

# The Circulating Inactive Form of Matrix Gla Protein (ucMGP) as a Biomarker for Cardiovascular Calcification

Ellen C.M. Cranenburg<sup>a,b</sup> Cees Vermeer<sup>a,b</sup> Ralf Koos<sup>d</sup> Marie-Louise Boumans<sup>a</sup>  
Tilman M. Hackeng<sup>a</sup> Freek G. Bouwman<sup>c</sup> Martijn Kwaijtaal<sup>a</sup>  
Vincent M. Brandenburg<sup>e</sup> Markus Ketteler<sup>f</sup> Leon J. Schurgers<sup>a,b</sup>

<sup>a</sup>VitaK, <sup>b</sup>Cardiovascular Research Institute CARIM, and <sup>c</sup>Department of Human Biology NUTRIM, Maastricht University, Maastricht, The Netherlands; Departments of <sup>d</sup>Cardiology and <sup>e</sup>Nephrology and Clinical Immunology, RWTH University Hospital Aachen, Aachen, <sup>f</sup>Kuratorium für Heimdialyse, Dialysis Center, Würselen, Germany

## Key Words

Arteries · Atherosclerosis · Calcification · Extracellular matrix · Matrix Gla protein · Vitamin K

## Abstract

**Objective:** Matrix  $\gamma$ -carboxyglutamate (Gla) protein (MGP) is a vitamin K-dependent protein and a strong inhibitor of vascular calcification. Vitamin K deficiency leads to inactive uncarboxylated MGP (ucMGP), which accumulates 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. **Methods and Results:** An ELISA-based assay was developed with which circulating ucMGP can be determined. Serum ucMGP levels were measured in healthy subjects (n = 165) and in four patient populations; patients who underwent angioplasty (n = 30), patients with aortic stenosis (n = 25), hemodialysis patients (n = 52), and calciphylaxis patients (n = 10). All four patient populations had significantly lower 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 ucMGP levels below the normal adult range. **Conclusion:** Serum 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. Copyright © 2008 S. Karger AG, Basel

## 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 hemostasis [1]. In recent years, one of these proteins has gained increasing attention: matrix  $\gamma$ -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 (VSMCs), respectively [2, 3]. MGP

contains 9 glutamate residues, 5 of which may be  $\gamma$ -carboxylated in a posttranslational step catalyzed by the vitamin K-dependent  $\gamma$ -glutamate carboxylase [1]. Deficiency of vitamin K, either due to nutritional deficiency or the use of coumarin-derivatives, results in undercarboxylation of MGP and impairment of its biological function [4].

With immunohistochemical techniques based on monoclonal antibodies specifically recognizing carboxylated and uncarboxylated MGP (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. [6] reported the accumulation of ucMGP at sites of arterial calcification in rats treated with vitamin D and warfarin and Sweatt et al. [7] showed that the aortic wall of aging rats contained elevated concentrations of ucMGP. 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  $\gamma$ -carboxylated [10], suggesting that MGP is a local inhibitor of vascular calcification and that its function depends on the presence of Gla residues, respectively. Gla residues 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. [14] proposed that this binding may assist cells in developing tissues to migrate and differentiate.

In humans, loss-of-function mutations in the MGP gene result in the Keutel syndrome [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 Keutel patients described 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 have significantly increased aortic valve calcification and coronary artery calcification [18, 19]. Patients with moderate to severe arterial calcification (as assessed by MSCT) 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]. Using this 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 MGP 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 MGP 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. 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 is 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 circulating fraction of ucMGP is decreased. To test our hypothesis and to evaluate the potential diagnostic utility of the assay, we have measured ucMGP in serum and plasma from different patient cohorts.

## 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 aor-

tic stenosis patients from the Department of Cardiology of the University Hospital Aachen, and 30 patients with a history of recent angioplasty from the University Hospital Maastricht. An exclusion criterion for both the reference population and the patient groups was the use of coumarin derivatives. In addition, we measured the ucMGP level in serum of a patient with Keutel syndrome (kind gift of Dr. M. Meier, Clinic of Pneumology, Immenhausen, Germany). 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) and stored for 20 min at room temperature before centrifugation (15 min 1,580 g). Serum and plasma were subsampled in 250- $\mu$ l aliquots and frozen at  $-80^{\circ}\text{C}$  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 uncarboxylated MGP sequences 35–49 and 35–54 (designated as ucMGP<sup>35–49</sup> and ucMGP<sup>35–54</sup>, respectively) and carboxylated MGP sequences 35–49 and 35–54 (cMGP<sup>35–49</sup> and cMGP<sup>35–54</sup>) were made by Pepsan (Lelystad, The Netherlands). All peptides were purified and analyzed by LC/MS (ion spray, positive ion detection) and their purity level was  $>98\%$  for all peptides. Synthetic full-length MGP, cMGP [28] or ucMGP was used for antibody screening and to define assay specificity. The tracer for the assay was prepared by biotinylating the noncarboxylated 35–54 peptide (ucMGP<sup>35–54</sup>) using the EZ-Link Maleimide PEO<sub>2</sub>-Biotin kit according to the manufacturer's instructions (Pierce, Etten-Leur, the Netherlands).

