. The latter two curves are based on competing-risks analysis.

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Table 2: Cox-regression analysis for the association of t-ucMGP tertiles with overall, CV and non-CV mortality in hemodialysis (A) and peritoneal dialysis (B) patients

A. HD patients		Overall mortality		Cardiovascular mortality		Non-cardiovascular mortality	
N		278		135		143	
t-ucMGP tertile		HR	95% CI	HR	95% CI	HR	95% CI
<u>Model 1</u>							
Low		1.06	0.80-1.42	1.09	0.71-1.67	1.03	0.70-1.53
Medium		0.89	0.65-1.21	1.04	0.67-1.62	0.76	0.49-1.17
High		1.0		1.0		1.0	
<u>Model 2</u>							
Low		1.05	0.78-1.41	1.11	0.72-1.70	1.00	0.67-1.50
Medium		0.87	0.64-1.19	1.05	0.67-1.63	0.72	0.46-1.12
High		1.0		1.0		1.0	
<u>Model 3</u>							
Low		0.96	0.68-1.36	1.01	0.61-1.68	0.93	0.57-1.53
Medium		0.79	0.54-1.15	0.83	0.49-1.40	0.76	0.45-1.31
High		1.0		1.0		1.0	

B. PD patients		Overall mortality		Cardiovascular mortality		Non-cardiovascular mortality	
N		89		44		45	
t-ucMGP tertile		HR	95% CI	HR	95% CI	HR	95% CI
<u>Model 1</u>							
Low		1.35	0.79-2.30	0.74	0.32-1.68	2.32	1.10-4.91
Medium		1.64	1.01-2.67	1.34	0.69-2.57	2.12	1.01-4.44
High		1.0		1.0		1.0	
<u>Model 2</u>							
Low		1.30	0.75-2.24	0.68	0.29-1.59	2.29	
Medium		1.72	1.04-2.84	1.52	0.77-2.99	2.05	
High		1.0		1.0		1.0	
<u>Model 3</u>							
Low		1.31	0.72-2.39	0.75	0.30-1.87	2.50	1.02-6.16
Medium		1.30	0.75-2.26	0.82	0.38-1.79	2.60	1.09-6.21
High		1.0		1.0		1.0	

Model 1, crude.

Model 2, age, sex, primary kidney disease, CVD, DM, smoking

Model 3, age, sex, primary kidney disease, CVD, DM, smoking, calcium, phosphorus, CRP, rGFR, malnourishment, BMI

Results of Cox-regression analysis showing the hazard ratio of the low and medium t-ucMGP tertile compared with the high t-ucMGP tertile for overall, CV and non-CV mortality for hemodialysis (A) and peritoneal dialysis (B) patients. Abbreviations used: CV, cardiovascular; HR, hazard ratio; CI, confidence interval.

Discussion

We determined serum t-ucMGP levels in a prospective, well-defined Dutch cohort of 949 incident dialysis patients to investigate the potential predictive value of circulating t-ucMGP for overall and cardiovascular mortality. Almost all patients (>96%) undergoing dialysis treatment for 3 months had circulating t-ucMGP levels far below the normal range. PD patients had significantly higher t-ucMGP levels compared to HD patients, but these levels were still very low when compared to healthy subjects (normal range 300–700 nmol/L). When patients were divided into tertiles according to t-ucMGP levels, we found no difference between high, medium, and low circulating t-ucMGP levels for mortality risk.

The finding of low t-ucMGP levels in dialysis patients is in accordance with previous reports [17–19]. However, this is the first study that demonstrates in a large cohort that patients who started recently with dialysis treatment (3 months) already have low t-ucMGP levels. This may be explained by the fact that all patients have reached the final stadium of chronic kidney disease. It has been shown that circulating t-ucMGP levels are inversely correlated with GFR in patients with stable CVD [27]. Moreover, in patients with aortic valve disease, those with CKD stage 4 had significantly lower t-ucMGP levels compared to patients with normal to moderately impaired renal function (CKD stage 1–3) [28]. It is therefore likely that circulating t-ucMGP levels are already lowered in the previous stages of CKD, and when patients have developed ESRD, apparently the majority of patients has very low t-ucMGP levels.

Since virtually all patients of the NECOSAD cohort had very low t-ucMGP levels, the t-ucMGP tertiles all represent relatively low t-ucMGP levels. This may contribute to the absence of a difference in mortality risk between the tertiles: whether a patient has a low or very low t-ucMGP level does not influence his or her mortality risk. However, the current finding of very low t-ucMGP levels in virtually all patients of the NECOSAD cohort supports the hypothesis that dialysis patients have a changed MGP status, which may contribute to the development of cardiovascular calcification in this patient population.

The present study is the first to investigate circulating t-ucMGP levels in a large group of PD patients and it is obvious that also patients undergoing this particular type of renal replacement therapy had low t-ucMGP levels. On average, HD patients had even lower t-ucMGP levels, but the difference was relatively small. It has been found in the NECOSAD cohort that patients with moderate to severe aortic arch calcifications were more often treated with HD than PD[29]. From the patients without calcification, the majority (55%) underwent PD treatment.

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The higher t-ucMGP levels in PD patients might therefore be explained by less (severe) arterial calcification found in these patients. Residual confounding may be another explanation of the found difference in t-ucMGP levels between PD and HD patients.

Patients in the low t-ucMGP tertile were significantly older, had more renal vascular disease, moderate co-morbidities, and malnourishment. There was no difference in the prevalence of CVD and DM between the t-ucMGP tertiles. It has been previously found, using the same MGP assay, that patients with CVD have significantly lower t-ucMGP levels compared to healthy subjects [17, 27, 28]. However, the t-ucMGP levels of these patients were higher compared to t-ucMGP levels of dialysis patients [17]. The presence of ESRD seemed to result in a more extensive lowering of t-ucMGP levels than the presence of CVD. In the present cohort, in which virtually all patients have very low t-ucMGP levels, the effect of CVD on t-ucMGP levels in the presence of ESRD might not be detected. Circulating t-ucMGP correlated inversely with pulse pressure in the total patient population; this association persisted after correction for age, rGFR, K_t/V , hsCRP, calcium, and phosphate levels. In agreement, t-ucMGP has been reported to correlate inversely with the aortic augmentation index and the coronary artery calcification score in dialysis patients [18, 20]. Altogether, this suggests that t-ucMGP may serve as a biomarker for prevalent arterial calcification.

In the PD population, there was a significant difference between the t-ucMGP tertiles for non-CV death, which remained statistically significant when adjusting for several clinical and biochemical variables in Cox regression analysis. It should be noted, however, that the 95% confidence intervals were broad and that only 45 deaths accounted for this observation. In addition, for some patients the cause of death was uncertain or could not be determined. These deaths were classified as cardiovascular deaths, since the majority of these patients died acutely, pointing to a cardiovascular cause. This might result in an overestimation of the number of CV deaths, and analyses were therefore repeated with these deaths classified as non-CV. The differences between the t-ucMGP tertiles in Cox-regression analysis for non-CV death among PD patients were then no longer significant (data not shown).

One of the limitations of the present study was that the mortality data relied on observations of treating physicians. This could induce some error in the definition of the exact cause of death. The classification of deaths with an uncertain or missing cause as CV mortality did influence our results, as previously described for PD patients. A major limitation was that no sensitive imaging technique such as multislice computed tomography scanning was applied to evaluate the precise amount of arterial calcification in the current population.

The relationship between t-ucMGP, the extent of vascular calcifications, and mortality could therefore not be evaluated in the present study.

In conclusion, this is the first study in which circulating t-ucMGP was determined in a large number of incident HD as well as PD patients. Serum t-ucMGP levels were extremely low in almost all patients, which may explain the fact that mortality risk did not differ between the t-ucMGP tertiles. Low circulating t-ucMGP levels indicate a changed MGP status, which may contribute to the high vascular calcification-risk in dialysis patients. However, further investigation is needed to confirm this.

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CHAPTER 5

Characterisation and potential diagnostic value of circulating Matrix Gla-protein (MGP) species

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Abstract

Matrix γ -carboxyglutamate (Gla)-protein (MGP) is an important local inhibitor of vascular calcification, which can undergo two post-translational modifications: vitamin K-dependent γ -glutamate carboxylation and serine phosphorylation. While carboxylation is thought to have effects upon binding of calcium ions, phosphorylation is supposed to affect the cellular release of MGP. Since both modifications can be exerted incompletely, various MGP species can be detected in the circulation.

MGP levels were measured with two commercially available competitive and two novel sandwich assays in healthy controls, in patients with rheumatic disease (RD), aortic valve disease (AVD), and end-stage renal disease (ESRD), as well as in volunteers after vitamin K supplementation (VKS) and treatment with vitamin K antagonists (VKA). Major differences were found between the MGP assays, including significantly different behaviour with regard to vascular disease and the response to VKA and VKS. The sandwich assay measuring non-phosphorylated, non-carboxylated MGP (dp-ucMGP) was particularly sensitive for these changes and would be suited to assess the vascular vitamin K status.

We conclude that the different assays for particular circulating MGP species allows the assessment of various aspects of the MGP system.

Introduction

Matrix γ -carboxyglutamate (Gla)-protein (MGP) has gained increasing attention as an important local inhibitor of vascular calcification [1]. MGP is a small secretory protein synthesized by vascular smooth muscle cells (VSMC) and chondrocytes [2–4]. It may undergo two post-translational modifications: vitamin K-dependent γ -glutamate carboxylation and serine phosphorylation [5–7]. MGP is thought to exert its function as a calcification inhibitor by binding to crystal nuclei in hydroxyapatite, thereby preventing crystal growth [8, 9]. Additionally, MGP is able to bind and inhibit bone morphogenetic protein-2, an osteogenic growth factor [10–12]. For both mechanisms of action, MGP has to be carboxylated [10, 13]. The exact function of the phosphoserine residues is unclear, but recent findings indicate that they may regulate the secretion of MGP into the extracellular environment [14]. These negatively-charged residues may also contribute to the binding of MGP to calcium, since MGP retained its affinity for hydroxyapatite after thermal decarboxylation and non-carboxylated MGP accumulated at sites of calcification [8, 15–17]. It has been shown in MGP-deficient mice that circulating MGP has little or none calcification-inhibitory activity [18].

MGP may exist as various species according to their state of phosphorylation and/or carboxylation (phosphorylated [pMGP], non-phosphorylated [desphospho, dpMGP], carboxylated [cMGP] or uncarboxylated [ucMGP]). Hence, in the circulation different MGP species may be detected including p-cMGP, p-ucMGP, dp-cMGP, and dp-ucMGP. In the vascular tissue, the affinity of these species for calcium salts and their calcification-inhibitory activity may differ widely. These various circulating MGP species may reflect the degree of calcification (inhibition) in the vascular wall and, since MGP carboxylation is vitamin K-dependent, certain species may also reflect the availability of vitamin K present in the vascular wall. Clinically, an insufficient vascular vitamin K status may result in an increased risk for arterial calcification [19–21]. Vitamin K occurs as vitamin K₁ (phylloquinone) and vitamin K₂ (menaquinones). Menaquinones are classified according to their aliphatic side chain length (MK-n) [22]. It has been demonstrated that MK-7 has a half-life time of several days and possess a higher efficacy in extra-hepatic Gla-protein carboxylation [23].

Unfortunately, there are currently no assays available to measure each circulating MGP species or even the total circulating MGP pool. In the literature, three different assays measuring circulating MGP have been described so far: one radio-immunoassay [24] and two competitive (mono-antibody) enzyme-linked immunosorbent assays (ELISA) [25, 26]. The first assay is not commercially available and was therefore not used in the current study.

The latter two MGP ELISA quantify total dpMGP (t-dpMGP; Biomedica, Vienna, Austria) and total ucMGP (t-ucMGP; VitaK, Maastricht, the Netherlands). With the t-ucMGP assay, uniformly low levels have been measured in cardiovascular disease (CVD) and chronic kidney disease patients [26–29]. In contrast, with the t-dpMGP assay, both low and high MGP levels have been measured in patients with CVD [17, 25, 30]. A direct comparison between these two assays has not yet been performed.

We aimed to develop new, sandwich (dual-antibody) MGP assays which could detect both uncarboxylated and carboxylated MGP species. We have developed two assays detecting circulating dp-ucMGP and dp-cMGP. Due to the absence of phosphoserine residues, we hypothesized that these MGP species may be more easily set free into the circulation, independent of the presence of calcification in the vascular wall, and may therefore mainly be influenced by the availability of vitamin K. Changes in systemic vitamin K status, for instance resulting from the use of vitamin K supplementation (VKS) or vitamin K antagonists (VKA, coumarin derivatives), should then be reflected in circulating MGP measurements. This was tested in the current study.

In recent years, biomarkers assessing either the risk to develop vascular calcification or the extent of prevalent calcification have become of substantial importance. The prognostic value of cardiovascular calcification has already been clearly established in patients with cardiovascular disease, including aortic valve disease (AVD), and in patients with end-stage renal disease (ESRD) [31–33]. Moreover, increasingly more high-risk patient populations are being identified, including patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [34–36]. In the present study, MGP levels were measured in these patient populations in order to compare the two available and the two newly-developed MGP assays and to evaluate their potential utility for disease monitoring and risk assessment. In addition, patients with cartilage calcification/destruction (chondrocalcinosis [CC] and gout) and soft tissue calcification (diffuse idiopathic skeletal hyperostosis [DISH], also known as Forestier's disease) were included to determine whether non-vascular pathologies would influence MGP levels. Finally, to test the suitability of the new dp-ucMGP and dp-cMGP assays for assessing vascular vitamin K status, MGP levels were measured in healthy subjects receiving VKS and VKA.

Materials and methods

Subjects

Seventy-five apparently healthy subjects, aged between 25 and 80 years (y), were recruited from the general Maastricht population to serve as reference population (RP) and to determine the characteristics of the dp-ucMGP and dp-cMGP assays. These subjects had no overt history of cardiovascular, chronic kidney, and/or rheumatic diseases and did not use VKS or VKA. Patient samples were acquired from a total of 146 patients diagnosed with either rheumatic disease (RD n=51), AVD (n=50), or ESRD (n=45) patients.

The RD population was diagnosed with either CC (n=9), gout (n=13), DISH (n=4), RA (n=16), and SLE (n=9) from the division Rheumatology of the University Hospital Maastricht (Maastricht, the Netherlands). RD patients with a history of CVD and/or CKD were excluded. Data were obtained on demographic and clinical characteristics during patient interviews as well as review of patients' files.

Patients with calcific AVD were recruited at the Echocardiography or Cardiology department of the Rheinisch-Westfälische Technische Hochschule (RWTH) University Hospital Aachen, Germany, as described previously [28].

The ESRD patient population consisted of 45 prevalent hemodialysis (HD) patients from three dialysis centers cooperating with the RWTH University Hospital Aachen, as described previously [27]. Etiologies for ESRD were glomerulonephritis/vasculitis (n=16), tubulointerstitial disease (n=3), amyloid nephropathy (n=2), vascular disease/hypertension (n=4), hereditary disease/cystic disease (n=6), diabetic kidney disease (n=4), and miscellaneous (n=10). From all patient populations, patients using VKA were excluded.

To determine the effect of changes in systemic vitamin K status on MGP levels, 2 groups of healthy volunteers were included: 52 subjects receiving VKS and 17 subjects receiving VKA. The VKS group consisted of 24 men and women aged between 20 and 40 y (mean±SD age 28±7 y) receiving 45, 90, 180 or 360 µg menaquinone-7 (MenaQ7, NattoPharma, Oslo, Norway) daily during 12 weeks and of 28 postmenopausal women aged between 55 and 75 s (70±11 y) receiving 45 mg menaquinone-4 (menatetrenone, Eisai Co, Tokyo, Japan) daily for 6 months. The subjects who received MK-7 as well as those receiving VKA were part of a separate study investigating supplementation with vitamin K2 and oral anticoagulant treatment (E. Theuvsen *et al.*, unpublished data). The postmenopausal women of the MK-4 group were part of a clinical trial investigating the effect of MK-4 on bone mineral density and bone strength [37]. For comparison, 11 women (76±14 y) from the same clinical trial receiving placebo were included.

The VKA group consisted of 17 subjects, aged between 20 and 45 y. These subjects received acenocoumarol (Sintrom mitis 1 mg, Novartis Pharma, Arnhem, the Netherlands) in a once-daily dose (mean±SD daily dose 2.8±1.0 mg mg) to achieve a target international normalized ratio (INR) of 2.0. Blood samples obtained at baseline and after 4 weeks, when a stable INR of 2.0 was reached, were used for MGP measurements. Subjects with a history of coagulation disorders, metabolic, gastrointestinal, and/or chronic inflammatory diseases and subjects using corticosteroids, VKS, or VKA were excluded.

The study was approved by the local Medical Ethics Committees and informed consent was obtained from all participants.

Imaging procedure

In 33 AVD patients, echocardiography was performed as described previously [28] and according to the ACC/AHA guidelines [38]. Patients with a transaortic flow velocity of 2.5–3.0 m/s were classified as mild aortic stenosis, those with a velocity of 3.0–4.0 m/s as moderate, and those with a velocity of >4.0 m/s as severe aortic stenosis [38]. Due to the low number of patients with severe aortic stenosis (n=3), the latter 2 groups were considered as one category for analysis (moderate to severe aortic stenosis).

Biochemical measurements

Citrated plasma and serum from all participants was prepared by standard centrifugation and stored at -80 °C until testing. From 4 healthy subjects (age 26±6 y), also EDTA and heparin plasma was prepared to determine the matrix effect on dp-ucMGP and dp-cMGP levels. Venous blood samples from the ESRD patients were taken at the start of HD after a long dialysis interval. Circulating MGP levels were measured with 4 different MGP assays, which are described below and in Table 1. Calcium, phosphate, hemoglobin, alkaline phosphatase, albumin, and C-reactive protein (CRP) were measured in the patients using standard laboratory techniques.

dp-ucMGP measurement

The method for dp-ucMGP measurement was a dual-antibody ELISA, with the capture antibody directed against the non-phosphorylated MGP sequence 3–15 (mAb-dpMGP; VitaK BV, Maastricht, the Netherlands) and the detecting antibody directed against the uncarboxylated MGP sequence 35–49 (mAb-ucMGP; VitaK BV). The same antibodies have been used previously for immunohistochemical staining [17, 39, 40].

The mAb-ucMGP was biotinylated using the EZ-Link Maleimide PEO₂-Biotin kit (Pierce, Etten-Leur, the Netherlands) according to the manufacturer's instructions. The synthetic peptide Acetyl-SHESMESYELNPF-(AADO)-VQERIRERSKPVHELNREAC-amide, designated as dpMGP^{3–15}-(AADO)-ucMGP^{35–54} (Proimmune, Oxford, UK), was used as standard. The first 13 amino acid residues of this peptide were homologous to the non-phosphorylated MGP sequence 3–15 and the last 20 amino acid residues to the uncarboxylated MGP sequence 35–54. AADO indicates the linker 8-amino-3,6-dioxaoctanoic acid.

The mAb-dpMGP was diluted 200-fold in carbonate buffer (33 mmol/l disodiumcarbonate, 66 mmol/l sodiumbicarbonate, pH 9.6) and used for coating of the microtiter plate (100 µl/well). After incubation for 4 hours at room temperature, the remaining binding sites were blocked with 200 µl blocking buffer (2% HNBSA, consisting of HEPES-NaCl buffer [25 mmol/l HEPES, 175 mmol/l NaCl, pH 7.7] and 2% bovine serum albumin [BSA; Sigma, St.Louis, MO, USA]). The plate was incubated overnight at 4 °C and subsequently washed 4 times with 300 µl washing buffer (0.05% Tween-20 in HEPES-NaCl buffer). Next, 90 µl of citrate plasma sample were diluted in 135 µl 0.2% Protifar (Nutricia, Zoetermeer, the Netherlands) and the dpMGP^{3–15}-(AADO)-ucMGP^{35–54} peptide was diluted 83,000 fold in 0.2% Protifar; 75 µl of either solution were transferred to the microtiter plate. Subsequently, 25 µL of the biotinylated mAb-ucMGP diluted 750-fold in 0.2% Protifar were added to the plate and the plate was incubated with shaking (150 rpm) overnight at 4 °C. After 4 washing cycles with washing buffer, the plate was incubated with 100 µl streptavidin-peroxidase (Zymed, Breda, the Netherlands) diluted in HEPES-NaCl buffer and 0.5% BSA during 30 min at room temperature. The plate was stained with 100 µl of 3,3',5,5'-tetramethylbenzidine (KLP, Gennep, the Netherlands) during 6–7 min. The staining process was stopped by adding 50 µl of 1.0 M H₂SO₄, and the plate was read at 450 nm.

dp-cMGP measurement

The method for dp-cMGP measurement was also a dual-antibody ELISA, in which mAb-dpMGP again served as a capture antibody. The detecting antibody was directed against the carboxylated MGP sequence 35–54 (mAb-cMGP; VitaK BV). The mAb-cMGP was biotinylated using the EZ-Link Maleimide PEO₂-Biotin kit (Pierce) according to the manufacturer's instructions. The standard peptide for the dp-cMGP assay, designated as dpMGP³⁻¹⁵-(AADO)-cMGP³⁵⁻⁵⁴ (Prolimmune), was a similar peptide as described for the dp-ucMGP assay, in which glutamate was replaced by γ -carboxyglutamate (Gla).

In brief, the mAb-dpMGP was diluted 200-fold in carbonate buffer and the mAb-cMGP 1500 times in 0.2% Protifar. The dpMGP³⁻¹⁵-(AADO)-cMGP³⁵⁻⁵⁴ synthetic peptide was diluted 83,000 fold in 0.2% in Protifar. From the standard or citrate plasma dilution (30 μ l of citrate plasma in 195 μ l of 0.2% Protifar), 25 μ l were added to the microtiter plate. The method was otherwise the same as described for the dp-ucMGP assay.

t-ucMGP measurement

The method for t-ucMGP measurement was a mono-antibody ELISA, as described previously [26]. The method has been modified, however, by using the new peptide dpMGP³⁻¹⁵-(AADO)-ucMGP³⁵⁻⁵⁴ as standard. Biotinylated synthetic MGP homologous to the uncarboxylated MGP sequence 35–54 (ucMGP³⁵⁻⁵⁴) was used as tracer (Pepscan, Lelystad, the Netherlands). In brief, mAb-ucMGP was coupled to the microtiter plate. Serum sample or standard was supplemented with tracer, transferred to the microtiter plate, and incubated overnight at 4 °C. The t-ucMGP concentration was calculated with the aid of a calibration curve of dpMGP³⁻¹⁵-(AADO)-ucMGP³⁵⁻⁵⁴. The intra-assay and inter-assay coefficients of variation (CV) have been found to be 8.9% and 11.4%, respectively (Table 1).

t-dpMGP measurement

Circulating t-dpMGP levels were measured with the commercially available kit from Biomedica (Vienna, Austria). The kit is based on the competitive (mono-antibody) ELISA principle, with mAb-dpMGP coated on the microtiter plate. Biotinylated synthetic MGP homologous to the non-phosphorylated MGP sequence 3–15 (dpMGP³⁻¹⁵) was used as tracer, which was simultaneously added to the wells with standard, samples or controls. The t-dpMGP concentration was calculated using a calibration curve of synthetic dpMGP³⁻¹⁵. The intra-assay and inter-assay coefficients of variation (CV) have been found to be 10.6% and 9.6%, respectively (Table 1).

Statistical analysis

Data are presented as mean $\pm$ SD, unless indicated otherwise. The RP was divided into 3 age groups: subjects aged 25–40 y, 41–65 y, and 66–80 y. Tukey post-hoc and Kruskal-Wallis tests were used to test for differences between age-groups and patient populations. Student's *t* test for independent samples, Mann-Whitney U tests, the χ -square test for homogeneity of proportions, and Fisher's exact test were used to test for differences between the patient populations and the concomitant healthy age groups. Student's *t* test for paired samples and Wilcoxon's sign-rank test were used to examine within-group differences. Bivariate correlation analysis was performed using the Pearson and Spearman correlation coefficient. A *p* value of ≤ 0.05 was considered to be statistically significant; reported *p* values are based on two-tailed tests of statistical significance. All statistical analyses were conducted using SPSS version 12.0 for Windows (SPSS Corp, Chicago, IL, USA).