#### *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 2,000-fold in carbonate buffer (0.1 M sodium carbonate, pH 9.6) and used for the coating of the microtiter plate (100  $\mu$ l/well). After incubation for 1.5 h at  $37^{\circ}\text{C}$ , the remaining protein-binding sites were blocked with 200  $\mu$ l 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., USA) and incubated for 2 h at room temperature. Next, the plate was washed 5 times with 300  $\mu$ l of washing buffer (0.05% Tween-20 in HEPES-NaCl buffer) and incubated overnight at  $4^{\circ}\text{C}$  with 100  $\mu$ l mAb-ucMGP (0.69  $\mu$ l/ml in 2% HNBSA; VitaK BV). Subsequently, the plate was washed 5 times with 300  $\mu$ l of HEPES-Tween washing buffer. 5  $\mu$ l of either serum sample or standard were diluted in 70  $\mu$ l of 2% HNBSA and 20  $\mu$ l of this solution were supplemented with 100  $\mu$ l of tracer (biotinylated ucMGP<sup>35–54</sup>, 0.7  $\mu$ g/ml). After gentle mixing, 100  $\mu$ l of this solution were transferred to the microtiter plate and incubated overnight at  $4^{\circ}\text{C}$ . Af-

ter 5 washing cycles with 300  $\mu$ l of HEPES-Tween washing buffer, the plate was incubated with 100  $\mu$ l streptavidin-peroxidase (Zymed, Breda, The Netherlands) diluted 10,000-fold with 0.5% HNBSA. The plate was subsequently washed 5 times with 300  $\mu$ l 0.05% HEPES-Tween washing buffer and stained with 100  $\mu$ l of 3,3',5,5'-tetramethylbenzidine (KPL, Gennep, the Netherlands). The staining process was stopped by adding 50  $\mu$ l of 1.0 M H<sub>2</sub>SO<sub>4</sub>, 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*

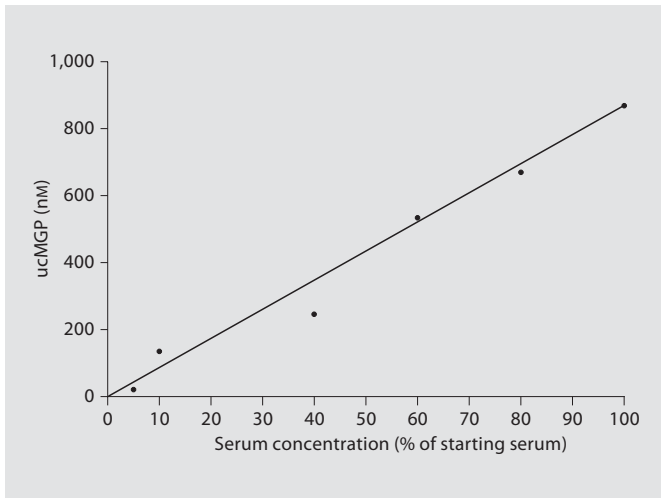
Serum MGP concentrations were quantified with the kit from Biomedica (Vienna, Austria). The kit is based on the competitive ELISA principle, with antibodies against non-phosphorylated MGP coated on the microtiter plate as has been described before [24].

#### *Immunoprecipitation*

ucMGP from plasma was purified by immunoprecipitation according to the method described by Michielsen et al. [30]. In brief, 200  $\mu$ g 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  $4^{\circ}\text{C}$ . 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-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-MGP<sup>61–79</sup>) or a monoclonal antibody raised against the MGP N-terminal peptide 3–15 (designated as mAb-MGP<sup>3–15</sup>; VitaK BV).

#### *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  $\mu$ l of the peptide mixture and 1.0  $\mu$ l matrix solution (2.5 mg/ml CHCA in 50% acetonitrile/0.1% trifluoroacetic acid; TFA) were spotted on a 384-well 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 Da and the MS/MS tolerance was set at 0.2 Da. No restrictions were made on the protein mass. A protein was regarded identified when it had a significant Mascot probability score ( $p < 0.05$ ).



**Fig. 1.** Recovery of ucMGP after dilution of serum. Serum from a healthy subject (ucMGP 869 nM) was diluted with 2% HNBSA. The theoretical (—) as well as the observed ucMGP concentration (●) is shown. The points represent means of duplicate measurements.

#### Statistical Analysis

Since 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 and  $\geq 60$  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, Ill., USA). Significance levels were based on two-tailed tests, with the alpha level set at 0.05.

## Results

#### Characteristics of the ucMGP Assay

The intra-assay variability was tested for two different serum samples (ucMGP levels: 697 and 303 nM) 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  $3 \times$  SD of the zero standard obtained from 21 replicates in one plate, and was found to be 21 nM. We compared the difference between citrated plasma and serum ucMGP measurements in paired samples from healthy subjects and patients, and found a good correlation ( $r = 0.897$ ;  $p < 0.001$ ).

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

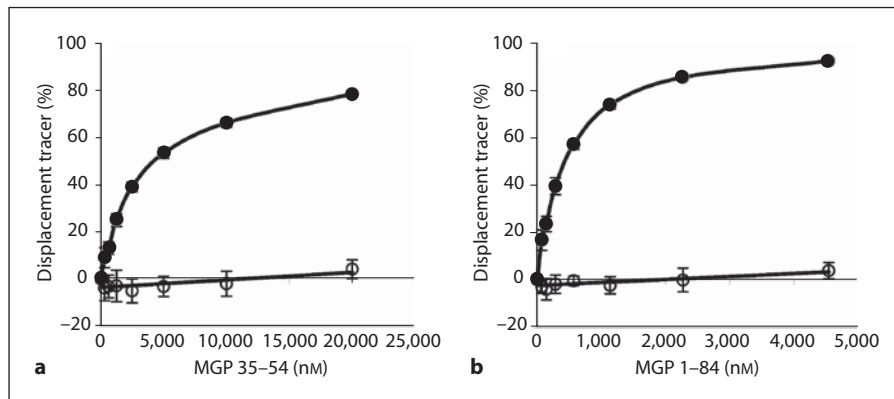
To assess the assay specificity, mAb-ucMGP was tested for its cross-reaction with synthetic MGP peptides (cMGP<sup>35-54</sup> and ucMGP<sup>35-54</sup>; fig. 2a) and synthetic full-length MGP (either cMGP or ucMGP; fig. 2b). These synthetic polypeptides were used in competitive inhibition experiments with the biotinylated ucMGP tracer (ucMGP<sup>35-54</sup>). As shown in figure 2, both ucMGP<sup>35-54</sup> and full-length ucMGP were competitive inhibitors in the assay, whereas cMGP<sup>35-54</sup> and full-length cMGP were not. Moreover, we tested the synthetic peptides cMGP<sup>35-49</sup> and ucMGP<sup>35-49</sup>, 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. Additionally, MGP was not detected in serum from a patient with Keutel syndrome with the ucMGP assay. 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  $\sim 11$  kDa, which reacted positively with a monoclonal antibody against the N-terminus and with a polyclonal antibody against the C-terminus of MGP (fig. 3). Analysis of the 11 kDa-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).

#### 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 ucMGP  $543 \pm 23$  nM). There was no measurable effect of

**Fig. 2.** 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 means  $\pm$  SD (n = 12).

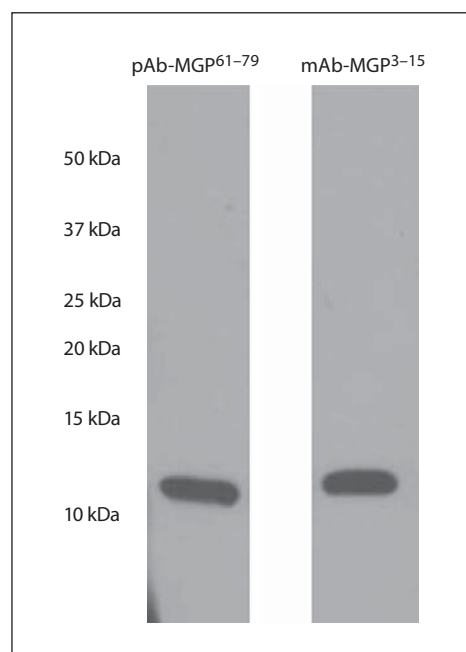


centrifugation speed (1,580 and 8,000 g) during serum preparation (mean ucMGP  $560 \pm 10$  nM and  $526 \pm 22$  nM, respectively). Freeze-thawing up to 10 cycles did not affect the observed ucMGP concentration (mean ucMGP after 10 cycles  $554 \pm 38$  nM).