Table 1: Characteristics of the MGP assays

Assay	dp-ucMGP	dp-cMGP	t-ucMGP	t-dpMGP
	This paper	This paper	Cranenburg <i>et al</i> , J Vasc Res 2008	Schurgers <i>et al</i> , Clin Chim Acta 2005
Reference values				
Healthy population 25-80 y (mean±SD; this paper)	447±188 pM	1763±478 pM	4704±1053 nM	14±3 nM
Standard range (according to manufacturer)	-	-	-	0-90 nM
Materials and methods				
Assay type	dual-antibody ELISA	dual-antibody ELISA	mono-antibody ELISA	mono-antibody ELISA
Antibodies	capture antibody mAb-dpMGP → non-phosphorylated MGP residues 3-15 Detecting antibody: biotinylated mAb-ucMGP → uncarboxylated MGP Gla-domain residues 35-49	capture antibody: mAb-dpMGP → non-phosphorylated MGP residues 3-15 Detecting antibody: biotinylated mAb-cMGP → carboxylated MGP Gla-domain residues 35-54	mAb-ucMGP → uncarboxylated MGP Gla-domain residues 35-49	mAb-dpMGP → non phosphorylated MGP residues 3-15
Tracer	-	-	biotinylated ucMGP ³⁵⁻⁵⁴	biotinylated MGP ³⁻¹⁵
Standard	synthetic dpMGP ³⁻¹⁵ -(AADO)- ucMGP ³⁵⁻⁵⁴	synthetic dpMGP ³⁻¹⁵ -(AADO)- cMGP ³⁵⁻⁵⁴	synthetic dpMGP ³⁻¹⁵ -(AADO)- ucMGP ³⁵⁻⁵⁴	synthetic MGP ³⁻¹⁵
Assay characteristics				
Intra-assay variation	5.6 %	3.7 %	8.9 %	10.6 %
Inter-assay variation	9.9 %	11.5 %	11.4 %	9.6 %
Lower detection limit	21 pM	60 pM	98 nM	0.14 nM
Within-day variation	5.5 %	7.4 %	10.8 %	-(no circadian rhythm)
Day-to-day variation	8.4 %	13.1%	11.3 %	-(no circadian rhythm)
Sample preparation				
Matrix effect	dp-ucMGP only measurable in plasma	dp-cMGP only measurable in plasma	Good correlation of serum and citrate plasma levels ($r=0.897$; $p<0.001$)	Concentration in citrate plasma (18.7±4.3 nM) high compared to serum concentration (8.2±1.7 nM)
Freeze-thawing	Up to 8 cycles stable (11% loss)	Up to 8 cycles stable (9% loss)	Up to 10 cycles stable	Up to 10 cycles stable (17% loss)
Storage at room temperature	Decrease of 3% after 3 hours; 20% after 6 hours	Decrease of 11% after 3 hours; 21% after 6 hours	-	Decrease of 20% temperature after 8 hours
Recommended				
Type of sample	Citrate or EDTA plasma	Citrate or EDTA plasma	Serum or plasma	Serum or plasma *
Centrifugation	1,520 <i>g</i>	1,520 <i>g</i>	1,520 <i>g</i>	-
Long-term storage	-80 °C (-112 ° F)	-80 °C (-112 ° F)	-80 °C (-112 ° F)	-70 °C (-94 F) *

Overview of the dual-antibody assays measuring desphosphorylated-uncarboxylated (dp-uc) MGP and desphosphorylated-carboxylated (dp-c) MGP, and the mono-antibody assays measuring total uncarboxylated (t-uc) MGP and total desphosphorylated (t-dp) MGP. In the section Antibodies, it is indicated by an arrow (→) against which MGP residues the described antibodies were raised. The recommended method of sample preparation by the manufacturer is indicated by an asterisk (*). Abbreviations used: ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody.

Results

Assay characteristics

The characteristics of the MGP assays are summarized in Table 1. The mean MGP levels of the total RP are given as reference values. We investigated the recovery after dilution for the newly-developed dp-ucMGP and dp-cMGP assays, by serial dilution of plasma from healthy subjects. For the dp-ucMGP assay, the optimal dilution of plasma was between 20 and 45% and for the dp-cMGP assay between 5 and 12%. Spiking recovery was determined in a pooled citrate plasma sample from healthy subjects. The sample was spiked with synthetic ucMGP³⁵⁻⁵⁴ and dp-ucMGP levels were measured; the recovery was 80%. The same was done for the dp-cMGP assay using the synthetic cMGP³⁵⁻⁵⁴ peptide; the recovery was 99%. No significant differences in dp-ucMGP and dp-cMGP concentrations were found when comparing citrate, EDTA and heparin plasma, although slightly lower levels were measured in heparin plasma (data not shown). Circulating dp-ucMGP and dp-cMGP levels were not measurable in serum.

Circulating MGP levels

Circulating MGP levels were measured in 75 healthy subjects of the RP and 146 patients, of whom the baseline characteristics are depicted in Tables 2A and 2B, respectively. In the RP, there was a significant difference in the prevalence of hypertension and hypercholesterolemia between the 3 age groups. Among the patients, the AVD population was significantly older compared to the RD and ESRD patients. Cardiovascular risk factors (hypertension, hypercholesterolemia, and smoking) were highly prevalent in the AVD and ESRD patients.

Table 2: Baseline characteristics of the healthy population (A) and the patient populations (B)

A Healthy reference population	Total population	Age-group			p
		25-40 years	41-65 years	66-80 years	
N	75	25	25	25	
Demographic characteristics					
Gender (m/f)	31/44	13/12 ^a	5/20 ^b	13/12 ^a	0.030
Age (years)	53 ± 17	31 ± 4 ^a	58 ± 6 ^b	70 ± 3 ^c	<0.001
BMI (kg/m ²)	26 ± 3	24 ± 3 ^a	27 ± 3 ^b	26 ± 2 ^b	<0.001
Clinical characteristics					
Hypertension (number [%])	15 (20)	1 (4) ^a	5 (20) ^b	9 (36) ^b	0.005
Hypercholesterolemia (number [%])	7 (9)	0 (0) ^a	2 (8) ^{ab}	5 (20) ^b	0.050
Current smoking (number [%])	7 (9)	1 (4)	5 (20)	1 (4)	ns
DM (number [%])	0 (0)	0 (0)	0 (0)	0 (0)	ns
CVD (number [%])	0 (0)	0 (0)	0 (0)	0 (0)	ns

B Patient population	RD	AVD	ESRD	p
N	51	50	45	
Demographic characteristics				
Gender (m/f)	27/24 ^a	9/11 ^b	20/25 ^a	0.002
Age (years)	56 ± 12 ^a	70 ± 10 ^b	59 ± 16 ^a	<0.001
BMI (kg/m ²)	27 ± 6 ^a	28 ± 4 ^b	26 ± 6 ^a	0.005
Clinical characteristics				
Hypertension (number [%])	17 (32) ^a	43 (86) ^b	40 (89) ^b	<0.001
Hypercholesterolemia (number [%])	9 (18) ^a	36 (72) ^b	Unknown	<0.001
Current smoking (number [%])	8 (16) ^a	25 (50) ^b	5 (11) ^a	<0.001
DM (number [%])	0 (0) ^a	10 (20) ^b	6 (13) ^b	0.005
CVD (number [%])	0 (0) ^a	50 (100) ^b	17 (38) ^c	<0.001

Demographic and clinical characteristics of the healthy population according to age groups (25–40, 41–65, and 66–80 years; A) and of the rheumatic disease (RD), aortic valve disease (AVD), and end-stage renal disease (ESRD) patients (B). Data are presented either as mean±SD or as numbers, with percentage of total between parentheses. P values for overall comparisons between healthy age-groups (A) and patient populations (B) are given as well as significant differences between 2 groups or populations, which are depicted by superscripts a, b, c, (p<0.05). Abbreviations used: BMI, body mass index; DM, diabetes mellitus (use of insulin or oral antidiabetic agents); CVD, cardiovascular disease (a history of coronary artery disease [myocardial infarction, angina pectoris or evidence of obstructive disease by angiography], cerebrovascular disease [thrombotic stroke or transient ischemic attack], calcific aortic valve disease, or peripheral artery disease [a history of claudication or lower extremity revascularization]).

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In Figure 1, the circulating concentrations of the various MGP species in the RP (according to age group) and in the patient populations are depicted. MGP levels in the RD and ESRD patients were compared with those in the concomitant healthy age group 41–65 y, and MGP levels in the AVD patients with those in the age group 66–80 y. In the RP, there was no difference in MGP levels between men and women. The dp-ucMGP and dp-cMGP levels significantly correlated with age in the RP ($r=0.263$, $p=0.022$ and $r=0.714$, $p<0.001$, respectively).

Figure 1: Circulating MGP levels in the reference and patient populations

Circulating dp-ucMGP (A), dp-cMGP (B), t-ucMGP (C), and t-dpMGP (D) levels in the reference population (RP, $n=75$) according to age-group (25–40, 41–65, 66–80 years) and in the rheumatic disease (RD, $n=51$), aortic valve disease (AVD, $n=50$), and end-stage renal disease (ESRD, $n=45$) patient populations. Median MGP levels are depicted as horizontal bars and mean $\pm$ SD MGP levels are given for each age group and patient population.

*: $p<0.05$, **: $p<0.001$ ns: non significant.

The **dp-ucMGP** levels (Figure 1A) in the RP were highest in the oldest age group. In all patient populations, dp-ucMGP levels were significantly higher than in the healthy group of comparable age. Particularly in the AVD and ESRD patients, very high dp-ucMGP levels were measured; mean dp-ucMGP levels were 2.4 and 5.0 times higher than in the corresponding age group, respectively. The **dp-cMGP** levels (Figure 1B) significantly increased with age in the RP. The dp-cMGP levels in AVD and ESRD patients were 1.4 and 2.6 times as high as those in the corresponding age-group, respectively. In the RD patients, dp-cMGP levels were not significantly different compared to the healthy age group 41–65 y. The **t-ucMGP** levels (Figure 1C) were independent of age in the RP. All patient populations had significantly lower ucMGP levels. The **t-dpMGP** levels (Figure 1D) were significantly higher in AVD patients compared to the healthy age-group 66–80 y. Otherwise, there were no significant differences.

MGP and clinical characteristics in patients

Among the total patient population (n=146), there was no significant correlation between circulating MGP and age. There was no difference between men and women, smoking and non-smoking patients, and patients with and without DM (data not shown).

Figure 2 shows the distribution of the MGP levels in the RD groups (CC, gout, DISH, and SLE) and in healthy subjects of the same age (41–65 y). Patients with gout had significantly higher **dp-ucMGP** levels (Figure 2A). Some individual RA and SLE patients had high dp-ucMGP levels (>700 pmol/l), but on a group level this was not significant. The **dp-cMGP** levels (Figure 2B) in the RD groups did not differ significantly from the healthy subjects, although again some RA patients had high dp-cMGP levels (>3000 pmol/l). Patients with CC had slightly lower **t-ucMGP** levels (Figure 2C) compared with the healthy subjects. The **t-dpMGP** levels (Figure 2D) in the RD groups were comparable with those of the healthy subjects. Unfortunately, only 4 DISH patients could be included, for which reliable statistical analysis could not be performed. Figure 2 shows that in general the MGP levels of these patients were comparable with those of healthy subjects. The range of dp-ucMGP levels in the DISH patients was relatively large (120–708 pmol/l).

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Figure 2: Circulating MGP levels in the rheumatic disease (RD) patient population

Circulating dp-ucMGP (A), dp-cMGP (B), t-ucMGP (C), and t-dpMGP (D) levels in RD patients with chondrocalcinosis (CC, n=9), gout (n=13), diffuse idiopathic skeletal hyperostosis (DISH, n=4), rheumatoid arthritis (RA, n=16), and systemic lupus erythematosus (SLE, n=9). For reference, MGP values in healthy subjects of comparable age (RP 41–65 years, n=25) are depicted. Median MGP levels are depicted as horizontal bars and mean±SD MGP levels are given for each group. *: p<0.05, **: p<0.001.

In 33 patients of the AVD population, there were no significant differences in MGP levels between those with aortic valve sclerosis (n=9), mild (n=6), and moderate to severe (n=18) aortic stenosis (data not shown). Circulating dp-ucMGP and dp-cMGP positively correlated with age ($r=0.401$, $p=0.004$ and $r=0.403$, $p=0.004$, respectively) in the AVD population. This was not seen in ESRD patients. There was also no correlation between circulating MGP and dialysis vintage in these patients. Circulating dp-ucMGP positively correlated with the calcium-phosphate product in ESRD patients ($r=0.473$, $p=0.006$).

Vitamin K status

In the both VKS groups (MK-7 and MK-4 supplementation), **dp-ucMGP** levels (Figure 3A) were significantly lower after supplementation. Remarkably, also the **dp-cMGP** levels (Figure 3B) were slightly lower after MK7 supplementation. The decrease in dp-cMGP levels was even more pronounced in the subjects receiving MK-4, which might be due to the fact that MK-4 was given in a much higher dose and 3 months longer than MK-7. Circulating **t-ucMGP** levels (Figure 3C) remained the same in all groups, indicating that these are not influenced by changes in systemic vitamin K status. Circulating **t-dpMGP** levels (Figure 3D) were significantly increased in the MK-7 group, but remained unchanged in the MK-4 group. In the placebo-group, all MGP levels were unchanged after 6 months with exception of the t-dpMGP levels, which were slightly increased.

The MGP measurements in the healthy subjects receiving VKA for 4 weeks are also depicted in Figure 3. The **dp-ucMGP** levels (Figure 3A) increased in all 17 subjects, and were 1.6 to 5.3 times higher after VKA. The **dp-cMGP** (Figure 3B) and **t-ucMGP** (Figure 3C) levels remained unchanged. There was a significant increase in **t-dpMGP** levels after 4 weeks (Figure 3D).

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Figure 3: Circulating MGP levels before and after vitamin K supplementation (VKS) and vitamin K antagonists (VKA) treatment

Circulating dp-ucMGP (A), dp-cMGP (B), t-ucMGP (C), and t-dpMGP (D) levels in healthy subjects before and after supplementation with menaquinone-7 for 3 months (MK-7, n=24), with menaquinone-4 (MK-4, n=28) or placebo (n=11) for 6 months, and after treatment with VKA for 4 weeks (n=17). Median MGP levels are depicted as horizontal bars and mean±SD MGP levels are given for each group. *: p<0.05, **: p<0.001.

Discussion

Measuring MGP levels in the circulation may be valuable in two different settings: assessing the amount of vascular damage and the vascular vitamin K status. In this paper, we report on the comparison of four different MGP assays: the available mono-antibody t-ucMGP and t-dpMGP assays, and the novel dual-antibody dp-ucMGP and dp-cMGP assays. Each assay detects a different fraction of the total circulating MGP pool, which explains the widely different results when used in patient populations (Table 3). This is illustrated by the finding of both high (dp-ucMGP and dp-cMGP) and low (t-ucMGP) MGP levels in patients with AVD and ESRD compared to healthy subjects of the same age. Another major difference between the assays was the response to changes in systemic vitamin K status, for which both the dp-ucMGP and dp-cMGP assays were sensitive. Particularly the dp-ucMGP levels showed a fast response to both VKS (both MK-7 and MK4 supplementation) and VKA (4 week-treatment with acenocoumarol). Circulating dp-ucMGP is clearly a marker capable of detecting changes in vitamin K status.

The measurement of circulating dp-ucMGP confirms the previous reported finding of uncarboxylated, inactive MGP species in the circulation of healthy subjects [26]. This has also been reported for osteocalcin, a vitamin K-dependent protein synthesized in bone, of which uncarboxylated levels are also present in the circulation of healthy subjects. Measurements of circulating uncarboxylated and carboxylated osteocalcin are commonly used as a marker for vitamin K status of bone [41, 42]. The dp-ucMGP levels decreased to almost immeasurably low levels after 6 months of VKS, indicating that the even in healthy subjects the vascular vitamin K status is insufficient for maximal MGP carboxylation. It is currently not known why also dp-cMGP levels decreased significantly after 6 months of MK-4 supplementation. The use of a ratio of uncarboxylated and carboxylated MGP may not be useful to assess vascular vitamin K status, since the ratio will not change upon long-term VKS as both dp-ucMGP and dp-cMGP levels decrease.

Vascular vitamin K deficiency may result in an increased risk for arterial calcification. Indeed, a low dietary intake of menaquinones was associated with increased cardiovascular mortality and arterial calcification in several population studies [20, 21, 43]. In animal models it has been a consistent finding that treatment with VKA (coumarin derivatives) results in extensive arterial calcification [8, 39]. Moreover, patients treated with coumarin were reported to have significantly more coronary artery and valve calcifications compared to those not treated with anticoagulation [44–46]. The newly-developed dp-ucMGP assay enables investigation of the potential correlation between vascular vitamin K status and (cardiovascular) risk, disease, or even mortality.

In the present study, the AVD and ESRD patients had very high circulating dp-ucMGP levels, indicating a poor vascular vitamin K status. Preliminary results of two large AVD and ESRD patient cohorts show that high dp-ucMGP levels predict mortality in these patients [47].

The RD patient population was included to assess whether patients with an increased risk for cardiovascular calcification without CVD (RA and SLE), and patients with non-vascular pathologies (CC, DISH, gout), would have different circulating MGP levels. Overall, patients with RD had significantly higher dp-ucMGP levels and significantly lower ucMGP levels. In the individual patient populations, these results were seen in patients with gout and CC, respectively. However, the differences were very small and the influence of cartilage disease on circulating MGP levels seems to be minimal compared to that of CVD. Patients with SLE and RA did not have significantly different circulating MGP levels, indicating that a poor vascular vitamin K status might not contribute to the increased arterial calcification risk in these patients.

The performance of the **dp-ucMGP** and **dp-cMGP** assays, the first dual-antibody MGP assays, was satisfactory with respect to intra- and inter-assay variation, stability upon repeated thawing and freezing cycles, and storage at room temperature during several hours. An important finding was that dp-ucMGP and dp-cMGP levels could not be measured in serum. Incubation of full-length synthetic MGP with thrombin, a circulating serine-protease, resulted in four MGP fragments as analyzed with mass-spectrometry (T. Hackeng *et al*, personal communication). MGP therefore appears to have several cleavage sites for thrombin, and might be degraded by the thrombin generated during serum preparation, resulting in non-measurable serum dp-ucMGP and dp-cMGP levels. More research is needed to confirm this hypothesis.

The method for **t-ucMGP** measurement has been improved by using a new synthetic standard, which is the same as used in the dp-ucMGP assay enabling comparison between the assays. Therefore, the t-ucMGP levels were found to be higher as compared to levels previously measured [26], but the differences in circulating t-ucMGP levels between healthy subjects, AVD, and ESRD patients have remained the same. Previously, we have demonstrated that circulating t-ucMGP is inversely correlated with the extent of coronary artery calcification in HDpatients [27]. Circulating t-ucMGP may be an important biomarker for prevalent vascular calcification in high-risk subjects. It can be measured both in serum and plasma [26] and it has the additional advantage of being virtually uninfluenced by systemic vitamin K status, as demonstrated in the present study.

The results of MGP measurements with the now commercially available **t-dpMGP** assay showed that there was a large overlap between t-dpMGP levels of healthy subjects and patients. In the past, low as well as high t-dpMGP levels have been reported in patients with atherosclerotic disease [25, 30]. Circulating t-dpMGP levels increased significantly after MK-7 supplementation, after coumarin treatment and also in the placebo group. The inconsistency of t-dpMGP results, together with the large overlap between patient and reference values, indicates that this assay is not capable of identifying patient populations and would not be suited for individual patient diagnosis.

The present study was primarily designed to compare the available MGP assays and to characterize the newly-developed MGP assays. It is therefore less suited to assess full clinical value of the individual MGP assays, which will require much further study. Another limitation was the inability to measure other circulating MGP species, including circulating phosphorylated MGP levels, which could potentially clarify the results obtained with the dp-ucMGP and dp-cMGP assay. We are aiming to develop measurements for phosphorylated MGP, but it has proven to be technically difficult to synthesize the relevant MGP sequence 3–15 in its phosphorylated form and to develop monoclonal antibodies against it. Therefore, there is no assay available to measure this fraction of MGP at this moment.

Not only the phosphorylation state of MGP, but also the synthesis and degradation of MGP may contribute to the variations in concentrations of MGP species within the patient populations. Measurements with both mono-antibody assays resulted in MGP levels within the nanomolar range, whereas with both dual-antibody assays levels within the picomolar range were measured. An explanation for the observed differences may be the breakdown of MGP into fragments. Although we have previously shown for the mono-antibody t-ucMGP assay that it is able to detect full-length MGP [26], fragments of MGP could in theory also contribute to measurements of MGP with the mono-antibody assays. It is less likely that the dual-antibody MGP assays detect MGP fragments, since these assays are based on two antibodies directed against residues in the N-terminal region (3–15) and in the central region of MGP (35–54). This may result in higher MGP levels measured with the mono-antibody assays. However, further fundamental research into the processes of MGP synthesis, transport, carboxylation, and phosphorylation is necessary to explain the results of the circulating MGP measurements on a cellular level.

In conclusion, we compared four different assays to measure circulating MGP, including the first dual-antibody assays. Based on our results, each assay may have its own application for research and/or for diagnostic use. From these assays, the dp-ucMGP assay seems to be particularly suited to assess vascular vitamin K status, and may therefore contribute to cardiovascular risk assessment. Further research is necessary to investigate the full diagnostic potential of the different MGP assays.

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CHAPTER 6

Rare mutations in the gene encoding Matrix Gla-protein (MGP): a new case of Keutel syndrome serves as a model for exploring MGP functions in humans

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Abstract

Matrix γ -carboxyglutamate (Gla)-protein (MGP), a vitamin K-dependent protein, is recognised as a potent local inhibitor of vascular calcification. Studying patients with Keutel syndrome (KS), a rare autosomal recessive disorder resulting from *MGP* mutations, provides an opportunity to investigate the functions of MGP.

We describe a novel homozygous *MGP* mutation (c.61+1G>A) in a newly identified KS patient, of whom the phenotype was carefully characterised. No signs of arterial calcification were found, in contrast to findings in *MGP* knockout mice that die within two months due to arterial calcification. This patient is the first in whom circulating MGP species were characterised, which revealed a high level of phosphorylated MGP and a low level of carboxylated MGP. Contrary to expectations, vitamin K supplements did not improve the circulating carboxylated MGP levels. In addition, we characterised MGP species in various affected tissues of the first KS patient originally described in 1971. Phosphorylated MGP was also found to be present in this patient.

The investigation of the phenotype and MGP species in the circulation and tissues of KS patients contributes to our understanding of MGP functions in various tissues and to further elucidation of the difference in arterial phenotype between MGP-deficient mice and humans.

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Introduction

Matrix γ -carboxyglutamate (Gla)-protein (MGP) is increasingly recognised as a potent inhibitor of arterial calcification with important clinical implications. MGP is present in the vascular wall and in cartilage, where it is synthesised by vascular smooth muscle cells and chondrocytes, respectively. It can undergo two post-translational modifications: γ -glutamate carboxylation, which is vitamin K-dependent, and serine phosphorylation. Uncarboxylated MGP is invariably found to accumulate in atherosclerotic lesions and areas of arterial calcification in tissue; high circulating levels are found in patients with a high risk of prevalent cardiovascular calcification [1-3]. The two post-translational modifications result in several MGP species (carboxylated, non-carboxylated, phosphorylated, and non-phosphorylated MGP). Carboxylation, which is vitamin K-dependent, enhances the binding of MGP to calcium nuclei in hydroxyapatite [4, 5] and is a prerequisite for its inhibition of bone morphogenetic protein-2 (BMP-2) [6-8], an osteogenic growth factor. Although the exact function of phosphorylation is unclear, it may regulate MGP secretion into the extracellular environment [9]. Several enzyme-linked immunoassays (ELISAs) have been developed to measure different MGP species in the circulation.

Genetically determined loss of MGP function may provide important insights into the functions of the protein. *MGP* knockout mice were born without abnormalities, but developed severe calcification of all elastic and muscular arteries, eventually resulting in rupture of the calcified aorta and subsequent death within two months [10]. Histological examination of *MGP* knockout mice revealed extensive calcification of the elastic lamellae at the age of two weeks, and the appearance of cartilage nodules in the calcified aorta at the age of one month [11]. They also displayed cartilage calcification at sites of proliferating chondrocytes: the lower end of the trachea, the main bronchi, and the growth plate, which resulted in short stature, osteopenia, and fractures [10].

Abnormal cartilage calcification is the hallmark of Keutel syndrome (KS), an extremely rare autosomal recessive disorder in humans, resulting from mutations in the *MGP* gene [12]. So far, only 26 patients with KS have been described in the literature [12-28]. In addition to abnormal cartilage calcification, KS is characterised by brachytelephalangism, peripheral pulmonary artery stenosis, hearing loss (sensorineural, mixed, and conductive), and facial abnormalities (i.e. mid-face hypoplasia and a depressed nasal bridge) [26]. Four *MGP* mutations have been reported thus far, which all predict absent or non-functional MGP [12, 26]. Remarkably, KS patients do not seem to suffer from extensive arterial calcification.

We describe a novel MGP mutation, found in a newly identified KS patient. His phenotype was carefully characterised, paying specific attention to signs of vascular calcification. In addition, this is the first patient in whom circulating MGP species have been characterised. These measurements were used to evaluate whether he would benefit from vitamin K supplements. In addition to these studies, we investigated the presence of MGP and other calcification-inhibitors in tissues from the first KS patient originally described by Keutel *et al.* [13].

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Materials and Methods

Subjects

A male patient was referred from the Department of Otorhinolaryngology to our clinical genetics outpatient clinic and subsequently diagnosed with Keutel syndrome. This patient and his first-degree relatives (parents and two siblings) were included in our study. All participants gave their informed consent. To assess the effect of increased vitamin K intake on his circulating MGP levels, he was prescribed 10 mg vitamin K₁ (phyloquinone; Konakion, Roche Nederland, Woerden, the Netherlands) daily for 3 months at the age of 21 years. This is the standard form and dosage of vitamin K which is prescribed in the Netherlands.

No tissue from our patient was available for immunohistochemical staining. Fortunately, tissues from another male patient diagnosed with Keutel syndrome were available. This was one of the two siblings originally described by Keutel *et al.* [13]. Meier *et al.* described the follow-up and post-mortem examination of this patient (their case 1) in 2001 [29]. He died at age 38 years due to right heart failure. He had previously suffered from increasing dyspnoea and coughing, seizures with ischemic cerebral lesions, and had been diagnosed with mediastinal seminoma, for which he had received chemotherapy.

Genetic analysis

Genomic DNA was isolated from blood samples (QIAamp DNA Blood Mini Kit) obtained from our newly identified patient with KS and his four first-degree relatives. The four coding exons, as well as exon-intron boundaries, of *MGP* were amplified by polymerase chain reaction (PCR) using the following oligonucleotides: exon1-F (CTCTCAACTGCTCTGGTTC) and exon1-R (AAGTAAGCCAAAGTCAGAGGC), exon2-F (TTTCCTCTCTCCATCCCTG) and exon2-R (CCCTCCCTGTTATATATCTTTC), exon3-F (TATTCACGGAAATATTTCCAGC) and exon3-R (CAGATCTGTGATCTACACTG), exon4-F (GTATTTTCCACTTTATCCTTC) and exon4-R (AAAATCAGGTGCCAGCCTC), and analysed by bidirectional fluorescent direct sequencing. Results were confirmed in a second amplification product.

A skin biopsy of the patient was performed to obtain fibroblasts, of which mRNA was extracted. The effect of the identified *MGP* mutation on mRNA splicing has been elucidated by performing RT-PCR on the obtained mRNA. The entire *MGP* coding sequence was amplified by RT-PCR and analyzed by bidirectional fluorescent direct sequencing.

Biochemical measurements

Blood samples were collected from our patient and, for comparison, from his first-degree relatives. For circulating MGP and osteocalcin measurements, blood was collected by venipuncture in serum (10 ml; BD Vacutainer systems, Plymouth, UK) and in sodium citrate tubes (10 ml; BD Vacutainer systems, Plymouth, UK) and stored for 20 minutes at room temperature before centrifugation (15 min, 1580 g). All data are means of duplicate measurements, unless stated otherwise.

Circulating MGP levels were quantified with four different MGP ELISA. Circulating non-phosphorylated MGP was measured with a kit from Biomedica (Vienna, Austria), in which the monoclonal antibody against the human non-phosphorylated MGP residues 3-15 is coated to the microtitre plate [30]. Circulating phosphorylated MGP was measured with a competitive ELISA, using a monoclonal antibody raised against the phosphorylated MGP residues 3-15 (VitaK BV, Maastricht, the Netherlands). The antibody was selected for its specificity towards phosphorylated MGP using standard techniques, as described for the other MGP antibodies [1]. For the phosphorylated MGP ELISA, the antibody was coupled to the microtitre plate. Serum sample or standard was supplemented with tracer (biotinylated phosphorylated MGP) and transferred to the microtitre plate and incubated overnight at 4°C. MGP concentration was calculated with the aid of a calibration curve of synthetic phosphorylated MGP. Biotinylated, synthetic phosphorylated MGP was made by Thinkpeptides (ProlImmune, Oxford, UK).

Circulating uncarboxylated and carboxylated MGP were quantified with two sandwich ELISA (VitaK BV). In these assays, the antibody against non-phosphorylated MGP residues 3-15 served as a detecting antibody. The biotinylated antibodies against the uncarboxylated MGP residues 35-49 and the carboxylated MGP residues 35-54 served as capture antibodies, respectively [31]. These assays seem to be particularly suited to assess the effect of changes in vitamin K status on MGP levels.

The carboxylation status of circulating osteocalcin (OC), a vitamin K-dependent protein present in bone, was assessed by measurements of uncarboxylated (ucOC) and carboxylated (cOC) osteocalcin using dual-antibody OC ELISAs (Takara, Shiga, Japan). The ratio of ucOC and cOC (ucOC/cOC) was calculated from these measurements. A decrease in ucOC/cOC over time indicates an improved vitamin K status [32].

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Immunohistochemistry

Immunohistochemistry for Keutel's original first male patient was performed on sections of lung (from trachea to bronchi of the third generation), aorta, skin, and heart tissue embedded in paraffin. All sections were stained for hematoxylin and eosin (HE). Von Kossa and Elastica von Gieson staining were performed to visualise calcification and elastic fibres, respectively. Immunostaining for MGP was performed as described previously [1], with the same monoclonal MGP antibodies as used in the MGP ELISA directed against phosphorylated MGP, non-phosphorylated MGP, carboxylated MGP, and non-carboxylated MGP. Sections were also stained with rabbit anti-human fetuin-A (BioVendor, Heidelberg, Germany) and rabbit anti-human osteopontin (Abcam, Cambridge, UK). For MGP, staining was performed using biotinylated sheep anti-mouse IgG (60 minutes at room temperature; Dako, Golstrup, Denmark) as a secondary antibody, followed by incubation with avidin-linked alkaline phosphatase complex (30 minutes at room temperature; Dako). Staining was then performed using the Vector Red Alkaline Phosphatase Substrate kit I (Vector Laboratories, Burlingame, CA, USA), yielding a red colour. For fetuin-A and osteopontin, biotinylated goat anti-rabbit IgG was used as a secondary antibody, followed by incubation with peroxidase-labelled streptavidin (LSAB2 System-HRP, Dako), according to the manufacturers' instructions. Staining was then performed by the Vector NovaRED substrate kit (Vector Laboratories), yielding a brown colour. Sections were counterstained with hematoxylin. Controls for the immunoreactions were performed by omitting the primary antibody.

Results

Phenotypic characterisation

A 16-year old male was referred to the Department of Otorhinolaryngology, University Medical Center Groningen, for evaluation of exertional dyspnoea. His medical history included mild congenital bilateral peripheral pulmonary stenosis and respiratory problems, refractive to the use of bronchodilators. Psychomotor development was normal. He is the second child of consanguineous Turkish parents, living in the Netherlands. He has two sisters and the younger one has a history of childhood epilepsy. All four first-degree relatives were apparently unaffected.

Physical examination of the patient revealed a slight inspiratory stridor on auscultation. There were no obvious dysmorphic facial features (Figure 1). However, his auricles were strikingly stiff, his chest was asymmetric, and his hands showed shortening of all distal phalanges. His height was -1 SD for age (with target height plus 1 SD) according to Turkish growth charts.

Lung function tests revealed obstructive upper airway disease. Laryngotracheoscopy showed a short and posteriorly tilted epiglottis, leading to difficult intubation. The vocal folds were remarkably short with a large posterior chink. The cricoid cartilage was stenosed (Figure 2A), providing the explanation for his exertional dyspnoea. The trachea showed an irregular appearance (Figure 2B). A CT scan revealed extensive cartilage calcification of his larynx, trachea, main bronchi, costochondral junctions, and auricles. The severity of the stenosis of the cricoid cartilage was clearly shown by three-dimensional CT scan reconstructions (Figure 3). Hearing thresholds were 10 dB (normal) for the right ear, with a 20 dB conductive hearing loss for the high tones in the left ear. An electrocardiogram showed no abnormalities. An echocardiogram showed normal dimensions and no functional abnormalities except for a small increase in the flow of the left pulmonary artery. Ophthalmological evaluation did not reveal any abnormalities and he did not have any skin abnormalities. No abnormalities were found with routine blood haematology and chemistry, including calcium, phosphate, liver function tests, and clotting tests. Overall, these features were best compatible with a diagnosis of Keutel syndrome (Table 1) and mutation analysis of the *MGP* gene was performed to confirm this diagnosis.

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Figure 1: Frontal and lateral view of our patient

Frontal (A) and lateral (B) view of patient. Note the calcification present in the patient’s auricle.

Figure 2: Laryngotracheoscopy

B

b

Laryngotracheoscopy images illustrating the stenosed cricoid cartilage (A) and irregular appearance of the trachea of the patient (B), with images of the normal configuration for comparison (a,b). (A) Patient's cricoids with irregular stenosed appearance. No visible tracheal rings due to stenosis. (a) Far left the left true vocal cord can be seen, and in the center the almost circular appearance of cricoids. Several patches of mucus are present. The trachea is visible distally. (B) Patient's irregular narrowed lumen of the trachea with tracheal rings shining through mucosa. (b) Regular smooth tracheal rings. Note: Do not compare picture size, but configuration.

Figure 3: Three-dimensional CT scan reconstructions

A

a

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B

b

Three-dimensional CT scan reconstructions illustrating the severity of the patient's cricoid cartilage stenosis (A), and calcifications of his rib cartilage (B), with images of the normal configuration for comparison (a, b).

(A) CT neck, volume dataset, virtual rendering mode, anterior-posterior view: pronounced ossification of hyoid and extensive calcification of cricoids, thyroid, and tracheal ring cartilage visible in white. Air band of hypopharynx and proximal trachea visible in red. (a) Patient of the same age with small left lateral laryngocele. Normal ossification of hyoid. No cartilage calcifications visible.

(B) CT thorax, volume dataset virtual rendering mode anterior-posterior view: Calcifications of bilateral rib arch cartilage (white) visible medial to the regularly ossified ventral ribs. (b) Patient of the same age (status after sternotomy). No rib cartilage calcifications visible.

Note the extensive calcification of the cricoid, thyroid, hyoid and tracheal rings.

Table 1: Clinical features of Keutel syndrome

Clinical features	Our patient	Literature (n=26)
Age at diagnosis (years [mean±SD])	16	8.3±4.8
Gender	M	11 M, 15 F
Consanguinity	+	19/21
Cartilage calcification		
Nose (septum, ala nasi)	-	8/8
Ear (outer and middle)	+	12/12
Larynx	+	13/13
Trachea and/or bronchi	+	20/20
Ribs	+	9/9
Epiphyseal stippling	-	3/5
Characteristic facial features		
Midface hypoplasia	-	14/14
Broad, depressed nasal bridge	-	21/21
Brachytelephalangism	+	25/25
Respiratory problems	+	14/18
Recurrent otitis media / sinusitis	-	9/13
Hearing loss	right -, left +	12/17
Peripheral pulmonary stenosis	+	14/17
Heart defects ^A	-	3/15
Cerebral defects ^B	-	4/12
Seizures	-	4/6
Developmental delay	-	5/15
Mild short stature (length ≤10 th percentile)	-	10/21

Summary of the clinical features of our patient and the patients with Keutel syndrome described in the literature

^A Reported heart defects are ventricular septal defect (n=1), patent ductus arteriosus and foramen ovale (n=1), and thick mitral valve leaflets (n=1).

^B Reported cerebral defects are multiple calcifications (n=2), leukodystrophy (with bilateral periventricular and subcortical with matter changes on brain MRI after 3 years; n=1), mild atrophy of the cerebral hemisphere (secondary to meningitis; n=1), and encephalomalacia (n=1).

Vascular evaluation was performed when the patient was 21 years old, to investigate whether any arterial calcification was present. He had no complaints consistent with cardiovascular disease. His blood pressure was 122/78 mm Hg, the ankle-brachial index was 108% on the right, 112% on the left side, and after exercise 82% and 79%, respectively (values above 140% may indicate arterial calcification [33]). Carotid intima-media thickness of the right and left common carotid arteries were both 0.45 mm; right and left internal carotid arteries 0.45 mm and 0.40 mm, respectively; bulbous right and left 0.5 mm and 0.4 mm, respectively (ultrasound upper limit of normal range for adolescents [34]). No arterial calcifications were seen on a chest X-ray of the upper abdomen (hepatic, lienal, renal arteries). A cranial CT scan did not reveal any arterial calcifications of intracranial arteries of the circle of Willis. Multi-slice CT (MSCT) scanning of the coronary arteries revealed no calcification (calcium score 0).

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MGP mutation

The clinical diagnosis was confirmed by the identification of a novel, homozygous *MGP* mutation in this patient: c.61+1G>A, indicating nucleotide substitution of guanine (G) by adenine (A) in cDNA of the first nucleotide in the introns after base 61 (donor splice site) ENTER weg (Figure 4). The effect of this mutation on mRNA splicing has been elucidated by performing RT-PCR. This identified mutation eliminates the consensus donor splice site at the end of exon 1 (TG-gt is replaced by TG-at). Instead of this mutated splice site, a new donor splice site is used, 19 bases upstream of the usual one. Thus the mutation results in the deletion of 19 bases in the cDNA, which gives rise to a frameshift. A novel stop codon in the 3'untranslated sequence of the *MGP* gene is used, 49 bases downstream of the normal stop codon. The new predicted protein is composed of 113 amino-acids (instead of 103) and only 14 amino-acids of the original sequence are conserved in the sequence upstream of the deletion. Both parents and the older sister of the patient are heterozygous carriers of the mutation. His younger sister does not carry the mutation.

Figure 4: *MGP* gene and identified mutations

The *MGP* gene in which exons are shown as boxes and introns as lines (not to scale). Oligonucleotides used for PCR and sequencing are noted as arrows. The start codon is indicated by an arrow and the stop codon by a line, at the top of the exons. Nucleotide 1 has been counted as the transcription initiation site. Mutations previously reported are indicated, and the mutation reported here is indicated in bold. Mutations affecting splice sites are indicated above the gene, other mutations are indicated below.

Circulating MGP species were measured in our patient's father (parent 1, age 43), mother (parent 2, age 42), our patient (age 19), his older sibling (sibling 1, age 20), and in his younger sibling (sibling 2, age 15). **Non-phosphorylated** MGP and **phosphorylated** MGP (A) as well as **uncarboxylated** and **carboxylated** MGP (B) could be detected in blood samples from the patient and his family members. For reference, the normal range for each measured MGP species is given in brackets. The normal range, defined as the mean $\pm$ 2 $\times$ standard deviation (SD), was based on MGP measurements in 50 healthy subjects. The mean $\pm$ SD age of this reference population (28 ± 7 years) was comparable with that of the patient and family members (28 ± 14 years).

Circulating MGP

Four different assays were used to characterise circulating MGP. The circulating non-phosphorylated and phosphorylated MGP levels (Figure 5A) of the patient and his first-degree relatives were within the normal range. He had the lowest concentration of non-phosphorylated MGP in comparison with his relatives. In contrast, he had the highest concentration of phosphorylated MGP, which was 1.5-2 times as high as those of his relatives. The circulating uncarboxylated and carboxylated MGP levels (Figure 5B) were low to very low in all family members, including our patient.

Since MGP carboxylation depends on the presence of vitamin K, we aimed to investigate whether increased intake of vitamin K would influence his MGP levels. In theory, increased MGP carboxylation would enhance its calcification-inhibitory activity, which would have a beneficial effect in preventing the development of arterial calcification. The measurements of uncarboxylated and carboxylated MGP are suited for detecting the effect of changes in vitamin K status on MGP levels. Uncarboxylated MGP, in particular, is sensitive for changes in vitamin K status and decreases significantly within 12 weeks upon vitamin K supplementation in healthy subjects [31]. The patient received 10 mg vitamin K₁ daily during 3 months. The decrease in the ucOC/cOC ratio from 0.70 at baseline to 0.08 after 3 months of supplements confirmed that his systemic vitamin K status had improved. The results of MGP measurements are shown in Figure 6. The uncarboxylated MGP levels remained unchanged; there was a decrease in carboxylated MGP levels after 3 months. This small decrease is within the intra-individual variation, and was not considered to be an effect of increased vitamin K intake. It was therefore concluded that the increased intake of vitamin K₁ during 3 months had not affected circulating MGP levels in our patient.

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Figure 5. Circulating MGP measurements

Circulating MGP species were measured in our patient’s father (parent 1, age 43), mother (parent 2, age 42), our patient (age 19), his older sibling (sibling 1, age 20), and in his younger sibling (sibling 2, age 15). **Non-phosphorylated** MGP and **phosphorylated** MGP (A) as well as **uncarboxylated** and **carboxylated** MGP (B) could be detected in blood samples from the patient and his family members. For reference, the normal range for each measured MGP species is given in brackets. The normal range, defined as the mean $\pm$ 2 $\times$ standard deviation (SD), was based on MGP measurements in 50 healthy subjects. The mean $\pm$ SD age of this reference population (28 ± 7 years) was comparable with that of the patient and family members (28 ± 14 years).

Figure 6. Effect of vitamin K supplementation on MGP levels

Circulating uncarboxylated and carboxylated MGP levels of the patient are shown at baseline and after 3 months of vitamin K₁ supplementation (10 mg daily). The **uncarboxylated** MGP level remained unchanged. Although there was a very small decrease in the **carboxylated** MGP level, this was not considered to be clinically significant.

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MGP in tissue

HE and Von Kossa staining of lung tissue from the first male patient described by Keutel *et al.* revealed concentric arterial calcification in the region of the internal elastic lamina of the small- and medium-sized pulmonary arteries, which was often accompanied by intimal hypertrophy (Figure 7A). This type of calcification was also seen in the coronary arteries and skin arteries. In the lung tissue, calcifications were also seen in the connective tissue surrounding the bronchi and vasculature. The presence of extra-vascular calcifications was also clearly demonstrated by Von Kossa staining of the skin, revealing ossifications and elastic fibre calcifications in the dermis (Figure 7A). Staining for MGP was found to be positive in the arteries of the different tissues, with the predominant MGP species being phosphorylated MGP (Figure 7B). Staining for fetuin-A was positive in the vasculature (not shown), as was osteopontin staining. Positive osteopontin staining was also seen in the aortic media, in which Von Kossa staining revealed minor calcifications along the elastic lamellae (Figure 7B).

Figure 7: Immunohistochemical stainings. (See next page.)

7A I, II: Hematoxylin and eosin (HE) and Von Kossa staining of lung tissue

7A III, IV: HE and Von Kossa staining of skin tissue

7B I, II: Phosphorylated MGP stainings of lung tissue

7B III, IV: Von Kossa and osteopontin staining of the aorta

Original magnifications x100 (AI, AII, AIV, BI) and x200 (AIII, BII-IV). HE staining of lung tissue obtained at post-mortem examination of the male patient originally described by Keutel *et al.* revealed extensive distortion of normal anatomy, indicating arterial calcification (**A I**; arrows). Specks of calcifications are also seen in the connective tissue surrounding the bronchi and arteries (**A II**; black, arrow). The presence of extra-vascular calcifications was also clearly demonstrated in the dermis of the right thigh, revealing ectopic calcifications (**A III and IV**; arrow) and elastic fibre calcifications (**A IV**; black, dotted arrow). The presence of MGP in the tissue was clearly demonstrated by staining of phosphorylated MGP in the media (M) of a pulmonary artery, which appeared to colocalize with vascular smooth muscle cells (**B I**; red, arrow). A band of tissue with the morphological appearance of cartilage (C) was present at the luminal side of an extensively calcified artery, with the chondrocyte-like cells positively stained for phosphorylated MGP (**B II**; red, arrow). Von Kossa staining of the aorta revealed calcium deposition along the elastic fibres in the aortic media (**B III**; black, arrows). Osteopontin staining was positive in the aortic media (**B IV**; brown, arrow). M: Media; L: Lumen; C: Cartilage.

Figure 7: Immunohistochemical stainings

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Discussion

This study was performed in two patients with Keutel syndrome (KS), a rare autosomal recessive disorder resulting from mutations in the gene encoding the protein MGP which inhibits calcification and is vitamin K-dependent. First we describe a novel homozygous *MGP* mutation in a newly identified KS patient. Second, this is the first report on circulating MGP species in the presence of a functional mutation and it demonstrates high levels of phosphorylated MGP and low levels of carboxylated MGP. Third, we found that vitamin K supplements did not improve the patient's MGP carboxylation. Finally, we were able to demonstrate the presence of phosphorylated MGP in the vasculature, obtained at post-mortem examination, of the first KS patient originally described by Keutel *et al.* [13].

Our patient presented with exertional dyspnoea refractive to bronchodilatory drugs. Laryngotracheoscopy revealed cricoid stenosis and an abnormal trachea, and CT scanning showed abnormal cartilage calcification. Other features, including brachytelephalangism and peripheral pulmonary artery stenoses, were consistent with the diagnosis of KS. Mutation analysis revealed a novel *MGP* mutation (c.61+1G>A). This is the fifth *MGP* mutation identified so far. This particular mutation results in the loss of the consensus donor splice site at the exon1-intron1 junction. The previously identified *MGP* mutations were two splice site mutations and two introducing a stop codon [12, 26]. Although all these mutations are expected to coincide with absent or non-functional MGP, circulating MGP species have not been previously characterised in KS patients.

In our patient, measurements of circulating MGP revealed a high level of phosphorylated MGP and a low level of carboxylated MGP compared to both his heterozygous first-degree relatives and to the sibling without the *MGP* mutation. The presence of different MGP species (non-phosphorylated, phosphorylated, non-carboxylated, carboxylated MGP) originates from two post-translational modifications that may occur: vitamin K-dependent carboxylation and serine phosphorylation. Phosphorylation of extracellular matrix proteins is thought to be important for control of biomineralization [35]. This has been extensively studied for osteopontin (OPN), a phosphorylated glycoprotein that inhibits soft tissue calcification. Non-phosphorylated OPN greatly reduces its ability to inhibit hydroxyapatite formation as well as smooth muscle cell calcification [36-38]. It has been postulated that phosphorylation results in a protein conformation change increasing the protein's affinity for calcium crystals thereby limiting their growth and proliferation [35]. Moreover, it may facilitate the recruitment and activation of macrophages that remove pathologic calcium deposits [39]. For MGP, a (additional) function for phosphorylation may be the regulation of MGP secretion from the cell [9]. Moreover, it has been shown in experiments with vascular smooth muscle cells that phosphorylated MGP residues (without carboxylated MGP residues) may also partly inhibit calcification [40].

The phosphoserine domain, the Gla domain, and the C-terminus seem to be important for normal MGP function. The high level of phosphorylated MGP in our patient may be explained by a relatively high synthesis of MGP, possibly induced by the high calcification load. The carboxylation of MGP is a prerequisite for its inhibition of BMP-2, and the low circulation levels of carboxylated MGP indicate that this mechanism of MGP action is likely to be impaired.

MGP acts as a local inhibitor of calcification, which has been illustrated by *MGP* knockout mice, of which the phenotype can be rescued if MGP expression is selectively restored locally in the vascular smooth muscle cell [41]. However, increasing the circulating MGP level did not rescue the calcifying phenotype [41]. The finding of MGP in the circulation of our patient is therefore not clear proof that MGP still has calcification-inhibitory in the patient. However, it does confirm that MGP is synthesized in the tissues of our patient. Of note, circulating MGP species may reflect various aspects of calcification inhibition by MGP in the tissue.