*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  $30 \pm 6$  years), those aged 40–60 years (mean age  $51 \pm 6$  years) and those aged 60 years or more (mean age  $68 \pm 6$  years). Baseline characteristics of the healthy subjects are shown in table 2. The normal range for ucMGP was established in the group of 54 healthy males and females under the age of 40 (reference population), with a mean serum ucMGP level of  $504 \pm 98$  nM. The normal range of ucMGP levels, defined as the mean  $\pm 2 \times$  SD, was calculated to be between 308 and 699 nM; no difference was observed between males and females. The mean level of serum ucMGP in the group of subjects aged 40–60 years was  $481 \pm 77$  nM; in the group of subjects aged 60 years or more it was  $441 \pm 97$  nM. The mean values of these two groups were within the normal range, with tailing towards lower values (fig. 4). There was a significant difference in 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 ( $r = -0.181$ ;  $p = 0.02$ ).

The time-related variability of serum ucMGP was established in 4 healthy subjects (between 25 and 35 years old, 2 males and 2 females; mean ucMGP  $578 \pm 233$  nM) from whom blood was taken by venipuncture at 9 time points on 1 day and subsequently on 5 different days at 9 a.m. with 1-week intervals. The within-day variation



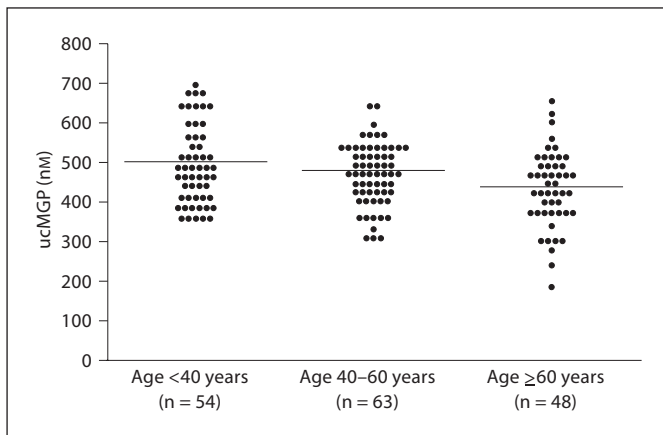
**Fig. 3.** Western blot analysis of the immunoprecipitated product shows a single band at  $\sim 11$  kDa as detected with both a polyclonal antibody raised against the MGP C-terminus (pAb-MGP<sup>61-79</sup>) and a monoclonal antibody raised against the MGP N-terminus (mAb-MGP<sup>3-15</sup>).

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 ucMGP  $476 \pm 31$  nM), 15.2% ( $470 \pm 71$  nM), 10.5% ( $1,067 \pm 112$  nM), and 10.9% ( $485 \pm 53$  nM). 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 coeffi-

**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 NANTFISPQQR	47

After excision of the 11-kDa 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 MS 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.



**Fig. 4.** Serum ucMGP levels in the reference subjects, divided in 3 groups: subjects aged less than 40 years, aged 40–60 years and aged 60 or more. Mean  $\pm$  SD ucMGP levels were  $504 \pm 98$ ,  $481 \pm 77$  and  $441 \pm 97$  nM, respectively (depicted as horizontal bars). The difference between the 3 subpopulations was significant at  $p = 0.02$ . The normal range, defined as the mean  $\pm 2 \times$  SD, was established in the group of subjects aged less than 40 years (reference population) and was calculated to be between 308 and 699 nM.

clients of variation were found to be 14.7% ( $513 \pm 75$  nM), 9.2% ( $449 \pm 41$  nM), 10.5% ( $1,016 \pm 107$  nM) and 10.7% ( $511 \pm 55$  nM).