Vitamin K deficiency, either due to nutritional deficit or the use of vitamin K antagonists (e.g. coumarin-derivatives), will result in undercarboxylation of MGP and thus impair its biological function [42]. In contrast, increased availability of vitamin K for MGP in tissue may enhance its calcification-inhibiting activity and possibly reduce the development or progression of calcification in cartilage, the arterial wall, or other soft tissues. We were able to measure circulating uncarboxylated MGP levels in our patient, which have previously been found to decrease with vitamin K supplements given to healthy subjects. We therefore aimed to assess whether vitamin K would also affect MGP levels in our patient. If so, this would be a first indication that the patient might benefit from vitamin K supplements and further exploration of the effect on clinical symptoms would be justified. However, no clinically relevant changes in either uncarboxylated or carboxylated MGP levels were seen after 3 months of vitamin K₁ supplements. The question arises as to why a beneficial effect from vitamin K supplements was lacking. We can speculate that carboxylation of MGP was not possible due to the mutation, since only the first 14 amino-acids of the mutated protein are comparable with those of the mature MGP protein. Alternatively, since the levels of uncarboxylated and carboxylated MGP were very low in our patient, a modest effect due to vitamin K supplements might not have been detected. Since the increased availability of vitamin K did not measurably affect MGP, it is unlikely that vitamin K supplements would improve the clinical symptoms or the long-term outcome in this particular KS patient.

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Additional phenotypic characterisation of our patient included a thorough vascular evaluation, which revealed no signs of arterial calcification. In contrast to the *MGP* knockout mice, which displayed severe arterial calcifications soon after birth, no clinical signs of vascular calcification have been reported in most KS patients. In 2001, Meier *et al.* reported on the follow-up of the siblings originally described by Keutel *et al.* [29]. The youngest sibling died at age 38 years from right heart failure, and a post mortem revealed concentric calcification in several vascular beds, including the coronary, hepatic, renal, meningeal, and cerebral arteries. This is the only KS patient in which extensive arterial calcification has been described. Tissues were available from this patient for immunohistochemical staining, although none were available from our own patient. We were able to confirm Meier *et al.*'s finding of concentric arterial calcification of several vascular beds. We also demonstrated the presence of MGP, predominantly in its phosphorylated form, in the vasculature of this patient. The finding of elastic fibre calcification in the dermis is in accordance with a report from Nanda *et al.*, who demonstrated fragmentation of mid-dermal elastic fibres in biopsies of normal-looking skin from two patients with KS [43]. These results suggest a possible role for MGP in preventing elastic fibre calcification in the skin.

The difference in the arterial phenotype between the *MGP* knockout mice model and the human KS is intriguing. It could be argued that the apparent absence of arterial calcifications in our patient is due to an insufficient sensitivity of the MSCT scanning to detect early calcifications. It has been shown in vascular tissue from young dialysis patients that apoptosis and damage of vascular smooth muscle cells, accompanied by increased tissue levels of uncarboxylated MGP, precede clinically overt calcification and even positive Von Kossa tissue staining for calcification [44]. Irrespective of this, the KS cases described so far may represent mutations accompanied with some residual calcification-inhibiting activity of MGP, which might be sufficient to prevent the development of arterial calcification. In both the patients presented here we found strong indications of the presence of phosphorylated MGP. Even if this is not the entire mature MGP protein, but fragments of MGP containing phosphoserine residues, binding of calcium crystals by MGP might still be possible.

In the *MGP* knockout mice, Speer *et al.* demonstrated that OPN was upregulated and accumulated in the calcified arterial wall [45]. In addition, mice deficient in both MGP and OPN showed twice as much calcification at the age of two weeks compared to *MGP* knockout mice, and they died approximately two weeks earlier due to vascular rupture [45]. It may therefore be hypothesised that other calcification inhibitors are upregulated in our patient that might prevent the development of arterial calcification.

Our study is the first to confirm Kaartinen *et al.*'s hypothesis that KS patients lacking functional MGP have arteries containing mineral-inhibiting OPN, possibly allowing for the extended survival of KS patients compared to *MGP* knockout mice [46]. Patients with KS may not develop overt arterial calcification unless other risk factors are present: either the loss of another inhibitor or the presence of a promoter of arterial calcification. These initiators might be absent in the relatively young KS patients described in the literature so far (mean age 8.3 years), which is supported by the absence of laboratory abnormalities in 19 patients. In contrast, several potential triggers were present in the only KS patient with extensive arterial calcifications described so far (chronic bronchitis, chemotherapy for recurrent malignancy) [29]. This patient was also older at the time of post-mortem examination than the described patients thus far (38 years).

In conclusion, we characterised the phenotype and circulating MGP species of a patient with KS, in whom we had identified a novel *MGP* mutation. In addition, we characterised MGP species in the tissues of the first KS patient originally described by Keutel *et al.*. Phosphorylated MGP was found to be present in both patients, indicating that some residual MGP activity might be present. Obviously, further investigation of the function of MGP phosphorylation is needed. Vitamin K supplements did not affect MGP activity in our patient, but might have a beneficial effect for patients in whom carboxylation of MGP is possible. The investigation of KS patients' phenotype and their circulating MGP species contributes to the understanding of MGP functions in various tissues, and helps to elucidate the difference in arterial phenotype between MGP-deficient mice and humans.

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CHAPTER 7

Vitamin K intake and (hepatic and extra-hepatic) vitamin K status in hemodialysis patients: a pilot study

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Abstract

Vitamin K is essential for the activity of γ -carboxyglutamate (Gla)-proteins, including matrix Gla-protein (MGP) and osteocalcin (OC). MGP is a potent local inhibitor of vascular calcification and OC is the most abundant non-collagenous bone matrix protein. Insufficient intake of vitamin K will lead to the production of uncarboxylated, inactive proteins. Vitamin K deficiency could contribute to the high cardiovascular calcification risk in hemodialysis (HD) patients. In this study we investigated vitamin K₁ and K₂ intake, as well as vitamin K status in a cohort of 40 Dutch HD patients.

Vitamin K intake was calculated using food diaries. Vitamin K status was assessed using measurements of circulating vitamin K, uncarboxylated MGP, uncarboxylated OC, and protein induced by vitamin K absence II (PIVKA-II; uncarboxylated prothrombin). Vitamin K intake was low in dialysis patients (median [range]; 140 [30–546] $\mu\text{g/day}$), especially on days of dialysis treatment and weekend days, as compared to intakes reported in Dutch population studies (>225 $\mu\text{g/day}$). PIVKA-II levels were elevated in 82.5% of patients, indicating hepatic subclinical vitamin K deficiency in the majority of patients. In addition, the very high uncarboxylated MGP levels found in all patients suggest vascular vitamin K deficiency.

It is concluded that vitamin K intake in HD patients is too low for maximal carboxylation of both hepatic and extra-hepatic Gla-proteins. Particularly the high inactive MGP levels may result in an increased risk for arterial calcification. Increasing the intake of vitamin K may therefore have significant health benefits for HD patients.

Introduction

Vitamin K is necessary for the function of proteins containing γ -carboxyglutamate (Gla) residues. Well-known vitamin K-dependent proteins (Gla-proteins) are the vitamin K-dependent coagulation factors, which are mainly synthesized in the liver. Extra-hepatic Gla-proteins are osteocalcin (OC), synthesized in bone, and matrix Gla-protein (MGP), synthesized in the vasculature and in cartilage. Gla-residues are formed during a vitamin K-dependent posttranslational carboxylation reaction and are essential for the activity of Gla-proteins [1]. Insufficient dietary intake of vitamin K will lead to the production of uncarboxylated (i.e. inactive) proteins [2].

MGP synthesized by vascular smooth muscle cells is the strongest physiological inhibitor of vascular calcification currently known. Deficiency of carboxylated MGP may contribute substantially to the development and progression of arterial calcification. Vascular calcifications are found in 60 to 80% of hemodialysis (HD) patients [3, 4] and are associated with a high cardiovascular risk, independent of traditional atherogenic risk factors [5, 6]. Areas of calcification in vascular tissue are associated with accumulation of uncarboxylated MGP species, which has also been found to precede the development of clinically overt calcification in children on dialysis [7].

Vitamin K intake may be differentiated for the intake of vitamin K₁ (phylloquinone) and vitamin K₂ (group name for menaquinones). The estimated daily vitamin K intake in a Western diet ranges from 60 to 200 μg , which is mainly determined by vitamin K₁ (phylloquinone) intake. Vitamin K₁ intakes reported in the Netherlands are typically higher (>200 $\mu\text{g}/\text{day}$) [8–13]. Vitamin K₁ and vitamin K₂ content of food products in the Netherlands has been extensively studied by our laboratory in the past [14], resulting in a comprehensive dietary vitamin K₁ and K₂ database. Previous studies using this database demonstrated that intake of vitamin K₂ was inversely associated with cardiovascular calcification and mortality [10, 12, 13].

Systemic vitamin K status can be determined by 1) measuring circulating vitamin K₁ and K₂ levels and 2) by measuring circulating inactive forms of vitamin K-dependent proteins. Intake of vitamin K-containing food products will readily influence measurements of circulating vitamin K levels. In contrast, measurements of uncarboxylated prothrombin (known as protein induced by vitamin K absence/antagonism II [PIVKA-II]), uncarboxylated OC, and dp-ucMGP will reflect utilization of vitamin K in tissues. We recently developed a new MGP assay, measuring desphospho-uncarboxylated (dp-uc) MGP species. First results show that this inactive, immature MGP species is suited to assess extra-hepatic vitamin K status, particularly in the vasculature [15].

In the present study, we therefore aimed to investigate vitamin K₁ and K₂ intake as well as vitamin K status in HD patients. Vitamin K intake was estimated using a comprehensive dietary vitamin K database, which has been previously used in Dutch population studies [12, 13]. Vitamin K status was evaluated with measurements of circulating vitamin K₁ and K₂ levels, as well as of hepatic and extra-hepatic vitamin K-dependent proteins, including PIVKA-II, MGP, and OC. With the present study we expected to obtain new insights in different aspects of vitamin K metabolism in HD patients, and to increase our knowledge on the risk factors and pathogenesis of arterial calcification in these patients. Additionally, the results of this study will give an indication whether HD patients may benefit from an increased dietary vitamin K intake.

Methods

Patients

For this explorative observational study, patients with end-stage renal disease who had been on regular HD treatment at the Dialysis Center Groningen for at least 3 months, were invited to participate. Patients with a minimum age of 18 years were eligible, irrespective of gender, the underlying primary renal disease, the presence of cardiovascular disease (CVD), diabetes mellitus (DM), or traditional atherogenic risk factors. Malignancy in the past 6 months, abnormal liver function, a history of gastrointestinal disease or metabolic disease, or an active infection were exclusion criteria. Additional exclusion criteria were use of coumarin derivatives, anticonvulsants (such as phenytoin, carbamazepine, and phenobarbital), which may influence vitamin K metabolism, and use of vitamin K supplements. Data were obtained on demographic, clinical, and dialysis-specific characteristics during interview and from review of patient's files. Complete data were acquired from 40 patients. Informed consent was obtained from all participants and the study was approved by the local Medical Ethics Committee.

Vitamin K intake

Energy and nutrient intake were estimated by registration of all food items consumed, including beverages, and the amount consumed during 4 non-consecutive days in food diaries by the patients, within a time-period of 2 weeks. All participants received verbal and written instructions before receiving the food diary. The diary consisted of 2 week days and 1 weekend day, during which the patient did not undergo HD treatment, and 1 week day during which the patient underwent HD treatment (dialysis day).

Diaries were analyzed using a nutrient software program (part of Evry dietist, Ensemble BV, Zoetermeer, the Netherlands), which is based on the Dutch national food compositions (NEVO) table. Energy and nutrient intake were calculated using the 2006 version of the NEVO table [16]. Concentrations of vitamin K₁ and K₂ (MK-4 through MK-10) of 260 foods have been added to the NEVO (2006) food database, as described previously [13]. All nutrients were adjusted for total energy intake by using the residual method [17]. Due to the variance intake on week, weekend, and dialysis days, intake was measured on non-consecutive days on 1 dialysis day, 2 separate week days, and 1 weekend day. These intake measurements were weighted to reflect their frequency in a 2-week period. This resulted in the following weightings: 6/14 for weekdays, 3/14 for weekend days, and 5/14 for dialysis days. In our study population, vegetables contributed 71% of vitamin K₁ intake, cheese contributed 59%, milk products 14%, and meat 18% of vitamin K₂ intake.

Vitamin K status

Vitamin K status was assessed by measurements of circulating vitamin K₁ and K₂ (menaquinones (MK)-4 through MK-10), and the vitamin K-dependent proteins PIVKA-II, dp-ucMGP, ucOC, and cOC. Venous blood samples were taken before the initiation of a HDsession after the two-day dialysis-free interval. Participants were asked to adhere to their dietary habits, but to refrain from consumption of vitamin K-containing food items (e.g. green vegetables and fermented food products) the evening before and the day of blood withdrawal. Before serum preparation, blood was kept for 20 min at room temperature. Plasma and serum were prepared by standard centrifugation and stored at -80 °C until testing.

The circulating concentrations of vitamin K species (vitamin K₁, MK-4 through MK-10) were measured using reversed phase high-performance liquid chromatography as described previously [14].

PIVKA-II levels were measured with the ASSERACHROM PIVKA-II kit (Diagnostica Stago, Asnières-sur-Mer, France). This enzyme-linked immunosorbent assay (ELISA) is based on mouse monoclonal F(ab')₂ fragments specific for PIVKA-II, without reactivity with native prothrombin.

Circulating dp-ucMGP levels were determined in plasma using a dual-antibody ELISA (VitaK BV, Maastricht, the Netherlands). In this assay, the capture antibody is directed against the non-phosphorylated MGP sequence 3–15 and the detection antibody against the uncarboxylated MGP sequence 35–49. This assay was shown to be particularly suited to assess vascular vitamin K status [15].

Markers used to evaluate the carboxylation status of circulating osteocalcin were uncarboxylated osteocalcin (ucOC), carboxylated osteocalcin (cOC), and the ratio between ucOC and cOC (ucOC/cOC). Both ucOC and cOC levels were measured with ELISAs based on conformation-specific OC antibodies (Takara, Shiga, Japan).

Other biochemical measurements, including calcium, phosphorus, cholesterol, and triglycerides, were performed using standard laboratory techniques.

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Statistical analysis

Normally and non-normally distributed continuous variables are presented as mean±standard deviation (SD) or as median [range], respectively, and categorical variables as numbers and frequencies. Differences in vitamin K intake between day types (week, weekend, and dialysis days) were analyzed with Kruskal-Wallis and Mann-Whitney U tests. Within-group differences in vitamin K intake and parameters of vitamin K status were analyzed with Kruskal-Wallis and Mann-Whitney U tests. Bonferroni correction was applied for multiple comparisons. Bivariate correlation analysis was performed using the Pearson correlation coefficient for normally distributed data and Spearman correlation coefficient for non-normally distributed data. A pvalue of ≤ 0.05 was considered to be statistically significant; reported p values are based on two-tailed tests of statistical significance. All statistical analyses were conducted using SPSS version 15.0 for Windows (SPSS Corp, Chicago, IL, USA).

Results

Patient population

Vitamin K intake and status were investigated in the entire cohort of 40 HD patients, consisting of 22 male and 18 female patients with a mean age of 62 ± 16 years. The median [range] dialysis vintage was 40 [4–385] months. All baseline characteristics of the patient population are presented in Table 1. As expected, the prevalence of CVD and hypertension was high. Control of calcium and phosphorus levels was quite well.

Vitamin K intake

Total vitamin K intake of patients, registered during 4 days, was 140 [30–546] $\mu\text{g}/\text{day}$, consisting of 118 [18–494] $\mu\text{g}/\text{day}$ vitamin K_1 and 21 [2–68] $\mu\text{g}/\text{day}$ vitamin K_2 (Table 2). As expected, total vitamin K intake was predominantly determined by intake of vitamin K_1 . One-third of the patients ($n=13$, 33%) had an average total vitamin K intake of less than 100 $\mu\text{g}/\text{day}$. Total vitamin K intake was considerably lower on weekend days and dialysis days (Table 2), as compared to week days ($p=0.003$ for both comparisons). These differences originated from differences in vitamin K_1 intake between these day-types ($p=0.003$ for both comparisons), without significant differences in vitamin K_2 intake.

There were no differences in total vitamin K, K_1 , or K_2 intake between males and females, or between patients with CVD or DM and patients without these conditions (Table 2). In correlation analysis, total vitamin K intake was not associated with age, BMI, dialysis vintage, or any of the biochemical characteristics.

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Table 1: Characteristics of the 40 HD patients

Demographic and clinical characteristics	HD population (n=40)
Age (years)	65 (23-86)
Sex (male/female ratio)	22/18
Dialysis vintage (months)	40 (4-385)
Presumed cause of ESRD (number of patients [%])	
Glomerular disease	9 [23]
Vascular disease, Hypertension	10 [25]
Polycystic kidney disease	9 [23]
Diabetic kidney disease	2 [5]
Miscellaneous	6 [15]
Unknown	4 [10]
Hypertension (number of patients [%])	27 [67.5]
Diabetes (number of patients [%])	6 [15.0]
Current smoking (number of patients [%])	6 [15.0]
BMI (kg/m ²)	24.6±3.7
Cardiovascular disease (number of patients [%])	15 [37.5]
Coronary artery disease	10 [25.0]
Peripheral artery disease	5 [12.5]
Cerebrovascular disease	4 [10]
History of renal transplantation	7 [17.5]
History of parathyroidectomy (number of patients [%])	5 [12.5]
Biochemical characteristics	
Hb (mmol/L)	7.2±0.8
Ca (mmol/L)	2.3±0.2
P (mmol/L)	1.6±0.3
Ca × P (nmol ² /L ²)	3.6±0.8
AP (U/L)	72±23
PTH (pmol/L)	15 (1-94)
Cholesterol (mmol/L)	4.0±1.0
Triglycerides (mmol/L)	1.9±0.8
Vitamin D supplementation (number of patients [%])	37 (92.5%)
Cinacalcet (number of patients [%])	4 (10)
Oral PB (number of patients [%])	35 (87.5)
>1 oral PB	20 (50)
Ca-containing PB	16 (40)
Non-Ca containing PB	29 (72.5)
Antihypertensive medication (number of patients [%])	28 (70)
>1 medication	10 (25)
Iron injections (number of patients [%])	38 (95)
Darbepoetin alfa (number of patients [%])	37 (92.5)
Dietary intake	
Total energy (kJ/d)	7502±2119
Total protein (g/d)	66±8.5
Total fat (g/d)	62±9
Mono-unsaturated fat (g/d)	21±4
Poly-unsaturated fat (g/d)	11±3
Saturated fat (g/d)	26±5
Fiber (g/d)	20±12
Calcium (mg/d)	814±235
Vitamin C (mg/d)	68±31

Data are given as mean±SD, median [range], or as absolute numbers with percentage of total between parentheses. Cardiovascular disease was defined as a history of coronary artery disease (myocardial infarction, angina pectoris or evidence of obstructive disease by angiography), cerebrovascular disease (thrombotic stroke or transient ischemic attack), calcific aortic valve disease, or peripheral artery disease (a history of claudication or lower extremity revascularization). Abbreviations used: BMI, body mass index; Ca, calcium; P, phosphorus.

Table 2: Vitamin K intake

Intake (µg/day) of energy-adjusted vitamin K			
	Total	Vitamin K ₁	Vitamin K ₂
Average all days	140 [30-546]	118 [18-494]	21 [2-68]
Day types			
Week (Monday-Friday)	171 [41-888]		
Weekend (Saturday, Sunday)	53 [13-1075]		
Hemodialysis	66 [5-452]		
	p <0.001	<0.001	0.624
Patients			
Male (n=22)	115 [42-393]	96 [33-360]	19 [2-48]
Female (n=18)	178 [30-546]	144 [18-494]	22 [4-68]
	p 0.289	0.302	0.471
with CVD (n=15)	113 [30-546]	93 [18-494]	18 [3-52]
without CVD (n=25)	162 [42-413]	134 [18-385]	22 [2-68]
	p 0.121	0.158	0.451
with DM	139 [66-292]	107 [47-272]	19 [4-48]
without DM	140 [30-546]	123 [18-494]	22 [2-68]
	p 0.791	0.910	0.691

Vitamin K intakes (total, K₁, and K₂) are given for different day types (week, weekend, and dialysis) and for different patient groups (female and male, with and without cardiovascular disease and diabetes mellitus). Data are given as median [range]. P-values are based on Kurskal-Wallis tests (for comparison of day-types) and Mann-Whitney U tests (for comparison of patient groups). Abbreviations used: CVD, cardiovascular disease; DM, diabetes mellitus.

Vitamin K status

Serum vitamin K₁ concentration showed a broad range (Table 3). Almost half of patients (n=18; 45%) had circulating vitamin K₁ levels below the normal range. Of the menaquinones (vitamin K₂), only MK-4 was measurable in low quantities in the blood. MK-5 through MK-10 were undetectable in the circulation of the patients (Table 3). Hepatic vitamin K status, as assessed with PIVKA-II measurement, was suboptimal in the patient cohort. Thirty-three patients (82.5%) had elevated PIVKA-II levels. The median level of 3.8 [1.4–12.4] ng/ml was almost twice the upper normal level. Plasma PIVKA-II levels were not associated with any of the baseline characteristics.

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Table 3: Vitamin K status

Vitamin K status	HD patients	Reference values
Circulating vitamin K (nmol/L)		
Vitamin K ₁	0.30 [0.00-5.71]	0.029-2.65
MK-4	0.00 [0.00-1.13]	< lower detection limit
MK-5 trough MK-10	0.00 [0.00-0.00]	< lower detection limit
Hepatic vitamin K status		
PIVKA-II (ng/ml)	3.8 [1.4-12.4]	<2
Extra-hepatic vitamin K status		
dp-ucMGP (pmol/l)	1868±870	50-700
ucOC (ng/ml)	6.1±28	2-4
cOC (ng/ml)	11.7±2.8	5-9
ratio ucOC/cOC	0.5 [0.1-0.9]	<1.0

Values of different markers for vitamin K status are given: circulating vitamin K₁ and K₂ levels, PIVKA-II levels for hepatic vitamin K status, and dp-ucMGP and OC levels for extra-hepatic vitamin K status. Reference values in healthy subjects are given based on literature (vitamin K₁ and K₂ levels), information provided by manufacturer of the assay (PIVKA-II), and measurements in healthy subjects aged above 40 years (dp-ucMGP and OC). Data are given as mean±SD or median [range].

Extra-hepatic vitamin K status was assessed by dp-ucMGP and OC measurements, reflecting vascular and bone vitamin K status, respectively. Plasma dp-ucMGP levels were elevated in all 40 patients (Table 3), indicating suboptimal vascular vitamin K status in the entire patient cohort. The dp-ucMGP levels were positively correlated with dialysis vintage (Spearman's $\rho=0.440$, $p=0.035$). In addition, patients with CVD had significantly higher dp-ucMGP levels (2226±1103 pmol/L) compared to patients without CVD (1653±626 pmol/L; $p=0.034$). The mean ucOC level was considerably increased compared with average levels found in healthy reference subjects aged >40 years (Table 3). However, since cOC levels were also elevated, the average ucOC/cOC ratio was 0.5, which is within the normal range.

When investigating the associations between the different parameters for vitamin K status, plasma dp-ucMGP levels were positively correlated with PIVKA-II levels (Spearman's $\rho=0.384$, $p=0.028$), depicted in Figure 1. There were no other significant correlations. Investigation of the associations between vitamin K intake (total, K₁, and K₂ intake) and measurements of vitamin K status, revealed no significant associations.

Figure 1: Correlation between dp-ucMGP and PIVKA-II measurements

Graph depicting the correlation between dp-ucMGP and PIVKA-II measurements (Spearman’s $\rho=0.384$, $p=0.028$). The dotted lines indicate the reference values of circulating dp-ucMGP (<700 pmol/L) and PIVKA-II (<2 ng/ml). It can be clearly seen that the majority of patients had elevated dp-ucMGP as well as PIVKA-II levels.

Discussion

This is the first study fully assessing vitamin K intake and vitamin K status in HD patients. The main finding was that vitamin K intake in the present HD patient cohort was relatively low. The second important finding is that both hepatic as well as extra-hepatic vitamin K status were strikingly poor, with the most extreme situation for the vascular wall.

Dietary intake of vitamin K in patients was below values previously found in our country (>225 $\mu\text{g/day}$) [8–13]. Low vitamin K intake of HD patients may be related to the dietary regimen generally prescribed for HD patients, which includes restriction of sodium and potassium intake [18]. Sodium is present in cheese and potassium is abundantly present in green vegetables, which are the food products containing abundant amounts of vitamin K₁ and K₂, respectively. Nevertheless, vitamin K intake of our patients was still higher than might have been expected since it was previously predicted that a typical renal diet would contain 80 μg or less of vitamin K per day [19]. This may have been related to a relatively high green vegetable and cheese intake in the Netherlands. Vitamin K intake was lower on weekend days and dialysis days as compared to week days, reflecting a different dietary pattern on these days. Consistent with the literature, intake of vitamin K₂ seems low compared to that of vitamin K₁ [8–13]. However, intake of vitamin K₂ is probably highly relevant for cardiovascular health. Vitamin K₂ intake (and not vitamin K₁) was inversely associated with cardiovascular calcification and mortality in the general population [10, 12, 13]. Vitamin K₂ is more uniformly distributed over the various tissues, whereas vitamin K₁ is preferentially targeted to the liver [20]. It has been shown for instance, that there is a higher accumulation and utilization of MK-4 in the vascular wall compared to vitamin K₁ [21].

Vitamin K status was investigated using measurements of circulating vitamin K, PIVKA-II, dp-ucMGP, and OC. Circulating vitamin K levels were below the normal range in almost half of the patients. The majority of the menaquinones (MK-5 through MK-13) were below the lower detection limit, which is usually found in humans [22]. It should be kept in mind that circulating vitamin K concentrations fluctuate, among others influenced by recent dietary intake. Also circulating vitamin K may not reflect the utilization of vitamin K in tissue. The high PIVKA-II levels found in the vast majority of patients indicate that vitamin K availability in the liver is too low to produce fully carboxylated clotting factors. Although an increased PIVKA-II level may not result in clinical haemostatic problems, it indicates that the hepatic vitamin K status is poor. The high dp-ucMGP levels found in all patients confirm previous findings in HD patients [15, 23] and indicate a poor vascular vitamin K status, which may well be a contributing factor in cardiovascular calcification.

Plasma dp-ucMGP was the only marker that correlated with PIVKA-II levels, stressing its value as a vascular biomarker for vitamin K status. With respect to osteocalcin, both the ucOC and cOC levels were high, resulting in a normal osteocalcin ratio. This may be explained by retention of circulating OC (fragments) in uremic serum [24, 25]. An alternative explanation may be that ESRD associated increased bone turnover resulted in an overall increased OC synthesis. Further investigations are needed to elucidate why both ucOC and cOC levels measured with ELISAs are high in HD patients. Overall, the application of OC measurements in HD patients seems to be complicated and OC carboxylation may not be a useful marker for vitamin K status in HD patients [25].

In general, dietary vitamin K intake in healthy subjects and in HD patients is sufficient to maintain normal haemostasis, but may not be sufficient for full carboxylation (activation) of the extra-hepatic Gla-proteins. Booth and coworkers showed in a cohort of 142 HD patients that vitamin K status in this patient population is suboptimal [25]. Low circulating vitamin K₁ levels and poor OC carboxylation were found in 29% and 93% of subjects, respectively. A recent study by the same group in 172 subjects with stage 3 to 5 CKD, showed that the criteria for subclinical vitamin K deficiency were met by 6% of the patients based on circulating K₁ measurements, by 60% based on OC carboxylation, and by 97% based on PIVKA-II levels [26]. The present study confirms these findings, with exception of poor OC carboxylation. It should be noted, however, that OC carboxylation was measured with a different technique in these studies (a radio-immunoassay based on the different affinities of ucOC and cOC for hydroxyapatite) than used in the present study (ELISA based on monoclonal antibodies against ucOC and cOC). Their outcomes may therefore not be comparable with ours. Due to its lipophilic characteristics and incorporation into lipoproteins, vitamin K is not expected to be removed by HD treatment [8]. It can therefore be hypothesized that vitamin K deficiency in HD patients is due to a diminished dietary intake, which may increase the risk of arterial calcification. Our study confirms that the dietary vitamin K intake in HD patients is low, although we could not demonstrate a correlation between vitamin K intake and markers for vitamin K status. This might be due to the relatively low number of patients.

The recommended daily allowance (RDA) for vitamin K of 1 µg/day/kg bodyweight is based on intakes of vitamin K₁ needed to maintain adequate synthesis of blood coagulation factors. The (higher) requirements of extra-hepatic tissues for maximal carboxylation of Gla-proteins were not taken into account in this recommendation, and although vitamin K intake met this intake level for the majority of patients, it is clear that it is too low for maximal carboxylation of extra-hepatic Gla-proteins. Based on the elevated plasma levels of PIVKA-II it may even be concluded that the hepatic vitamin K status was not adequate to fully support the carboxylation of the vitamin K-dependent clotting factors.

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The number of patients included in our study is too low to investigate whether factors other than low dietary intake may cause insufficient vitamin K status and to evaluate its long-term consequences. Further studies need to be performed to confirm the present results in larger cohorts of CKD and dialysis patients, including peritoneal dialysis patients. Also, it may be relevant to evaluate kidney transplant recipients, because these patients may well maintain their previous dietary habits as dialysis patient.

What may be the clinical implications of our findings? Improvement of vitamin K status in HD patients may readily be achieved by food supplements. The bioavailability of vitamin K from supplements is probably better than that from most foods [22]. For OC it is known that, although ucOC is responsive to increased dietary vitamin K, a maximal response can only be achieved with pharmacological vitamin K intakes [22]. The most common vitamin K form in food supplements and multivitamins is K₁. Two commercially available K₂ vitamins are MK-4 and MK-7. The half-life of MK-7 (approximately 3 days), is much longer compared to that of MK-4 and K₁, with a steady state plasma level reached after 2 weeks of daily supplementation [27]. In a pilot study among 53 HD patients, the administration of MK-7 (dosage 45, 135, or 360 µg/day) resulted in significantly decreased ucOC and dp-ucMGP levels within 6 weeks (Westenfeld R *et al.*: ASN Renal Week 2008, Abstract Book). In the highest dosage-group, the response-to-treatment for dp-ucMGP levels was even 100%. To improve the vitamin K status of HD patients, supplementation may be indeed be the best method, since increasing the dietary intake of vitamin K is probably not possible due to the generally prescribed dietary regimen, as discussed previously. Since HD patients are generally prescribed multivitamins, it may be relatively easy to add vitamin K to these preparations. A contra-indication for vitamin K supplementation is the use of vitamin K-antagonists (oral anticoagulants).

In conclusion, this is the first study demonstrating that HD patients have a vitamin K intake too low to prevent poor overall vitamin K status. The high dp-ucMGP levels are strongly suggestive for vascular vitamin K insufficiency to a level that the calcification-inhibitory activity of MGP is impaired, which may contribute to the extremely high risk for arterial calcification in HD patients. These data therefore warrant vitamin K intervention studies to elucidate the potential clinical benefit of vitamin K supplementation in HD patients.

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CHAPTER 8

General Discussion

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1. Introduction

Matrix γ -carboxyglutamate (Gla)-protein (MGP) is the most potent local inhibitor of arterial calcification currently known. The vascular calcification phenotype of *MGP* null mice could not be rescued by raising systemic MGP levels [1]. However, restoration of MGP expression in the vascular smooth muscle cells (VSMC), prevented the development of arterial calcification. These experiments show that MGP exerts its function as a calcification-inhibitor locally in its tissue of synthesis. Although MGP may not be active in the circulation, circulating MGP species may reflect the (inhibition of) calcification processes in the vascular wall.

MGP can undergo two post-translational modifications: γ -carboxylation of specific glutamate (Glu) residues and phosphorylation of serine residues. The conversion of Glu residues into Gla residues confers calcium-binding properties to vitamin K-dependent proteins, also called Gla-proteins. γ -Carboxylation is thought to be essential for the function of these proteins [2]. Indeed, it was demonstrated in the *MGP* null mice that MGP required at least 4 Gla residues to prevent extra-cellular matrix calcification [1]. In addition, the Gla-domain of MGP is involved in binding of bone morphogenetic protein-2 (BMP-2), an osteogenic growth factor [3]. As a result, binding of BMP-2 to its receptor is blocked, thereby inhibiting its action [4]. Next to binding of MGP to crystal nuclei to prevent their growth, this is considered a mechanism of action of MGP.

The exact role of phosphoserine residues remains to be elucidated, but it has been suggested that they play a role in the secretion of MGP into the extracellular matrix. We have hypothesized that these negatively-charged groups are also able to bind to insoluble calcium salts [5]. In addition, experiments with VSMC have shown that phosphorylated MGP residues may also contribute to its calcification-inhibitory activity [6]. As a result of both post-translational modifications, several MGP species may be synthesized: phosphorylated (pMGP), non-phosphorylated (desphospho, dpMGP), carboxylated (cMGP), or uncarboxylated (ucMGP). With the development of species-specific antibodies, it became possible to detect MGP species in tissues by immunohistochemical staining and in the circulation by enzyme-linked immunoassays (ELISA).

Vitamin K serves as a cofactor for the enzyme γ -glutamyl carboxylase, thereby promoting the carboxylation reaction that is coupled to vitamin K recycling. Vitamin K is a group name for several K vitamins, with vitamin K₁ (phylloquinone) and vitamin K₂ (menaquinones) being responsible for the dietary vitamin K intake. Vitamin K₁ is present in green leafy vegetables and vitamin K₂ in cheese, curd, and the Japanese food natto. The recommended daily allowance (RDA) is 1 $\mu\text{g}/\text{day}/\text{kg}$ bodyweight, which is based on requirements for normal haemostasis. The requirements for carboxylation of extra-hepatic Gla-proteins, including MGP, has not been taken into consideration in establishing the RDA [7].

Arterial calcification is associated with cardiovascular morbidity and mortality in the general population, as well as in high-risk patient populations [8-11]. These include patients with chronic kidney disease (CKD) [8, 12], diabetes mellitus (DM) [10], and inflammatory diseases including rheumatic diseases [13, 14]. Arterial calcification is currently regarded as an active, cell-mediated process. The VSMC and their potential to secrete matrix vesicles that can form the nidus for calcification, and to transform from a contractile phenotype into osteoblastic/chondrocytic-phenotype, play a central role in this process [15]. The balance between promoters and inhibitors of calcification is thought to play an important role in the development and progression of arterial calcification [16]. A general literature overview on MGP, vitamin K, and arterial calcification is given in **Chapter 1**.

Circulating MGP levels may serve as a biomarker for prevalent arterial calcification both in high-risk patient populations and the general population. Measurements of MGP may also contribute to the identification of those patients at high risk to develop arterial calcification, due to an impaired MGP as a consequence of vascular vitamin K deficiency. Within this context, we developed ELISA to measure circulating MGP species and investigated their potential for research and clinical use.

2. Circulating MGP measurements

2.1. The mono-antibody MGP assay measuring t-ucMGP

Before we started the work described in this thesis, there were two assays described in the literature to measure circulating MGP species. One is a radio-immunoassay, not commercially available, that is based on MGP polyclonal antibodies purified from human bone [17]. The second assay is a single-antibody (competitive) enzyme-linked immunosorbent assay (ELISA), that is based on a monoclonal antibody raised against the non-phosphorylated MGP sequence 3–15 [18, 19]. This assay therefore measures circulating levels of total desphospho MGP species (t-dpMGP) and does not reflect the carboxylation status of MGP.

In **Chapter 2**, we report on the development of a single-antibody ELISA for human circulating total ucMGP (t-ucMGP) species. Using a monoclonal ucMGP antibody (directed against the sequence 35–49) in immunohistochemical techniques, it was shown that ucMGP species accumulated at sites of arterial calcification in the vascular wall [20]. Circulating ucMGP species may therefore be particularly suited to reflect the presence of arterial calcification. The accumulation of ucMGP species at sites of arterial calcification and its probable high affinity for hydroxyapatite [20–22], suggest that ucMGP species are not easily set free into the circulation. We hypothesize therefore that in the presence of arterial calcification, the levels of circulating ucMGP species are decreased. The specificity of the t-ucMGP assay was demonstrated by using synthetic MGP peptides; only the ucMGP peptides reacted with the monoclonal ucMGP antibody, whereas no affinity was observed for cMGP peptides. Full length ucMGP was also identified by mass spectrometry as the major MGP species recognized by the monoclonal ucMGP antibody.

Circulating t-ucMGP levels were measured in coronary angioplasty, aortic valve stenosis, dialysis, and calciphylaxis patients. All patient populations had significantly lower t-ucMGP levels compared to healthy subjects of the same age. There was some overlap between the t-ucMGP values of the coronary angioplasty and aortic stenosis patients, which may be explained by the expected larger inter-individual variation in the extent of vascular calcification in these patient populations. In addition, aortic stenosis patients can be free from atherosclerosis, whereas coronary angioplasty patients suffer from intimal (atherosclerotic) calcification. Almost all hemodialysis and calciphylaxis patients had lower t-ucMGP levels than the healthy subjects, indicating that t-ucMGP is a marker capable of discriminating between healthy subjects and patients with end-stage renal disease (ESRD; CKD stage V). These results support our hypothesis of low circulating t-ucMGP levels in the presence of arterial calcification and indicate that circulating t-ucMGP has the potential to become a non-invasive biomarker for cardiovascular calcification.

However, one of the major limitations of this study was the absence of arterial calcification quantification. Nevertheless, we measured CAC in our second study to investigate the potential of circulating t-ucMGP as a biomarker for cardiovascular calcification.

Circulating t-ucMGP levels were measured in a population of hemodialysis (HD) patients, who underwent multislice CT scanning (MSCT) to assess the extent of coronary artery calcification (CAC), described in **Chapter 3**. In HD patients, CAC has been associated with the severity of coronary artery disease and the presence of stenotic lesions [23] as well with increased mortality [24]. This study enabled us to correlate t-ucMGP measurements with the extent of CAC in a high-risk patient population. Two reference populations were used for comparison of t-ucMGP levels: healthy subjects and patients with rheumatoid arthritis (RA) who had no CAC (CAC score 0 on MSCT scan). The healthy subjects did not undergo MSCT scanning. We therefore included the group of RA patients, although it was at that time unknown if RA itself influenced t-ucMGP levels. The HD patient cohort had significantly lower t-ucMGP levels compared to both the healthy subjects and the RA patients without CAC. CAC scores in the HD patients ranged from 0 (no CAC) to 2928 (extreme CAC). When the HD patients were divided into tertiles according to CAC score (CAC ≤ 103.0 , 103.1 to 600.0, and ≥ 600.1), patients in the low CAC tertile had significantly higher t-ucMGP levels compared to the other tertiles. In addition, t-ucMGP levels were inversely correlated with CAC in the HD patients. This association persisted after correction for age, dialysis vintage, and hs-CRP in multivariate regression analysis. The maximal number of possible confounders which could be included in the regression analysis was four (including t-ucMGP), due to the limited number of patients ($n=40$). However, with the variables age, dialysis vintage, and hs-CRP included, we could adjust at least for three of the most important potential confounders regarding CAC development in ESRD patients. This study confirms the finding that t-ucMGP levels are low in patient populations with a high burden of vascular calcification (HD and calciphylaxis patients). Moreover, t-ucMGP measurements may not only be used for inter-group comparisons of arterial calcification, but also for estimating the individual extent of CAC.

In cooperation with the Department of Cardiology, RWTH Aachen University, t-ucMGP levels were also measured in patients diagnosed with aortic valve stenosis [25]. t-ucMGP levels in these patients were significantly lower compared to a control group, which is in accordance with our findings. Serum t-ucMGP did not correlate with either severity of aortic stenosis or extent of aortic valve calcification.

To investigate the potential predictive value of circulating t-ucMGP for overall and cardiovascular mortality, we determined serum t-ucMGP levels in a prospective, well-defined Dutch cohort of 949 incident HD and peritoneal dialysis (PD) patients (NECOSAD; **Chapter 4**). Circulating t-ucMGP levels were measured in serum samples obtained at the time of 3 months of dialysis treatment. Almost all patients (>96%) had circulating t-ucMGP levels below the normal range. PD patients had significantly higher t-ucMGP levels compared to HD patients, but these levels were still well below those of healthy subjects. When patients were divided into tertiles of t-ucMGP levels, no difference was found between high, medium, and low circulating t-ucMGP in predicting mortality risk. The circulating low t-ucMGP levels are in accordance with our previous results. This study demonstrated that patients who started recently with dialysis treatment (3 months) already have low t-ucMGP levels. This may be explained by the fact that all patients have reached the final stadium of chronic kidney disease (ESRD).

Circulating t-ucMGP levels were shown to inversely correlate with glomerular filtration rate (GFR) in patients with stable cardiovascular disease (CVD) [26]. Moreover, in patients with aortic valve disease, those with CKD stage 4 had significantly lower t-ucMGP levels than patients with normal to moderately impaired renal function (CKD stage 1–3) [25]. It is therefore likely that circulating t-ucMGP levels are already lowered in the previous stages of CKD.

This was the first study investigating t-ucMGP levels in a large group of PD patients. These patients also had low t-ucMGP levels. On average, HD patients had even lower t-ucMGP levels, but the difference was relatively small. It has been found in the NECOSAD cohort that patients with moderate to severe aortic arch calcifications were more often treated with HD than PD[27]. From the patients without calcification, the majority (55%) underwent PD treatment. The higher t-ucMGP levels in PD patients might therefore be explained by less (severe) arterial calcification found in these patients. Residual confounding may be another explanation of the found difference in t-ucMGP levels between PD and HD patients.

There was no difference in t-ucMGP levels between patients with and without CVD. This might be due to the fact that the range of t-ucMGP levels in CVD patients is relatively wide (Chapter 2; coronary angioplasty and aortic stenosis patients). The presence of ESRD seemed to result in a more extensive lowering of t-ucMGP levels than the presence of CVD (Chapter 2). In the NECOSAD cohort, in which virtually all patients had very low t-ucMGP levels, the effect of CVD on t-ucMGP levels might therefore not have been detected. Circulating t-ucMGP correlated inversely with pulse pressure in the total patient population; this association persisted after correction for several variables, including age, hsCRP, calcium, and phosphate levels. This is in agreement with our findings of an inverse correlation between t-ucMGP and CAC in HDpatients (Chapter 3). These results support the hypothesis that t-ucMGP may serve as a biomarker for prevalent arterial calcification.

The mortality data in the NECOSAD study relied on observations of treating physicians, which could induce some error in the definition of the exact cause of death. The classification of deaths with an uncertain or missing cause as CV mortality did influence our results, as previously described for PD patients.

The major limitation of this study was that no sensitive imaging technique such as MSCT scanning was applied to evaluate the precise amount of arterial calcification in the current population. The relationship between t-ucMGP, the extent of vascular calcifications, and mortality could therefore not be evaluated in the present study. However, this was the first study in which circulating t-ucMGP levels were determined in a large number of incident HD as well as PD patients.

The extent of aortic calcification was investigated in 384 patients of the NECOSAD cohort[27]. This was done by screening annual chest X-rays for calcification in the aortic arch and patients were categorised as having no, moderate, or severe calcification. The progression of aortic calcification was significantly related to an increased mortality risk. Together, we investigated the association between t-ucMGP levels (measured at 3 months of dialysis) and aortic calcification in the NECOSAD cohort. Although patients without aortic calcification had somewhat higher t-ucMGP levels (191 ± 87 nmol/L; $n=83$) compared to patients with moderate (176 ± 69 nmol/L; $n=205$) and severe calcification (172 ± 62 nmol/L; $n=96$), the difference was not statistically significant. It is possible that the screening of X-rays is too insensitive to detect small arterial calcifications, resulting in misclassification of patients as having no aortic calcification.

2.2. The dual-antibody assays measuring dp-ucMGP and dp-cMGP

Since MGP carboxylation is vitamin K-dependent, certain species may also reflect the availability of vitamin K present in the vascular wall. Clinically, an insufficient vascular vitamin K status may result in an increased risk for arterial calcification. We aimed to develop MGP assays which would reflect the availability of vitamin K in the vascular wall for MGP carboxylation (vascular vitamin K status).

We developed two assays detecting circulating desphospho-uncarboxylated (dp-uc) MGP and desphospho-carboxylated (dp-c) MGP. Due to the absence of phosphoserine residues, we hypothesized that these MGP species may be more easily set free into the circulation than the corresponding phosphorylated species, independent of the presence of calcification in the vascular wall. They may therefore mainly be influenced by the availability of vitamin K (see also paragraph 2.3). Changes in systemic vitamin K status, for instance resulting from the use of vitamin K supplementation or vitamin K antagonists (coumarin derivatives), should then be reflected in circulating MGP measurements.

This was tested in the study described in **Chapter 5**, which revealed that particularly dp-ucMGP is sensitive to changes in vitamin K status. Healthy subjects receiving vitamin K supplementation during several weeks had significantly decreased dp-ucMGP levels, whereas subjects who received vitamin K antagonists (acenocoumarol) during 4 weeks had significantly increased dp-ucMGP levels. The dp-cMGP levels also decreased after high-dosage vitamin K supplementation (45 mg MK-4 during 6 months), it is currently not known why (see also paragraph 2.3). Treatment with acenocoumarol had no significant effect on dp-cMGP levels. Circulating dp-cMGP seems therefore less suited as a marker for vitamin K status. The use of a ratio of uncarboxylated and carboxylated MGP may not be useful to assess vascular vitamin K status, since the ratio will not change upon long-term vitamin K supplementation as both dp-ucMGP and dp-cMGP levels will decrease.

Since a direct comparison between the commercially available MGP assay (t-dpMGP assay) and the assays developed by our group (t-ucMGP, dp-ucMGP, and dp-cMGP assay) had not yet been performed, we also measured MGP levels in several patient populations with these assays. The patient populations included patients with aortic valve disease (AVD) and ESRD, in which the prognostic value of cardiovascular calcification has been clearly established [8, 12, 28]. Additionally, a rheumatic disease (RD) patient population consisting of patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), chondrocalcinosis (CC), gout, and diffuse idiopathic skeletal hyperostosis (DISH) was included.

Overall, patients with RD had significantly higher dp-ucMGP levels and significantly lower t-ucMGP levels. In the RD patient groups (SLE, RA, CC, gout, and DISH), these results were seen in patients with gout and CC, respectively. However, the differences were very small and the influence of cartilage disease on circulating MGP levels seems to be minimal compared to that of CVD. Patients with SLE and RA did not have significantly different circulating MGP levels, indicating that a poor vascular vitamin K status might not contribute to the increased arterial calcification risk in these patients.

Patients with ESRD and AVD had very high circulating dp-ucMGP levels, indicating a poor vascular vitamin K status. In a preliminary report, the group of Massy in cooperation with VitaK described dp-ucMGP measurements in 107 patients with different stages of chronic kidney disease (CKD stage 2–5D) [29]. It was found that circulating dp-ucMGP levels increased progressively with CKD stage. The use of coumarins was also independently associated with plasma dp-ucMGP levels.

Moreover, dp-ucMGP was positively and independently associated with the severity of aortic calcification. In a crude analysis, a plasma dp-ucMGP level above the median level (>921 pmol/L) predicted overall mortality (log rank test $p=0.006$). Ueland *et al.* from the Rikshospitalet Radiumhospitalet Medical Centre, University of Oslo, investigated dp-ucMGP and dp-cMGP levels in 147 patient with calcific valvular aortic stenosis (submitted for publication). Patients with high levels of dp-ucMGP (>950 pmol/L) had an increased all-cause mortality risk compared to patient with low dp-ucMGP levels (HR 9.2, $p<0.001$). For dp-cMGP, there were no significant associations with mortality. In this study there was also a positive correlation between dp-ucMGP and warfarin use ($r=0.65$, $p<0.001$). A poor vascular vitamin K status, indicated by high dp-ucMGP levels, may therefore be associated with an increased mortality. Importantly, in both of these studies the dp-ucMGP levels of the patients were significantly higher compared to normal controls, which is in agreement with our findings in ESRD and AVD patients.

The dp-cMGP levels in AVD and ESRD patients were 1.4 and 2.6 times fold higher than those in the corresponding age-groups, respectively. The t-ucMGP levels were significantly lower in these patient populations, which is in accordance with the results described in Chapter 2. The t-dpMGP levels were significantly higher in AVD patients; otherwise there were no significant differences between patient populations and the healthy reference groups of comparable age. In the past, low as well as high t-dpMGP levels have been reported in patients with atherosclerotic disease [18, 19]. Circulating t-dpMGP levels increased significantly after MK-7 supplementation, after coumarin treatment and also in the placebo group.

The inconsistency of t-dpMGP results, together with the large overlap between patient and reference values, indicates that this assay is not capable of identifying patient populations and would not be suited for individual patient diagnosis.

An important finding was that dp-ucMGP and dp-cMGP levels could not be measured in serum. We hypothesized that the thrombin generated during serum preparation could be the cause of this. Previous experiments with incubation of full-length synthetic MGP with thrombin, a circulating serine-protease, resulted in four MGP fragments as analyzed with mass-spectrometry (T. Hackeng, personal communication). MGP therefore appears to have several cleavage sites for thrombin, and might be degraded by the thrombin, resulting in non-measurable serum dp-ucMGP and dp-cMGP levels. More research is needed to confirm this hypothesis.

The measurement of circulating dp-ucMGP confirms the previous finding of uncarboxylated, inactive MGP species in the circulation of healthy subjects, measured with the t-ucMGP assay (Chapter 2). This has also been reported for osteocalcin, a vitamin K-dependent protein synthesized in bone, of which uncarboxylated levels are also present in the circulation of healthy subjects. The decrease of dp-ucMGP levels to almost immeasurably low levels after 6 months of VKS, indicates that the even in healthy subjects the vascular vitamin K status might be insufficient for maximal MGP carboxylation. Based on our results, each assay may have its own application for research and/or for diagnostic use. From these assays, the dp-ucMGP assay seems to be particularly suited to assess vascular vitamin K status.

2.3. Hypothesis regarding circulating MGP species

In Figure 1, our general hypothesis regarding circulating MGP species and vitamin K-dependent regulation of vascular calcification is illustrated. This hypothesis does not explain the results obtained with the dp-cMGP assay. Since carboxylation of MGP is vitamin K-dependent, we expected that dp-cMGP levels would increase upon high intake of vitamin K (use of vitamin K supplementation) and decrease upon vitamin K deficiency (use of vitamin K antagonists). Our expectation was that the dp-cMGP species would respond to changes in vitamin K status in an opposite direction as the dp-ucMGP species. However, circulating dp-cMGP levels decreased upon high-dosage vitamin K supplementation and remained unchanged upon (short-term) use of vitamin K antagonists (Chapter 5). One possible explanation for these results may be found in the second post-translational modification: phosphorylation. It is possible that upon (high-dosage) vitamin K-supplementation, dp-cMGP species are not only better carboxylated but that the carboxylated precursor proteins are also better substrates to be phosphorylated. This would result in formation of p-cMGP species, which remain undetected with the dp-cMGP assay. The dp-cMGP levels would thus be decreased. Another explanation could be that the dp-cMGP species that are synthesized upon vitamin K supplementation remain in the tissue and are not readily secreted into the circulation. It is clear that more fundamental research into MGP phosphorylation is necessary to investigate this hypothesis and to investigate why dp-cMGP species do not respond to lower vitamin K dosages and to short-term vitamin K antagonist use.

Concerning the binding of MGP to areas of calcification in tissue, we expect that the negatively-charged phosphoserine residues contribute significantly to the calcium-binding capacity of MGP. The binding of ucMGP species to areas of calcification in tissue, as demonstrated with immunohistochemical staining, is most probably due to the presence of phosphoserine residues. The role of phosphoserine residues has been examined in a cell model of calcification [6]. This model consisted of VSMC exposed to high extracellular calcium concentrations. The synthetic MGP peptides pMGP³⁻¹⁵ and dpMGP³⁻¹⁵, contain three serine residues that are phosphorylated or non-phosphorylated, respectively. These peptides do not contain Gla-residues. After addition of the (biotinylated) peptides to the calcifying VSMC, staining revealed that pMGP³⁻¹⁵ specifically adhered to vesicular structures derived from the VSMC. These vesicles were presumably matrix vesicles, preceding the formation of calcification [30]. In contrast, dpMGP³⁻¹⁵ did not bind to either the cell surface or the vesicles. pMGP³⁻¹⁵ was also able to inhibit the calcification process, whereas dpMGP³⁻¹⁵ had no measurable effect. These results indicate that MGP containing phosphoserine residues, but no Gla residues, is able to bind to (initial) sites of calcification.

The presence of Gla-residues does not seem to be a prerequisite for MGP's binding to calciumsalts and/or -crystals. Indeed, Price *et al.* had showed previously with in vitro experiments that thermal decarboxylation of MGP did not affect its calcium-binding properties [21]. The majority of the uncarboxylated MGP species measured with the t-ucMGP assay may therefore be phosphorylated (p-ucMGP), whereas only a minor part may be non-phosphorylated (dp-ucMGP). The t-ucMGP levels would therefore behave as the p-ucMGP levels indicated in Figure 1D. The phosphorylated, uncarboxylated species will adhere to the sites of calcification (low circulating t-ucMGP levels) whereas non-phosphorylated, uncarboxylated MGP species will be easily set free in the circulation (high dp-ucMGP levels). Taken also into account that dp-ucMGP species may constitute only a minor part of the total uncarboxylated MGP species, this may result in a combination of high circulating t-ucMGP levels and low dp-ucMGP levels in certain patient populations. Since phosphorylation is often associated with cellular secretion of proteins, it is at least feasible that in the diseased vessel MGP phosphorylation is hampered, leading to decreased circulating levels. This could be an alternative explanation for the low circulating t-ucMGP levels in patients with vascular calcifications. Other alternative explanations may be lower synthesis of MGP and increased degradation of MGP before carboxylation in these patients.

Figure 1: Circulating MGP species and vitamin K-dependent regulation of vascular calcification

A. Vitamin K sufficiency

In the case of sufficient vitamin K supply (via dietary intake) and in the absence of vascular disease, all MGP species synthesized in the VSMC are activated to prevent vascular calcification. Macrophages and/or surrounding VSMC will clear matrix vesicles and apoptotic bodies (MV/AB) that have the potential to form the nidus for calcification through phagocytosis. The nidus for calcification is then absent. There is no formation of hydroxyapatite in the tunica media, to which MGP can bind. MGP species that are set free into the circulation are desphospho-carboxylated (dp-c) and phospho-carboxylated (p-c) MGP.

B. Vitamin K insufficiency

Vitamin K insufficiency results from a low dietary vitamin K intake or the use of vitamin K antagonists (coumarins). The expression of MGP is normal. Uncarboxylated, inactive MGP will be synthesized, possibly leading to decreased clearance of the calcification vesicles. This will result in deposition of negatively charged phospholipid-remnants in the arterial media. These remnants have the capacity to nucleate calcium and phosphate, and subsequently calcify in the absence of the calcification-inhibitory function of MGP. The phosphorylated-uncarboxylated (p-uc) MGP species will bind to vascular calcifications, and thus p-ucMGP in the circulation is lowered. The dp-ucMGP species are easily set free into the circulation, since there are no calcium-binding groups present in these proteins, resulting in increased levels in the circulation. In the majority of healthy subjects (and patients) the vitamin K status seems to be insufficient for MGP carboxylation, as deduced from inactive ucMGP species in the circulation.

C. Vascular calcification present

In diseases leading to the shedding of high numbers calcification-vesicles, the development of calcification is triggered and the need for active MGP is high, to counteract the nidus for calcification. This includes diseases with a disordered mineral balance (high calcium-phosphate product) and inflammation (leading to apoptosis). Therefore, the demand for vitamin K is increased. The average dietary vitamin K intake is insufficient to address this higher demand of vitamin K required for the activation of all newly synthesized MGP. Therefore, a major part will be synthesized as ucMGP species, unable to stop the calcification process. The ucMGP species with phosphoserine residues (p-ucMGP) will bind to local vascular calcifications, which will be measured by the lower plasma p-ucMGP levels.

D. Increasing the vitamin K intake (high vitamin K intake)

When increasing the vitamin K intake, all newly synthesized MGP will be activated via the γ -carboxylation reaction. This will result in synthesis of dp-cMGP and p-cMGP species. However, since we found that dp-cMGP levels decreased rather than increased upon vitamin K supplementation, it is possible that dp-cMGP is converted to p-cMGP, thereby decreasing the dp-cMGP levels (see paragraph 2.3.). 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.

3. Keutel syndrome investigations

Keutel syndrome (KS) is a rare autosomal recessive disorder resulting from mutations in the *MGP* gene. We performed a study in two patients with KS, which included a characterization of the KS phenotype to further elucidate the function of MGP, as well as investigation of the presence of MGP in the circulation and tissues of KS patients (**Chapter 6**).

The first patient presented with exertional dyspnoea at the Department of Otorhinolaryngology of the University Medical Center Groningen. Laryngotracheoscopy revealed cricoid stenosis and an abnormal trachea, and subsequent CT scanning showed abnormal cartilage calcification. Cartilage calcification is the hallmark of KS and other clinical features, including brachytelephalangism and peripheral artery stenoses, were consistent with the diagnosis of KS [31]. Mutation analysis revealed a novel *MGP* mutation (c.61+1G>A), which is the fifth *MGP* mutation identified thus far. This particular mutation results in the loss of the consensus donor splice site at the exon1-intron1 junction. The previously identified *MGP* mutations were two splice site mutations and two mutations introducing a stop codon. Although all these mutations are expected to coincide with absent or non-functional MGP, circulating MGP species have not been characterized previously in KS patients.

The development of our species-specific MGP assays allowed us to investigate the presence of different circulating MGP species in the KS patient described above. We used the dp-ucMGP and dp-cMGP assays to characterize circulating MGP species. In addition, we used a very recently developed mono-antibody assay to measure phosphorylated MGP species (t-pMGP). The patient had low dp-ucMGP levels, which were comparable to those of his first-degree relatives, one of whom was a younger sibling without the *MGP* mutation (non-carrier). However, he had the lowest dp-cMGP levels and, surprisingly, the highest t-pMGP levels, compared to his relatives and healthy subjects. The high level of t-pMGP may be explained by a relatively high synthesis of MGP, possibly induced by the high calcification load.

Vitamin K supplementation was shown to decrease dp-ucMGP levels in healthy subjects, probably indicating an improved vascular vitamin K status and improved calcification inhibition by MGP (**Chapter 6**). We therefore aimed to assess whether vitamin K also affects MGP levels in our patient. If so, this would be a first indication that a KS patient may benefit from vitamin K supplements. Further exploration of the effect on clinical symptoms would then be justified. The patient received a high dose of 10 mg vitamin K₁, which is the dosage and type of vitamin K that can be prescribed as a medical drug in the Netherlands (Konakion, Roche Nederland, Woerden, the Netherlands).

No clinically relevant changes in either ucMGP or cMGP levels were seen after 3 months of supplementation. Probably, carboxylation of MGP was not possible due to the mutation, since only the first 14 amino-acids of the mutated protein are comparable with those of the mature MGP protein. In addition, dp-ucMGP and dp-cMGP levels were low in our patient, and therefore a modest effect due to vitamin K supplementation may not have been detected. Since the increased availability of vitamin K did not measurably affect MGP levels, it is unlikely that vitamin K supplementation would improve the clinical symptoms or the long-term outcome of this particular KS patient. MGP is a local inhibitor of calcification in the vascular wall. Increasing the circulating MGP level in the *MGP* null mice did not rescue the vascular calcification phenotype of these mice. The finding of MGP in the circulation of our KS patient is therefore not clear proof that MGP still has calcification-inhibitory activity. However, it does confirm that MGP is synthesized in the tissues of our patient.

Vascular evaluation of the patient, including MSCT scanning of the coronary arteries, revealed no signs of arterial calcification. This is consistent with a report by Hur *et al.*, who describe that body CT scan of a KS patient (8 years) revealed cartilage calcifications, without involvement of the soft tissue [31]. KS patients seem to survive well into adulthood, and extensive vascular calcification has been reported only in one patient (see below). It should be noted, however, that vascular evaluation has not been performed in the majority of patients described in the literature [31-47]. Overall, the phenotype of KS patients, mainly characterized by cartilage calcification, seems to be in contrast with the arterial calcification phenotype of the *MGP* null mice.

There is one paper describing extensive arterial calcification in a KS patient. This paper describes the post-mortem examination of the first patient described by Keutel *et al.* in 1971. This examination revealed concentric arterial calcification in several vascular beds, including the coronary, hepatic, renal, meningeal, and cerebral arteries [48]. This is to our knowledge the only described post-mortem examination of a KS patient. Some tissues of this patient were available to examine the presence of MGP species and other calcification inhibitors. Using the species-specific MGP antibodies, the presence of MGP - predominantly in its phosphorylated form - was shown in the vasculature. Elastic fiber calcification was seen in the skin. This is in accordance with a report from Nanda *et al.*, who demonstrated fragmentation of mid-dermal elastic fibres in biopsies of normal-looking skin from two KS patients. These results suggest a possible role for MGP in preventing elastic fiber calcification in the skin (see also paragraph 5.1.). Unfortunately, no tissues were available from our Dutch KS patient.

The difference in the arterial phenotype between the *MGP* null mice model and the human KS is intriguing. The apparent absence of arterial calcifications in our patient may be due to an insufficient sensitivity of the MSCT scanning. It has been shown in vascular tissue from young dialysis patients that apoptosis and damage of vascular smooth muscle cells is accompanied by increased tissue levels of ucMGP. This preceded clinically overt calcification and even positive Von Kossa tissue staining for calcification [49]. Irrespective of this, the KS cases described so far may represent mutations accompanied with some residual calcification-inhibiting activity of MGP, which might be sufficient to prevent the development of arterial calcification. In both the patients presented here we found strong indications of the presence of phosphorylated MGP. Even if this is not the entire mature MGP protein, but fragments of MGP containing phosphoserine residues, binding of calcium crystals by MGP might still be possible.

It may be hypothesized that other calcification inhibitors are upregulated in our patient that might prevent the development of arterial calcification. Our study is the first to confirm Kaartinen *et al.*'s hypothesis that KS patients lacking functional MGP have arteries containing mineral-inhibiting osteopontin, possibly allowing for the extended survival of KS patients compared to *MGP* null mice [50]. Patients with KS may not develop overt arterial calcification unless other risk factors are present: either the loss of another inhibitor or the presence of a promoter of arterial calcification. These initiators might be absent in the relatively young KS patients described in the literature so far (mean age 8.3 years), which is supported by the absence of laboratory abnormalities in 19 KS patients. In contrast, several potential triggers may be present in the only KS patient with extensive arterial calcifications described so far (chronic bronchitis, chemotherapy for recurrent malignancy) [48].

Phosphorylated MGP was found to be present in both patients, indicating that some residual MGP activity might be present. Obviously, further investigation of the function of MGP phosphorylation is needed. Vitamin K supplements did not affect MGP activity in our patient, but might have a beneficial effect for patients in whom carboxylation of MGP is possible. The investigation of KS patients' phenotype and their circulating MGP species contributes to the understanding of MGP functions in various tissues, and helps to elucidate the differences in arterial phenotype between MGP-deficient mice and humans.

4. Vitamin K and vascular health

4.1. Vitamin K intake

Low dietary vitamin K intake may be a risk factor for arterial calcification, probably due to decreased activity of MGP. The population-based Rotterdam study was the first to show an inverse correlation between dietary vitamin K₂ intake (and not vitamin K₁ intake) with cardiovascular calcification and death in men and women aged 55 years and over [51]. The correlation between vitamin K₂ intake and CVD was further investigated in three observational studies conducted in Dutch female participants (aged 49–70 years) of the PROSPECT study. The first study among 1,689 women showed no association between dietary vitamin K₁ and K₂ intake and calcification of breast arteries. In the second study among 16,057 women, it was found that vitamin K₂ intake (especially MK-7, MK-8, and MK-9 intake) was associated with a reduced risk for coronary heart disease (CHD) (HR 0.91, 95% CI 0.85–1.00) [52]. A third study among post-menopausal participants showed that a high dietary vitamin K₂ intake was associated with reduced coronary calcification [53]. In these latter two studies, vitamin K₁ intake was not related to the described outcomes.

The majority of observational studies investigating only vitamin K₁ intake have not shown an association between vitamin K₁ intake and cardiovascular outcomes. Jie *et al.* found in women aged 60–69 years that those with abdominal aortic calcific lesions (n=34) had a lower vitamin K₁ intake than those without these lesions (n=79) [54]. This association was not confirmed in women aged between 70 and 79 years. In a cohort of 807 men and women aged 39–45 years without known CHD, Villines *et al.* found no significant correlation between vitamin K₁ intake and CAC [55]. In 72,874 female nurses aged 38–65 years of the Nurses' Health Study (1984–2000), it was concluded that high vitamin K₁ intake (300 µg/day) is a marker for slightly reduced risks of total and non-fatal CHD. These associations were independent of established CVD risk factors, but were attenuated after adjustment for dietary variables associated with CHD (for instance fat, cereal fiber, and folate). The authors hypothesized that dietary and lifestyle patterns associated with K₁ intakes, rather than the intake of the nutrient itself, may account for all or part of the weak association. In a second study by Erkkila *et al.* in 40,087 men aged 40–75 years of the Health Professionals' Follow-up Study (1986–2000), no significant associations were found between vitamin K₁ intake and CHD events and strokes after adjustment for lifestyle and dietary factors. When controlling for lifestyle and other dietary factors associated with CVD in statistical analysis, the association between vitamin K₁ intake and CVD is often lost. The main source of dietary vitamin K₁ is formed by green leafy vegetables, which indicate generally healthier diets and lifestyle [53, 56]. Indeed, a strong overlap was found between low vitamin K₁ intake and other dietary CVD risk factors in the Framingham Offspring Cohort [57]. Vitamin K₁ can therefore be seen as an intake marker for a heart-healthy diet [53].

HD patients are at high risk to develop arterial calcification [8, 12]. Based on a typical renal diet, it can be predicted that the vitamin K intake of HD patients is low (80 μg or less of vitamin K per day) [58]. Booth *et al.* reported low circulating K_1 levels and a poor carboxylation grade of osteocalcin (OC) in 29% and 93%, respectively, of a cohort of 142 HD patients [59]. A recent study by the same group in 172 subjects with stage 3 to 5 CKD, showed that the criteria for subclinical vitamin K deficiency were met by 6% of the patients based on circulating K_1 measurements, 60% based on the carboxylation grade of OC, and 97% based on protein induced by vitamin K absence II levels (PIVKA-II; uncarboxylated prothrombin) [60]. Due to its lipophilic properties and incorporation into lipoproteins, vitamin K is not expected to be removed by HD [61]. It can therefore be hypothesized that the vitamin K deficiency seen in HD patients may be due to a diminished dietary intake, which may increase the risk of arterial calcification. We have therefore investigated vitamin K intake as well as vitamin K status in a HD patient population (**Chapter 7**). We found a relatively low vitamin K intake in 40 HD patients compared to intakes previously found in the Netherlands (>225 $\mu\text{g}/\text{day}$) [51-54, 61, 62]. The second important finding was that both hepatic as well as extra-hepatic vitamin K status were strikingly poor, with the most extreme situation for the vascular wall. Low vitamin K intake of HD patients may be related to the dietary regimen generally prescribed for HD patients, which includes restriction of sodium and potassium intake [63]. Vitamin K intake was lower on weekend days and dialysis days as compared to week days, reflecting a different dietary pattern on these days.

Vitamin K status was investigated using measurements of circulating vitamin K, PIVKA-II, dp-ucMGP, and OC. Circulating vitamin K levels were below the normal range in almost half of the patients. The majority of the menaquinones (MK-5 through MK-13) were below the lower detection limit, which is usually found in humans [7]. The high PIVKA-II levels found in the vast majority of patients indicate that vitamin K availability in the liver is too low to produce fully carboxylated clotting factors. The high dp-ucMGP levels found in all patients confirm our previous findings in HD patients and indicate a poor vascular vitamin K status. Plasma dp-ucMGP was the only marker that correlated with PIVKA-II levels, stressing its value as a vascular biomarker for vitamin K status. With respect to osteocalcin, both the uncarboxylated OC (ucOC) and carboxylated OC (cOC) levels were high, resulting in a normal osteocalcin ratio. This may be explained by retention of circulating OC (fragments) in uremic serum [59, 64]. An alternative explanation may be that ESRD-associated increased bone turnover resulted in an overall increased OC synthesis. Further investigations are needed to elucidate why both ucOC and cOC levels measured with ELISA are high in HD patients. Overall, the application of OC measurements in HD patients seems to be complicated and OC carboxylation may not be a useful marker for vitamin K status in HD patients [59].

In general, dietary vitamin K intake in healthy subjects and in HD patients is sufficient to maintain normal haemostasis, but may not be sufficient for full carboxylation (activation) of the extra-hepatic Gla-proteins. The present study confirms the findings of Booth and coworkers [59, 60], with exception of poor OC carboxylation. It should be noted, however, that OC carboxylation was measured with a different technique in these studies than used in the present study. Their outcomes may therefore not be comparable with ours. It can be hypothesized that vitamin K deficiency in HD patients is due to a diminished dietary intake, which may increase the risk of arterial calcification. Our study confirms that the dietary vitamin K intake in HD patients is low, although we could not demonstrate a correlation between vitamin K intake and markers for vitamin K status. This might be due to the relatively low number of patients.

The recommended daily allowance (RDA) for vitamin K of 1 µg/day/kg bodyweight is based on intakes of vitamin K₁ needed to maintain adequate synthesis of blood coagulation factors. The (higher) requirements of extra-hepatic tissues for maximal carboxylation of Gla-proteins were not taken into account in this recommendation, and although vitamin K intake met this intake level for the majority of HD patients, it is clear that it is too low for maximal carboxylation of extra-hepatic Gla-proteins. Based on the elevated plasma levels of PIVKA-II it may even be concluded that the hepatic vitamin K status was not adequate to fully support the carboxylation of the vitamin K-dependent clotting factors. The number of patients included in our study is too low to investigate whether factors other than low dietary intake may cause insufficient vitamin K status and to evaluate its long-term consequences. Further studies need to be performed to confirm the present results in larger cohorts of CKD and dialysis patients, including peritoneal dialysis patients. Also, it may be relevant to evaluate kidney transplant recipients, because these patients may well maintain their previous dietary habits as dialysis patient.

What may be the clinical implications of our findings? Improvement of vitamin K status in HD patients may readily be achieved by food supplements. The bioavailability of vitamin K from supplements is probably better than that from most foods [7]. For OC it is known that, although ucOC is responsive to increased dietary vitamin K, a maximal response can only be achieved with pharmacological vitamin K intakes [7]. The most common vitamin K form in food supplements and multivitamins is K₁. Two commercially available K₂ vitamins are MK-4 and MK-7. The half-life of MK-7 (approximately 3 days), is much longer compared to that of MK-4 and K₁, with a steady state plasma level reached after 2 weeks of daily supplementation [65]. To improve the vitamin K status of HD patients, supplementation may be indeed be the best method, since increasing the dietary intake of vitamin K is probably not possible due to the generally prescribed dietary regimen, as discussed previously.

Since HD patients are generally prescribed multivitamins, it may be relatively easy to add vitamin K to these preparations. A contra-indication for vitamin K supplementation is the use of vitamin K-antagonists (oral anticoagulants).

In conclusion, this is the first study demonstrating that HD patients have a vitamin K intake too low to prevent poor overall vitamin K status. The high dp-ucMGP levels are strongly suggestive for vascular vitamin K insufficiency to a level that the calcification-inhibitory activity of MGP is impaired, which may contribute to the extremely high risk for arterial calcification in HD patients. These data therefore warrant vitamin K intervention studies to elucidate the potential clinical benefit of vitamin K supplementation in HD patients.

4.2. Vitamin K antagonists

Vitamin K antagonists, also known as oral anticoagulants (OAC), are widely prescribed for the treatment and prevention of thromboembolic diseases. Coumarin derivatives remain the only licensed oral anticoagulant agents for the treatment of venous thromboembolism to this day. Warfarin, a coumarin derivative, was approved in 1954 for medical use. Limitations of OAC include their slow onset of action and the variability in effectiveness between subjects which results in part from genetic polymorphisms in the metabolic pathways of OAC [66]. Moreover, frequent monitoring and dose adjustments are required to maintain the international normalized ratio (INR) within the therapeutic range. OAC block vitamin K recycling, resulting in undercarboxylated Gla-proteins. Besides coagulation factors, OAC treatment can also affect the extra-hepatic Gla-proteins, including MGP (in cartilage and vasculature) and OC(in bone).

It has been shown in animal models that vitamin K antagonists induce vascular calcification and cartilage calcification [21, 67, 68]. Several independent human studies investigated the potential association between OAC use and arterial/valvular calcification in humans. Schurgers *et al.* first addressed this question in patients undergoing aortic valve replacement. Patients treated pre-operative with marcoumar (target INR 2–3, mean duration 25 months) had significantly more valve calcification compared to non-treated patients (mean calcified area 16% and 37%, respectively; $p < 0.02$) [69]. Koos *et al.* showed that patients undergoing long-term OAC treatment (mean duration 88 months) also had increased CAC (mean score 2,410), compared to non-treated patients (mean score 1,070; $p = 0.024$) [70]. The association between OAC use and soft tissue calcification was confirmed in two other studies. Lerner *et al.* showed a significant association between the use of warfarin and the risk of calcification in 1155 patients with atrial fibrillation (OR unadjusted 1.71, 95% CI 1.34–2.18), of whom 63% was treated with warfarin and 37% without warfarin.

The association remained significant after adjustment for various confounding risk factors. Further, Rennenberg *et al.* found a high odds ratio of 8.5 (95% CI 2.01–35.95) for calcification in 19 patients receiving coumarins for more than 10 years as compared to 18 matched controls.

The association between OAC treatment and soft tissue calcification has also been investigated in HD patients. Holden *et al.* assessed the extent of aortic valve calcification in 108 HD patients with echocardiography [71]. The 18 patients who received warfarin for more than 6 months were more likely to have severe aortic valve calcification ($p=0.04$). There was also a significant association between lifetime months of warfarin exposure and severity of aortic valve calcification in these patients ($p=0.004$). Long-term warfarin treatment therefore appeared to be associated with aortic valve calcification in HD patients. However, the results of these studies need to be confirmed in prospective randomized, placebo-controlled clinical trials.

The most common indication for OAC treatment in HD patients is to maintain vascular access for dialysis treatment [72]. The role of low-intensity OAC treatment (target INR 1.4–1.9) for prevention of vascular access-thrombosis has been investigated in a randomized placebo-controlled trial in 107 patients [73]. This study, however, was ceased after the occurrence of serious bleedings in the warfarin group. The use of warfarin for vascular access therefore seems to confer little benefit to these patients and appears to expose them to an unacceptably high risk of major bleedings [71]. Moreover, the safety and efficacy of OAC treatment in dialysis patients still has to be demonstrated in randomized clinical trials.

Another indication for OAC treatment in dialysis patients is atrial fibrillation. A recent paper by Chan *et al.* showed in a large retrospective cohort of 1671 incident HD patients (average follow-up 1.6 years) that warfarin use is associated with a significantly increased risk for new (ischemic and hemorrhagic) stroke (HR 1.93 compared to non-use; 95% CI 1.29–2.90). Warfarin use was not associated with increased all-cause mortality or hospitalization. The authors discussed that in addition to a hemorrhagic mechanism for stroke, warfarin may potentiate vascular calcification to increase the risk for ischemic stroke. Wizemann *et al.* also found that warfarin use was associated with a significantly higher stroke risk in HD patients, particularly in those over 75 years of age ($n=171$; HR 2.17, 95% CI 1.04–4.53, $p=0.04$). In addition, OAC therapy (warfarin) was not associated with fewer cerebrovascular events. It can be concluded that the effectiveness and safety of warfarin use in HD patients should be further investigated.

Cozzolino and Brandenburg have recently reviewed the potential adverse effect of OAC therapy in CKD patients [74]. Patients with calciphylaxis are described as striking evidence for the deleterious role of OAC in inducing ectopic calcification. These patients develop calcification of the skin arteries, resulting in necrotic ulcers, and eventually often death. Brandenburg has registered the use of warfarin in these patients as part of the German section of the International Collaborative Calciphylaxis Network (ICNN) registry. He found that about 50% of all calciphylaxis patients reported to the registry received treatment with OAC before the onset of symptoms (V. Brandenburg, unpublished data). In cooperation with Brandenburg, we found that calciphylaxis patients had the lowest t-ucMGP levels (**Chapter 2**) and the highest dp-ucMGP values (unpublished data). These results indicate that MGP may be involved in the development of arterial calcification in these patients. Moreover, the high dp-ucMGP levels indicate a poor vascular vitamin K status, which would be worsened by OAC therapy. It could be hypothesized that these patients would benefit from vitamin K supplementation. Circulating MGP measurements may contribute to elucidation of the underlying mechanism and to investigation of other treatment strategies.

4.3. Vitamin K supplementation

Whereas vitamin K antagonists may increase the risk of soft tissue calcification, the use of vitamin K supplementation may reduce the risk of vascular calcification. The vitamin K forms that are available in multivitamins or food supplements are vitamin K₁ (synthetic and the predominant form used), MK-4 (prepared by organic synthesis and almost exclusively used in Japan), and MK-7 (a natural form prepared by extraction of natto food) [65]. After intestinal absorption, K vitamins are mainly transported in the circulation by lipoproteins to their target tissues. In animal experiments, vitamin K₁ has been found to preferentially accumulate in the liver, whereas menaquinones had a more widespread distribution pattern [75, 76]. Schurgers *et al.* demonstrated that during the first hours after oral intake, vitamin K₁ and K₂ (MK-4 and MK-9, all vitamins dissolved in corn oil) predominantly associated with the triacylglycerol-rich lipoprotein fraction, suggesting that initially most K vitamins are first transported to the liver [77]. Both menaquinones were also found in the low density-lipoprotein fraction, which is equally transported to extra-hepatic tissues. In the warfarin rat-model, it was shown that MK-4 and not vitamin K₁ could inhibit warfarin-induced arterial calcification [78]. There was also a higher accumulation of MK-4 in the aortic tissue and a more efficient utilization of MK-4 by the aorta. These results suggest tissue-specific uptake of K vitamins, in which menaquinones could be more distributed to the extra-hepatic tissues.

Moreover, Buitenhuis *et al.* showed that vitamin K₂, especially the long chain K₂ vitamins such as MK-7, have lower KM values for the enzyme γ -glutamyl carboxylase. Menaquinones may therefore be the preferred cofactors for vascular carboxylase [79]. Recently, Wallin *et al.* showed that specifically vitamin K₂ 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 [3]. Menaquinones may therefore have the potential to more effectively influence MGP and arterial calcification than vitamin K₁.

Thus far, only two clinical trials have been conducted investigating vitamin K supplementation and vascular health. The first trial examined the effects of vitamin K₁ and D supplementation on vascular properties among 181 post-menopausal women aged 55–65 years [80]. Women received placebo, a supplement containing minerals and vitamin D (MD-group), or the same supplement with 1 mg vitamin K₁ (MDK-group). The elastic properties of the common carotid artery, assessed with echography, remained unchanged during the three-year study-period, but decreased in the MD- and placebo-group. However, the change in intima-media thickness did not differ between the groups. Shea *et al.* investigated the effect of 500 μ g vitamin K₁ in a multivitamin (as compared to a multivitamin alone) on the progression of CAC during 3 years in 388 healthy men and postmenopausal women [81]. In a subgroup analysis of participants with an adherence of $\geq 85\%$ to supplementation (n=367), CAC progression was 6% less in those who received vitamin K₁. MGP was also measured with a MGP radio-immunoassay, which cannot determine the carboxylation status of MGP. Neither baseline or changes in MGP concentrations predicted the change in CAC. The first placebo-controlled clinical trial investigating the effect of MK-7 supplementation on bone health as well as vascular characteristics is currently being conducted .

Adequate intakes of vitamin K, particularly of menaquinones, could be of importance for the prevention of cardiovascular disease. However, there are only very low amounts of vitamin K in the diet, with only a few food products containing sufficient amounts of particularly menaquinones. Supplementation could therefore be of major importance. In a comparison of synthetic K1 and natto-derived MK-7, it was found that the half-life time of MK-7 is very long (several days) compared to that of K1 (several hours), resulting in more stable circulating levels and accumulation of MK-7 to higher levels during prolonged intake [65]. MK-7 is therefore longer available for uptake by extra-hepatic tissues. In addition, MK-7 was more effective in both catalyzing osteocalcin carboxylation and counter-acting coumarin-derivatives in the liver.

The finding of circulating dp-ucMGP confirms the previous reported finding of uncarboxylated, inactive MGP species in the circulation of healthy subjects [82]. This may indicate that the majority of the healthy population is not optimally protected against calcification, since part of their MGP occurs in an uncarboxylated, inactive form. This has also been reported for OC, of which uncarboxylated levels are also present in the circulation of healthy subjects. The dp-ucMGP levels decreased to almost immeasurably low levels after 6 months of vitamin K supplementation, indicating that the even in healthy subjects the vascular vitamin K status is insufficient for maximal MGP carboxylation.

5. Value of circulating MGP measurements

5.1. For research

With the t-ucMGP assay, the total pool of uncarboxylated MGP can be measured both in plasma and serum, regardless of the phosphorylation status (Chapter 2). We found consistently low t-ucMGP levels in dialysis patients (Chapter 2 and 4), and showed that t-ucMGP is inversely correlated with arterial calcification in this patient population (Chapter 3). The search for biomarkers reflecting or predicting (the risk of) arterial calcification has led to the development of ELISA measuring proteins involved in the calcification process. Circulating t-ucMGP as a biomarker could easily be included in such studies, since it is an important local inhibitor of arterial calcification. Moreover, MGP is the only calcification inhibitor of which the function of MGP can be modulated by either the use of vitamin K supplementation or vitamin K antagonists.

For studies in which vascular vitamin K status needs to be measured, dp-ucMGP seems to be particularly suited as a biomarker (Chapter 5). The major drawback of this assay is that dp-ucMGP levels are only measurable in plasma samples. We demonstrated in healthy subjects that dp-ucMGP levels decrease upon vitamin K supplementation and increase upon vitamin K antagonist use. In collaboration with Dr. Westenfeld from the department of Cardiology, RWTH Aachen University, a pilot study was performed among 50 HD patients, who received MK-7 supplementation in a dosage of 45, 135, or 360 µg/day (R. Westenfeld, submitted for publication). The dp-ucMGP levels significantly decreased after only 6 weeks of supplementation. In the highest dosage-group, the response-rate for dp-ucMGP levels was even 100%. Circulating dp-ucMGP may therefore serve as a biomarker to follow-up vitamin K status not only in healthy subjects, but also in patient populations. Additionally, dp-ucMGP could serve as a marker for investigating the effect of high vitamin K intake on the development or progression of arterial calcification. Any changes in dp-ucMGP levels could then be correlated with (changes in) clinical calcification scores. Investigating the effect of vitamin K intake on vascular calcification has been performed previously [81], however at time no species-specific MGP assay was available. Using dp-ucMGP as a biomarker, it can be investigated whether any (beneficial) effect of high vitamin K intake works via MGP. Currently, trials investigating the effect of vitamin K supplementation on dp-ucMGP and vascular calcification are in progress. Measurements of dp-ucMGP levels could also contribute to gaining insight in the underlying mechanism between OAC therapy and arterial calcification. Clinical trials investigating this association, can directly link any changes in dp-ucMGP levels with clinical markers for arterial calcification.

Thus far, tools to investigate the vascular vitamin K status were unavailable. Such investigations would contribute to the elucidation of the role of MGP in the association between OAC therapy and arterial calcification.

The development of the MGP assays described in this thesis has led to many collaborations in which we measured circulating MGP species in different patient cohorts. An overview of these and our studies and their major findings is given in Table 1. These studies included investigations of MGP as a biomarker in vascular pathologies, but also of MGP's role in non-vascular pathologies. Moreover, MGP levels are currently being measured in a large population-based study investigating vitamin K status, intake, and cardiovascular morbidity and mortality (collaboration between VitaK in Maastricht and the Julius Center in Utrecht, for which a grant of the Dutch Heart Foundation has been received).

Pseudoxanthoma elasticum (PXE) is an outstanding example of a disease in which measurements of MGP have contributed to the knowledge of the calcification process in this pathology. PXE is an autosomal recessive disorder caused by mutations in the *ABCC6* gene (ATP-binding cassette subfamily C member 6). A characteristic feature of this disease is mineralization of connective tissues, particularly of elastic fibers, affecting mainly the skin, eyes, and cardiovascular system. PXE-like clinical findings have been seen in patients suffering from mutations in either the *GGCX* or *VKOR1* genes [83], which are normally associated with deficiency of the vitamin K-dependent coagulation factors. Staining of skin samples from patients with mutations in the *GGCX* gene, revealed predominantly the presence of ucMGP species in areas of mineralization in the dermis [83]. Li *et al.* hypothesized that reduced carboxylase activity in these patients leads to a reduction of MGP carboxylation, allowing peripheral mineralization to occur [83]. The same group demonstrated low t-ucMGP values and high dp-ucMGP levels in a family with *ABCC6* and *GGCX* mutations [84] (Table 1). The involvement of MGP in classic PXE was also demonstrated by this group and confirmed by the group of Gheduzzi, showing that fibroblasts from PXE patients almost exclusively produce the inactive ucMGP, which is not able to block or inhibit calcification [85, 86]. The very low cMGP production in pathological fibroblasts compared to controls suggests that these cells have an impaired vitamin K metabolism, which may play an important role in the ectopic calcification in PXE.

Table 1: Circulating MGP measurements with the t-ucMGP, dp-ucMGP, and dp-cMGP assays

t-ucMGP assay	Results
Hermans <i>et al.</i> , Blood Purif 2007	Dialysis patients (n=120) had significantly lower t-ucMGP levels as compared to healthy controls. t-ucMGP was negatively correlated with the aortic augmentation index, but not with pulse wave velocity or pulse pressure.
Van Summeren <i>et al.</i> , Pediatr Nephrol 2008	Pediatric renal transplant recipients (n=29) had no different t-ucMGP levels as compared to healthy controls. t-ucMGP was not associated with vascular parameters.
Shroff <i>et al.</i> , Nephrol Dial Transplant 2008	Pediatric dialysis patients (n=61) had significantly lower MGP levels as compared to healthy controls. t-ucMGP was not associated with vascular parameters, including CAC scores.
Cranenburg <i>et al.</i> , J Vasc Res 2008	Angioplasty (n=30), aortic stenosis (n=25), hemodialysis (n=52), and calciphylaxis (n=10) patients had significantly lower t-ucMGP levels as compared to healthy controls.
Cranenburg <i>et al.</i> , Thromb Haemost 2009	Hemodialysis patients (n=40) had significantly lower t-ucMGP levels as compared to healthy controls and patients without CAC. t-ucMGP was inversely associated with CAC scores in hemodialysis patients.
Parker <i>et al.</i> , Nephrol Dial Transplant 2009	Among stable CVD patients (n=842) decreased t-ucMGP levels were associated with a reduced glomerular filtration rate.
Koos <i>et al.</i> , Thromb Haemost 2009	Aortic valve disease patients (n=191) had significantly lower t-ucMGP levels compared to control subjects. t-ucMGP was not associated with the severity of aortic valve disease or calcification.
Schlieper <i>et al.</i> , Kidney Blood Press Res 2009	Among hemodialysis patients (n=188) t-ucMGP was not associated with semiquantitative cardiovascular calcification scores.
Parker <i>et al.</i> , Atherosclerosis 2010	Among stable CVD patients (n=839) higher t-ucMGP was associated with lower odds of mitral annular calcification in patients without diabetes, and higher odds of calcification in patients with diabetes.
Parker <i>et al.</i> , Ann Intern Med 2010	Among stable CAD patients (n=833) those in the highest t-ucMGP tertile had a ~50% lower mortality risk compared to those in the lowest tertile.
Rennenberg <i>et al.</i> , Eur J Clin Invest 2010	Among hypertensive patients (n=36) t-ucMGP was not associated within calcification scores in univariate analysis.
Noordzij <i>et al.</i> , Nephrol Dial Transplant 2010	Among hemodialysis and peritoneal dialysis patients (n=160) t-ucMGP was not associated with (progression of) aortic calcification.
Cranenburg <i>et al.</i> , submitted for publication	Among hemodialysis and peritoneal dialysis patients (n=952) there was no difference between t-ucMGP tertiles in overall, cardiovascular, or non-cardiovascular mortality. t-ucMGP was inversely associated with pulse pressure.

dp-ucMGP and/or dp-cMGP assay	Results
Schurgers <i>et al.</i> , Clin J Am Soc Nephrol 2010	Among CKD patients (n=107) dp-ucMGP levels increased with CKD stage and were positively associated with aortic calcification score.
Cranenburg <i>et al.</i> , Thromb Haemost 2010	Aortic valve disease (n=50) and hemodialysis (n=45) patients had significantly higher dp-ucMGP and dp-cMGP levels as compared to healthy controls. dp-ucMGP decreased upon vitamin K supplementation and increased upon vitamin K antagonist use in healthy subjects (n=52 and n=17). dp-cMGP decreased upon high-dosage vitamin K supplementation (n=28).
Schlieper <i>et al.</i> , submitted for publication	Hemodialysis patients (n=188) had significantly higher dp-ucMGP and dp-cMGP levels compared to controls. dp-ucMGP was positively associated with PIVKA-II. Patients with low dp-cMGP levels had higher vascular scores and an increased all-cause and cardiovascular mortality risk.
Cranenburg <i>et al.</i> , submitted for publication	Hemodialysis patients (n=40) had high dp-ucMGP levels, which were positively associated with PIVKA-II levels.
Ueland <i>et al.</i> , submitted for publication	Patients with calcific valvular aortic stenosis (n=147) had significantly higher dp-ucMGP and dp-cMGP levels compared to controls. dp-ucMGP was associated with cardiac function and long-term mortality in multivariate analysis.
Westenfeld <i>et al.</i> , submitted for publication	Hemodialysis patients (n=50) had 4.5 fold higher dp-ucMGP levels compared to controls. dp-ucMGP levels decreased upon 6 weeks of MK-7 supplementation (45, 135, or 360 µg/day), with a response rate of 80% in 45 µg group and of 100% in the 360 µg group.

t-ucMGP, dp-ucMGP and dp-cMGP assays	Results
Li <i>et al.</i> , J Invest Dermatol 2009	A family with PXE-like syndrome had low-normal to low t-ucMGP levels as compared to unrelated controls. Two family members with mutations in the <i>GGCX</i> gene had high dp-ucMGP levels and a high dp-ucMGP/dp-cMGP ratio (>1.0), whereas the other family-members had low dp-ucMGP levels and a ratio of <1.0.
Vanakker <i>et al.</i> , Lab Invest 2010	PXE patients (n=16) had significantly lower t-ucMGP levels as compared to controls. PXE-like patients (n=4) had lower t-ucMGP and higher dp-ucMGP levels as compared to controls. dp-cMGP levels were not different in patients.

Abbreviations used: CAC, coronary artery calcification; CVD, cardiovascular disease; CKD, chronic kidney disease; PIVKA-II, protein induced by vitamin K absence II; MK-7, menaquinones-7; PXE, pseudoxanthoma elasticum; GGCX, γ-glutamyl carboxylase.

5.2. For clinical application

Circulating t-ucMGP was inversely associated with arterial calcification in the dialysis patients (Chapter 3) and could therefore contribute to the identification of patients with high probability of cardiovascular calcification and who would benefit from a more thorough diagnostic work-up (e.g. non-invasive ischemia testing or coronary angiography). However, this has not yet been confirmed in patient populations besides. More research needs to be performed before any clinical application can be considered.

Measurements of dp-ucMGP could contribute to the identification of patients with vascular vitamin K-deficiency, possibly at high risk for developing arterial calcification (Chapter 5). First, however, it should be investigated whether subjects with high dp-ucMGP levels are indeed at risk to develop arterial calcification, independent of the presence of traditional atherogenic risk factors or known promoters of arterial calcification (for instance high calcium and phosphate levels). Studies investigating the effect of both vitamin K supplementation and vitamin K antagonists on the development of arterial calcification, could help in establishing the clinical value of dp-ucMGP measurements. In these intervention studies, it should be investigated whether any decrease or increase in dp-ucMGP is associated with changes in clinical markers for arterial calcification. Until such studies have been conducted, dp-ucMGP measurements cannot be applied in clinical practice. A possible clinical application for dp-ucMGP measurements is in CKD patients to identify those patients who are at high risk for warfarin-induced vascular calcification [74].

Lateral flow tests are now being developed for ucMGP species. Such tests may be used for point of care or even home diagnostics providing information to the consumer about his/her vitamin K status and the potential benefit of taking vitamin K supplements. By monitoring MGP carboxylation on a regularly basis, this system would provide a unique possibility to visualize short-term benefits of vitamin supplements. Without doubt, such system should not be brought to the market before the health benefits of nutritional doses of supplemental vitamin K have been confirmed in long-term clinical trials.

Besides clinical research to further explore the diagnostic value of MGP measurements, more fundamental research is needed to unravel the processes of underlying MGP synthesis, transport, carboxylation, and phosphorylation. Such basic knowledge is needed to explain the value of circulating MGP measurements as marker for local vascular processes. Animal experiments using the *MGP* null mouse and knocked-in constructs of MGP in which serine residues have been replaced could for instance be conducted to further investigate the role of MGP phosphorylation. On short term, paired plasma and tissue samples would contribute to full understanding of results of the MGP assays.

Regarding measurements of circulating MGP species, the mono-antibody t-pMGP assay is currently further investigated in different patient populations. The development of an assay to measure the total circulating MGP pool, using for instance antibodies directed against the C- terminal end of MGP, would contribute significantly to our understanding of the circulating MGP species.

6. 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. The measurement of biomarkers, which can reflect the early signs of vascular disease, could be of great importance. We have developed three different MGP assays to measure various circulating MGP species. The first studies investigating MGP levels in different patient populations are described in this thesis. The t-ucMGP assay has the potential to become a biomarker for prevalent arterial calcification, although this has to be further confirmed in ESRD and other patient populations. The dp-ucMGP assay seems to be particularly suited to assess vascular vitamin K status and may therefore contribute to cardiovascular risk assessment. In conclusion, based on our results, each MGP assay may have its own merit for research and/or for diagnostic use. Further research is necessary to investigate the full diagnostic potential of the different MGP assays.

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Samenvatting

Matrix γ -carboxyglutamate (Gla)-proteïne (MGP) is een klein eiwit dat het proces van arteriële verkalking (vaatverkalking) remt (**Hoofdstuk 1**). Vaatverkalking leidt tot cardiovasculaire morbiditeit en mortaliteit in de gezonde populatie alsook in hoog-risico patiëntenpopulaties, zoals patiënten met chronische nierinsufficiëntie.

MGP is aanwezig in de vaatwand en in kraakbeen, waar het gesynthetiseerd wordt door vasculaire gladde spiercellen en chondrocyten, respectievelijk. MGP kan twee post-translationele modificaties ondergaan: γ -carboxylering van glutamaat residuen en fosforylering van serine residuen. Door γ -carboxylering kunnen vitamine K-afhankelijke eiwitten (zgn. Gla-eiwitten) calcium binden, hetgeen essentieel is voor de functie van deze eiwitten. De precieze rol van de phosphoserine residuen is onbekend, maar deze lijken een rol te spelen in de secretie van MGP en in het binden van onoplosbare calciumzouten. Als een resultaat van deze modificaties, kunnen er verschillende MGP species onderscheiden worden: gefosforyleerd MGP (phosphorylated, pMGP), niet-gefosforyleerd MGP (desphospho, dpMGP), gecarboxyleerd MGP (cMGP) en niet-gecarboxyleerd (uncarboxylated, ucMGP) MGP. Door de ontwikkeling van antilichamen gericht tegen specifieke MGP species, werd het mogelijk om MGP species in weefsel en bloed te meten met behulp van immunohistochemie en enzyme-linked immunoassays (ELISA), respectievelijk.

Vitamine K is een cofactor voor het enzyme γ -glutamyl carboxylase, waardoor de carboxyleringsreactie kan plaatsvinden. Deze reactie is gekoppeld aan de recycling van vitamine K. Vitamine K is aanwezig in groene bladgroenten (vitamine K₁, fylochinon) en in kaas, kwark en het Japanse natto (vitamine K₂, menachinonen). Gla-eiwitten hebben verschillende functies in het lichaam, waaronder als bloedstollingsfactoren in de lever. In de extra-hepatische weefsels zijn ook Gla-eiwitten aanwezig, zoals MGP in de vaatwand en osteocalcine in bot. De aanbevolen dagelijkse hoeveelheid van vitamine K is 1 $\mu\text{g}/\text{dag}/\text{kg}$ lichaamsgewicht; deze aanbeveling is gebaseerd op de benodigde hoeveelheid vitamin K voor een adequate bloedstolling.

Arteriële verkalking wordt tegenwoordig als een actief, cel-gemedieerd proces beschouwd. De vasculaire gladde spiercel, welke kan transformeren in een cel gelijkend op de osteoblast/chondrocyt, speelt een centrale rol in dit proces. De balans tussen promotors en remmers van calcificatie lijkt een belangrijke rol te spelen in de ontwikkeling en het voortschrijden van arteriële verkalking. Circulerend MGP is mogelijk een biomarker voor reeds aanwezige vaatverkalking en kan mogelijk patiënten identificeren met een hoog risico op het ontwikkelen hiervan. Het doel van de studies beschreven in dit proefschrift was om ELISA's te ontwikkelen waarmee circulerende MGP species gemeten kunnen worden en om de toepassingsmogelijkheden hiervan te onderzoeken.

In onze eerste studie (**Hoofdstuk 2**) ontwikkelden we een ELISA voor het meten van totaal circulerend ucMGP (t-ucMGP). ucMGP species accumuleren op plaatsen van arteriële verkalking in de vaatwand en hebben waarschijnlijk een hoge affiniteit voor hydroxyapatiet. Circulerend t-ucMGP kan daardoor verlaagd zijn in de aanwezigheid van vaatverkalking. De onderzochte patiëntenpopulaties waren patiënten die recent percutane transluminale coronaire angioplastiek (PTCA) hadden ondergaan, patiënten met aortaklep stenose, hemodialyse (HD) patiënten en patiënten gediagnosticeerd met calciphylaxis. Circulerend t-ucMGP was verlaagd in al deze populaties, waarbij er bij de PTCA en aortaklep stenose patiënten een duidelijke overlap was met de t-ucMGP waarden van de gezonde populatie. Bijna alle HD en calciphylaxis patiënten hadden lagere t-ucMGP waarden in vergelijking met de gezonde populatie. Circulerend t-ucMGP is daardoor een marker die in staat is een onderscheid te maken tussen gezonde personen en patiënten met eindstadium nierfalen. Onze hypothese van verlaagd circulerend t-ucMGP bij de aanwezigheid van vaatverkalking werd door deze resultaten ondersteund. Deze studie gaf een eerste aanwijzing dat circulerend t-ucMGP een niet-invasieve biomarker voor cardiovasculaire verkalking kan zijn.

Om de waarde van t-ucMGP als een biomarker voor cardiovasculaire verkalking vast te stellen, onderzochten we de correlatie tussen circulerend t-ucMGP en de ernst van vasculaire verkalking. Daarvoor maten we de t-ucMGP concentraties in het serum van 40 hemodialyse (HD) patiënten (**Hoofdstuk 3**). Bij deze patiënten werd de hoeveelheid verkalking van de coronairarteriën (coronary artery calcification, CAC) vastgesteld door middel van een multislice CT (MSCT) scan. Een hoge CAC score is geassocieerd met de aanwezigheid van significante, stenotische laesies in de coronairarteriën alsmede met toegenomen mortaliteit in HD patiënten.

De hoeveelheid circulerend t-ucMGP was significant lager in HD patiënten in vergelijking met gezonde personen van dezelfde leeftijd en (reumatoïde artritis) patiënten zonder CAC. De HD patiënten werden ingedeeld in tertielen van CAC score ($CAC \leq 103.0$, $103.1-600.0$ en ≥ 600.1). De patiënten in het laagste tertiel (dus met de minste cardiovasculaire verkalking) hadden significant hogere t-ucMGP concentraties in vergelijking met patiënten in het intermediaire en hoogste tertielen ($p=0.022$ en $p=0.021$, respectievelijk). Tevens bestond er een inverse correlatie tussen t-ucMGP en CAC scores ($r=-0.414$, $p=0.009$). Deze correlatie persisteerde na correctie voor leeftijd, duur van dialyse en ultra-sensitief C-reactief proteïne, drie belangrijke factoren die een rol spelen bij de ontwikkeling van CAC in patiënten met eindstadium nierfalen. Deze studie bevestigt dat t-ucMGP concentraties laag zijn in patiëntenpopulaties met een hoog risico op cardiovasculaire verkalking (HD en calciphylaxis patiënten). Bovendien laat deze studie zien dat t-ucMGP mogelijk als maker kan dienen om de individuele hoeveelheid CAC in te schatten.

In **Hoofdstuk 4** beschrijven we t-ucMGP metingen in 949 patiënten van NECOSAD, een groot prospectief Nederlands cohort van nieuwe HD en peritoneaal dialyse (PD) patiënten. Deze patiënten werden gevolgd vanaf de start van dialyse tot aan transplantatie of overlijden (inclusieperiode tussen 1997 en 2005, eind van studieperiode Mei 2009). Circulerend t-ucMGP werd gemeten in serum afgenomen na drie maanden dialyse. Patiënten werden ingedeeld in tertielen van t-ucMGP concentraties (hoog, midden en laag) om te onderzoeken of er een verschil in mortaliteit bestond tussen deze tertielen. Bijna alle patiënten (>96%) hadden t-ucMGP concentraties onder de normaal range, waarbij HD patiënten de laagste concentraties hadden. De gemeten lage t-ucMGP concentraties zijn in overeenstemming met onze eerdere resultaten. Er werd geen significant verschil in totale mortaliteit, cardiovasculaire en niet-cardiovasculaire mortaliteit gevonden tussen de t-ucMGP tertielen. Deze resultaten duiden erop dat patiënten die recent met dialyse begonnen zijn (3 maanden geleden), al lage t-ucMGP concentraties hebben. Circulerend t-ucMGP is daarom minder geschikt als voorspeller voor mortaliteit in deze patiëntenpopulatie.

De carboxylering van MGP is afhankelijk van de aanwezigheid van vitamine K. Bepaalde MGP species zouden daarom de beschikbaarheid van vitamine K in de vaatwand (vasculaire vitamine K status) kunnen weerspiegelen. Klinisch zou een insufficiënte vasculaire vitamine K status kunnen leiden tot een verhoogd risico op arteriële verkalking. In **Hoofdstuk 5** beschrijven we twee nieuwe ELISA's welke dp-ucMGP en dp-cMGP meten. Deze MGP species bevatten geen fosfoserine residuen en lijken daarom geschikt te zijn om de vasculaire vitamine K status te weerspiegelen. Door de afwezigheid van deze residuen komen dp-ucMGP en dp-cMGP gemakkelijker in de circulatie, onafhankelijk van de aanwezigheid van arteriële verkalking. Voornamelijk dp-ucMGP bleek inderdaad gevoelig te zijn voor veranderingen in de vitamine K status: gezonde personen die vitamine K supplementen ontvingen hadden na enkele weken significant afgenomen dp-ucMGP concentraties. Proefpersonen die vitamine K antagonisten (acenocoumarol) kregen gedurende vier weken, hadden daarentegen significant toegenomen dp-ucMGP concentraties.

We vergeleken ook de commercieel verkrijgbare MGP assay (t-dpMGP assay) en de assays ontwikkeld door onze onderzoeksgroep (t-ucMGP, dp-ucMGP en dp-cMGP assays) met elkaar. Hiervoor werden MGP concentraties in gezonde proefpersonen en in een aantal patiëntenpopulaties gemeten met deze assays. Er werden grote verschillen gevonden tussen de MGP assays, wat verklaard kan worden door de verschillende MGP species die door de assays gemeten worden. Hierdoor kunnen er verscheidende aspecten van het MGP systeem onderzocht worden en mogelijk heeft iedere MGP assay een eigen toepassing voor onderzoek en/of diagnostiek. Van alle MGP assays was de dp-ucMGP assay het meest geschikt om de vasculaire vitamine K status te onderzoeken.

Keutel syndroom (KS) is een zeldzame, autosomaal-recessieve aandoening veroorzaakt door mutaties in het *MGP* gen. Wij onderzochten twee patiënten met KS, waaronder een recent geïdentificeerde Nederlandse KS patiënt bij wie een nieuwe homozygote *MGP* mutatie (c.61+1G>A) gevonden werd (**Hoofdstuk 6**). Onderzoek van het fenotype van deze patiënt liet abnormale kraakbeenverkalking zien, het belangrijkste kenmerk van KS. Vasculair onderzoek, waaronder een MSCT scan van de coronairarteriën, liet geen aanwijzingen zien voor cardiovasculaire verkalking. Het fenotype van KS patiënten, voornamelijk gekarakteriseerd door kraakbeenverkalking, is niet overeenkomstig met het vaatverkalkings-fenotype van *MGP* knockout muizen.

Circulerende *MGP* species werden gemeten in de Nederlandse KS patiënt met behulp van onze *MGP* assays, waaronder een zeer recent ontwikkelde assay om totaal gefosforyleerd (t-pMGP) te meten. De patiënt had lage dp-ucMGP concentraties, maar de hoogste t-pMGP concentraties in vergelijking met zijn familieleden en gezonde personen. Vitamine K supplementatie had geen significante invloed op de gemeten *MGP* concentraties. Dit kan verklaard worden door de *MGP* mutatie die resulteert in een splice-site mutatie, waarbij alleen de eerste 14 aminozuren van het gemuteerde eiwit overeenkomstig het normale *MGP* eiwit zijn. Hierdoor is γ -carboxylering waarschijnlijk niet mogelijk. Gefosforyleerd *MGP* was ook aanwezig in de weefsels van de tweede onderzochte KS patiënt verkregen bij post-mortem onderzoek. Deze bevinding wijst op mogelijk resterende *MGP* activiteit en kan een verklaring zijn voor het verschil tussen het fenotype van KS patiënten en van *MGP* knockout muizen. Er is echter duidelijk meer onderzoek nodig naar de functie van *MGP* fosforylering.

In onze laatste studie onderzochten we de vitamine K inname en status van 40 Nederlandse HD patiënten (**Hoofdstuk 7**). Gebaseerd op eerdere studies, was de verwachting dat de vitamine K inname van deze patiënten laag zou zijn, wat kan bijdragen aan een slechte vitamine K status en mogelijk aan de ontwikkeling van arteriële verkalking. Er werd inderdaad een lage vitamine K inname gevonden in HD patiënten vergeleken met de eerder beschreven vitamine K inname van de algemene Nederlandse bevolking (>225 $\mu\text{g}/\text{dag}$). Dit kan gerelateerd zijn aan het dieet dat vaak aan HD patiënten voorgeschreven wordt, wat o.a. een restrictie van natrium en kalium omvat.

De vitamine K status van zowel de lever als van extra-hepatische organen werd onderzocht door middel van verschillende serum en plasma bepalingen, waaronder protein induced by vitamin K absence II (PIVKA-II) en dp-ucMGP. Hoge PIVKA-II concentraties werden gevonden in het merendeel van de patiënten, duidend op een relatief tekort aan vitamine K in de lever om volledig gecarboxyleerde stollingsfactoren te produceren.

De hoge dp-ucMGP concentraties gevonden in alle HD patiënten bevestigen onze eerdere bevindingen in deze patiëntenpopulatie en wijst op een slechte vasculaire vitamine K status. Dus zowel de vitamine K status van de lever als extra-hepatische organen was niet goed.

De vitamine K inname van gezonde personen en van HD patiënten lijkt voldoende te zijn voor normale hemostase, maar is waarschijnlijk onvoldoende voor volledige carboxylering (activering) van extra-hepatische Gla-eiwitten. Onze studie bevestigt dat de vitamine K inname van HD patiënten laag is, hoewel er geen correlatie tussen vitamine K inname en markers voor vitamine K status aantoonbaar was. De hoge dp-ucMGP concentraties zijn sterk suggestief voor vasculaire vitamine K insufficiëntie waardoor de activiteit van MGP beperkt wordt, wat kan bijdragen aan het hoge risico op arteriële verkalking van HD patiënten. Er is meer onderzoek met supplementatie van vitamine K nodig om de klinische effecten hiervan te beoordelen.

Samenvattend, is vaatverkalking gecorreleerd aan cardiovasculaire mortaliteit en een van de belangrijkste remmers aanwezig in de vaatwand is MGP. Het bepalen van biomarkers, die de eerste tekenen van vaatziekte kunnen weergeven, is hierbij belangrijk. Wij hebben drie verschillende MGP assays ontwikkeld om verschillende circulerende MGP species te meten (**Hoofdstuk 8**). De t-ucMGP assay kan een biomarker voor prevalentie vaatverkalking worden, hoewel dit bevestigd moet worden in patiënten met eindstadium nierfalen en andere patiëntenpopulaties. De dp-ucMGP assay is met name geschikt om vasculaire vitamine K status te beoordelen en kan hierdoor bijdragen aan het bepalen van het cardiovasculaire risico. In conclusie, gebaseerd op onze resultaten heeft iedere MGP assay zijn eigen waarde voor onderzoek en/of voor klinische diagnostiek. Meer onderzoek is nodig om deze waarde voor de individuele MGP assays vast te stellen.

Summary

Matrix γ -carboxyglutamate (Gla)-protein (MGP) is a small protein that inhibits arterial calcification (**Chapter 1**). Arterial calcification is associated with cardiovascular morbidity and mortality in the general population, as well as in high-risk patient populations including patients with chronic kidney disease.

MGP is present in the vascular wall and in cartilage, where it is synthesized by vascular smooth muscle cells (VSMC) and chondrocytes, respectively. MGP can undergo two post-translational modifications: γ -carboxylation of specific glutamate (Glu) residues and phosphorylation of serine residues. γ -Carboxylation confers calcium-binding properties to vitamin K-dependent proteins and is thought to be essential for the function of these proteins. The exact role of phosphoserine residues is unknown, but it has been suggested that they play a role in the secretion of MGP into the extracellular matrix. We have hypothesized that these negatively-charged groups are also able to bind to insoluble calcium salts. As a result of both post-translational modifications, several MGP species can be synthesized: phosphorylated (pMGP), non-phosphorylated (desphospho, dpMGP), carboxylated (cMGP), or uncarboxylated (ucMGP). With the development of species-specific antibodies, it became possible to detect MGP species in tissues by immunohistochemical staining and in the circulation by enzyme-linked immunoassays (ELISA).

Vitamin K serves as a cofactor for the enzyme γ -glutamyl carboxylase, thereby promoting the carboxylation reaction that is coupled to vitamin K recycling. Vitamin K is present in green leafy vegetables (vitamin K₁, phylloquinone) and in cheese, curd, and the Japanese food natto (vitamin K₂, menaquinones). Vitamin K-dependent proteins (Gla proteins) exert a broad range of functions, for example as blood coagulation factors in the liver. Gla-proteins are also present in extra-hepatic tissues, including the vasculature (MGP) and bone (osteocalcin, OC). The recommended daily allowance of vitamin K is 1 $\mu\text{g/day/kg}$ bodyweight, which is based on requirements for normal haemostasis.

Arterial calcification is currently regarded as an active, cell-mediated process. The VSMC with its ability to transform into osteoblastic/chondrocytic-like cell plays a central role in this process. The balance between promoters and inhibitors of calcification is thought to play an important role in the development and progression of arterial calcification.

Circulating MGP levels may serve as a biomarker for prevalent arterial calcification and for identification of patients at high risk to develop arterial calcification. The aim of the studies described in this thesis was to develop enzyme-linked immunosorbent assays (ELISA) to measure circulating MGP species and to investigate their potential for research and clinical use.

In our first study (**Chapter 2**) we developed a single-antibody ELISA for circulating total ucMGP (t-ucMGP) species using a monoclonal ucMGP antibody. ucMGP species accumulate at sites of arterial calcification in the vascular wall and probably have a high affinity for hydroxyapatite. We therefore hypothesized that circulating t-ucMGP levels may be decreased in the presence of arterial calcification. All patient populations, which included coronary angioplasty, aortic valve stenosis, dialysis, and calciphylaxis patients, had significantly lower t-ucMGP levels compared to healthy subjects of the same age. In the coronary angioplasty and aortic stenosis patients there was some overlap with t-ucMGP levels of healthy subjects, which may be explained by the expected larger inter-individual variation in the extent and type of vascular calcification in these patient populations. In the hemodialysis and calciphylaxis patient populations, almost all patients had lower t-ucMGP levels compared to the healthy subjects. This indicated that t-ucMGP is a marker capable of discriminating between healthy subjects and patients with end-stage renal disease (ESRD). Our hypothesis of low circulating t-ucMGP levels in the presence of arterial calcification was supported by these results. This study indicates that circulating t-ucMGP has the potential to become a non-invasive biomarker for cardiovascular calcification.

To further establish the potential of t-ucMGP as a biomarker for cardiovascular calcification, we aimed to investigate the correlation between t-ucMGP levels and the extent of vascular calcification. We therefore measured t-ucMGP levels in 40 patients undergoing regular hemodialysis (HD) treatment (**Chapter 3**). The extent of coronary artery calcification (CAC) was quantified in these patients using multislice computed tomography (MSCT) scanning. A high CAC score is associated with the severity of coronary artery disease and the presence of significant, stenotic lesions as well as with increased mortality in HD patients. Circulating t-ucMGP levels were significantly lower in HD patients compared to healthy subjects of the same age and patients who had no CAC. This latter group consisted of patients with rheumatoid arthritis in whom MSCT scanning excluded the presence of CAC. HD patients were divided into tertiles according to CAC score ($\text{CAC} \leq 103.0$, 103.1 to 600.0, and ≥ 600.1). Those in the low CAC tertile had significantly higher t-ucMGP levels compared to patients in the intermediate and high tertile ($p=0.022$ and $p=0.021$, respectively). In addition, t-ucMGP levels correlated inversely with CAC scores ($r=-0.414$, $p=0.009$). This correlation persisted after adjustment for age, dialysis vintage, and high-sensitivity C-reactive protein, three important confounders regarding CAC development in ESRD patients. This study confirms that t-ucMGP levels are low in patient populations with a high burden of vascular calcification (HD and calciphylaxis patients). Moreover, t-ucMGP measurements may not only be used for inter-group comparisons of arterial calcification, but also for estimating the individual extent of CAC.

In **Chapter 4** we describe t-ucMGP measurements in 949 patients from NECOSAD, a large prospective Dutch cohort of incident HD and peritoneal dialysis (PD) patients. These patients were followed from initiation of dialysis treatment until transplantation or death (enrolment between 1997 and 2005, end of study period May 2009). Circulating t-ucMGP levels were measured in serum samples obtained at the time of 3 months of dialysis treatment. Patients were divided into tertiles according to t-ucMGP levels (high, medium, and low) to determine whether there was a difference in mortality risk between the tertiles. Almost all patients (>96%) had circulating t-ucMGP levels below the normal range, with HD patients having the lowest t-ucMGP levels. The circulating low t-ucMGP levels were in accordance with our previous results. No significant differences were found between the high, intermediate, and low t-ucMGP tertiles in overall, cardiovascular, and non-cardiovascular mortality. These results indicate that patients who started recently with dialysis treatment (3 months) already have low t-ucMGP levels. Circulating t-ucMGP is less suited for predicting mortality in this patient population, probably due to very low t-ucMGP levels in dialysis patients.

Since MGP carboxylation is vitamin K-dependent, certain species may also reflect the availability of vitamin K present in the vascular wall (vascular vitamin K status). Clinically, an insufficient vascular vitamin K status may result in an increased risk for arterial calcification. In **Chapter 5** we describe two novel dual-antibody assays detecting circulating dp-ucMGP and dp-cMGP. These species may be suited to reflect the vascular vitamin K status, since phosphoserine residues are absent in these MGP species. We hypothesized that they would therefore be more easily set free into the circulation, independent of the presence of calcification and mainly influenced by the availability of vitamin K. Particularly circulating dp-ucMGP was found to be sensitive for changes in vitamin K status: healthy subjects receiving vitamin K supplementation during several weeks had significantly decreased dp-ucMGP levels, whereas subjects who received vitamin K antagonists (acenocoumarol) during 4 weeks had significantly increased dp-ucMGP levels.

We also compared the commercially available MGP assay (t-dpMGP assay) and the assays developed by our group (t-ucMGP, dp-ucMGP, and dp-cMGP assay) by measuring MGP levels in healthy subjects and several patient populations with these assays. Overall, major differences were found between the MGP assays, which may be explained by the different fractions detected by the assays of the total circulating MGP pool. These different MGP assays allow the assessment of various aspects of the MGP system and each assay may have its own application for research and/or for diagnostic use. From these assays, the dp-ucMGP assay was particularly suited to assess the vascular vitamin K status.

Keutel syndrome (KS) is a rare autosomal recessive disorder resulting from mutations in the *MGP* gene. We performed a study in two patients with KS, including a newly identified Dutch patient (**Chapter 6**). A novel homozygous *MGP* mutation (c.61+1G>A) was identified in this patient, of whom the phenotype was carefully characterized. CT scanning showed abnormal cartilage calcification, which is the hallmark of KS. Vascular evaluation of the patient, including MSCT scanning of the coronary arteries, revealed no signs of arterial calcification. The phenotype of KS patients, mainly characterized by cartilage calcification, seems to be in contrast with the arterial calcification phenotype of the *MGP* null mice.

We characterized circulating MGP species in the Dutch KS patient, using our species-specific MGP assays, including a very recently developed mono-antibody assay to measure phosphorylated MGP species (t-pMGP). The patient had low dp-ucMGP levels, but the highest t-pMGP levels, compared to his relatives and healthy subjects. Vitamin K supplementation did not measurably affect MGP levels. This may be explained by the finding that the *MGP* mutation results in a splice-site mutation, with only the first 14 amino-acids of the mutated protein being comparable with those of the mature MGP protein. γ -Carboxylation may therefore not be possible. Phosphorylated MGP was also found to be present in tissues of the second KS patient, obtained at post-mortem examination. This finding indicates that some residual MGP activity might be present, which could one explanation for the difference between the phenotype of KS patients and *MGP* null mice. However, further investigation of MGP phosphorylation is needed.

In our last study we investigated the vitamin K intake and status in a Dutch cohort of 40 HD patients (**Chapter 7**). Based on previous studies, it was expected that vitamin K intake of these patients was low, which could contribute to a poor vitamin K status and possibly to the development of arterial calcification. We found a relatively low vitamin K intake in the HD patients compared to intakes previously found in the general population of the Netherlands (>225 $\mu\text{g/day}$). This may be related to the dietary regimen generally prescribed for HD patients, which includes restriction of sodium and potassium intake.

Hepatic as well as extra-hepatic vitamin K status was investigated with several serum and plasma measurements, including protein induced by vitamin K absence II (PIVKA-II) and dp-ucMGP. High PIVKA-II levels were found in the vast majority of patients, indicating that vitamin K availability in the liver is too low to produce fully carboxylated clotting factors. The high dp-ucMGP levels found in all patients confirm our previous findings in HD patients and indicate a poor vascular vitamin K status. Plasma dp-ucMGP was the only marker that correlated with PIVKA-II levels, stressing its value as a vascular biomarker for vitamin K status. Thus both hepatic as well as extra-hepatic vitamin K status were strikingly poor.

In general, dietary vitamin K intake in healthy subjects and in HD patients is sufficient to maintain normal haemostasis, but may not be sufficient for full carboxylation (activation) of the extra-hepatic Gla-proteins. Our study confirms that the dietary vitamin K intake in HD patients is low, although we could not demonstrate a correlation between vitamin K intake and markers for vitamin K status. The high dp-ucMGP levels are strongly suggestive for vascular vitamin K insufficiency to a level that the calcification-inhibitory activity of MGP is impaired, which may contribute to the extremely high risk for arterial calcification in HD patients. These data therefore warrant vitamin K intervention studies to elucidate the potential clinical benefit of vitamin K supplementation in HD patients.

In conclusion, vascular calcification is a major determinant of cardiovascular mortality and one of the main calcification inhibitors in the vasculature is MGP. The measurement of biomarkers, which can reflect the early signs of vascular disease, could be of great importance. We have developed three different MGP assays to measure various circulating MGP species (**Chapter 8**). The t-ucMGP assay has the potential to become a biomarker for prevalent arterial calcification, although this has to be further confirmed in ESRD and other patient populations. The dp-ucMGP assay seems to be particularly suited to assess vascular vitamin K status and may therefore contribute to cardiovascular risk assessment. In conclusion, based on our results, each MGP assay may have its own merit for research and/or for diagnostic use. Further research is necessary to investigate the full diagnostic potential of the different MGP assays.

Curriculum vitae

Ellen Cranenburg was born on the 22th of January 1982 in Boxmeer. She completed high school (VWO Gymnasium) at Maurick College Vught in 2000. She moved to Maastricht in the same year to study Medicine at Maastricht University. During her study she was assigned as student assistant to the Department of Anatomy / Embryology of the Faculty of Medicine where she assisted in practical lessons for students. In the 4th year of her study, Ellen was introduced to dr. Cees Vermeer, which eventually resulted in a 3-month research project under his supervision in 2006. In the same year, she spent her last clinical internship of 3 months at the division of Rheumatology at the Academic Hospital Maastricht, which was her first introduction to this specialization. In August 2006 she graduated from her study Medicine and started her work in September of that year on her PhD thesis at the Department of Biochemistry within VitaK under supervision of dr. Cees Vermeer, dr. Leon Schurgers, and prof. dr. Jan Rosing. Her thesis describes several studies in relation to the role of circulating Matrix Gla-protein as a biomarker for vascular disease. Ellen started her pre-training in Internal Medicine for her specialization in Rheumatology in August 2010 at Máxima Medisch Centrum in Veldhoven, under supervision of dr. Harm Haak. She lives together with Arthur Denissen in Middelbeers (Noord-Brabant).

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Submitted

Circulating desphospho-carboxylated Matrix Gla Protein predicts survival in HD patients.

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Submitted

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