#### Patient Samples

To evaluate the potential clinical utility of the ucMGP assay, we measured serum ucMGP in 4 different patient populations. The demographic and clinical characteris-

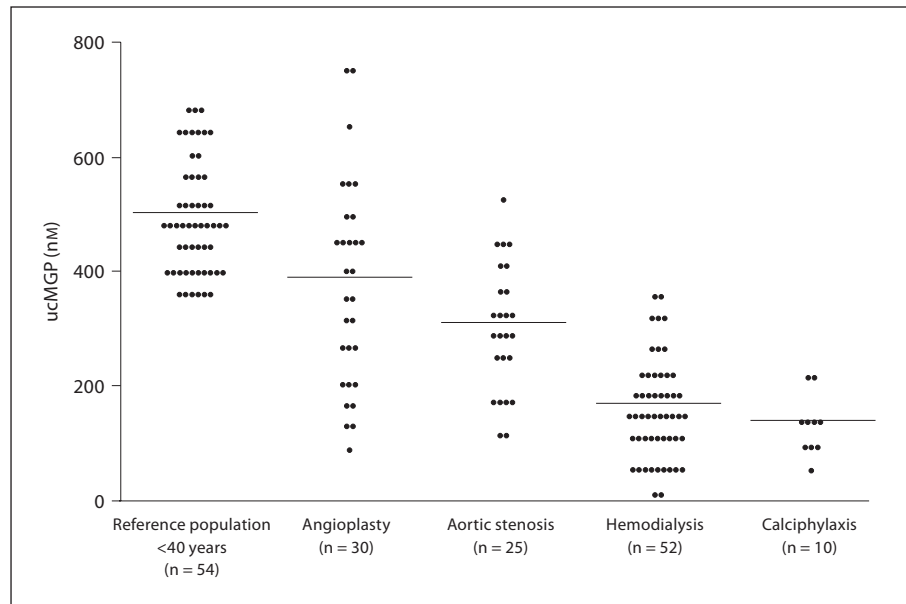
**Table 2.** Demographic and medical characteristics of healthy subjects

Demographics		Medical characteristics	
All			
Number	165	Diabetes	0
Age, years	$49 \pm 16$	Hypertension	0
BMI	$23 \pm 4$	History of heart disease	0
Gender, M/F	91/74	Medication	0
<40 years			
Number	54	Diabetes	0
Age, years	$30 \pm 6$	Hypertension	0
BMI	$22 \pm 3$	History of heart disease	0
Gender, M/F	29/25	Medication	0

Data are given either as absolute values  $\pm$  SD or as numbers.

tics of the patient populations are summarized in table 3. Figure 5 shows the serum ucMGP distribution in the reference (mean ucMGP  $504 \pm 98$  nM) and angioplasty ( $389 \pm 182$  nM), aortic stenosis ( $312 \pm 109$  nM), dialysis ( $172 \pm 82$  nM) and calciphylaxis ( $140 \pm 55$  nM) population. Obviously, all 4 patient populations had significantly lower circulating 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 ucMGP levels in hemodialysis as well as in calciphylaxis patients showed virtually no overlap with the normal range (fig. 5).

**Fig. 5.** Serum ucMGP levels in patients compared to the reference population (healthy subjects aged less than 40 years). Mean  $\pm$  SD ucMGP values of angioplasty, aortic stenosis, dialysis and calciphylaxis patients were  $389 \pm 182$ ,  $312 \pm 109$ ,  $172 \pm 82$ , and  $140 \pm 55$  nM, 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.



Additionally, ucMGP levels of the patient populations were compared with the group of healthy subjects of the same age. The ucMGP level of the angioplasty population was significantly lower in comparison with the group of healthy subjects aged 40–60 years ( $p = 0.003$ ). The 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, MGP 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 MGP 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 ucMGP. To test the assay for its diagnostic utility, we analyzed ucMGP in 4 different patient populations. Circulating ucMGP levels were significantly lower in both groups of cardiovascular

**Table 3.** Demographic and medical characteristics of patients

Demographics		Medical characteristics	
<b>Angioplasty</b>			
Number	30	Diabetes	5 (15)
Age, years	$52 \pm 8$	Hypertension	6 (20)
BMI	$27 \pm 4$	CAD	30 (100)
Gender, M/F	18/12	CKD	0 (0)
<b>Aortic stenosis</b>			
Number	25	Diabetes	7 (28)
Age, years	$72 \pm 10$	Hypertension	16 (64)
BMI	$28 \pm 4$	CAD	15 (60)
Gender, M/F	14/11	CKD	0 (0)
<b>Hemodialysis</b>			
Number	52	Diabetes	18 (35)
Age, years	$64 \pm 13$	Hypertension	46 (88)
BMI	$27 \pm 4$	CAD	28 (54)
Gender, M/F	28/24	CKD <sup>1</sup>	52 (100)
<b>Calciphylaxis</b>			
Number	10	Diabetes	4 (40)
Age, years	$63 \pm 14$	Hypertension	9 (90)
BMI	n.d.	CAD	8 (80)
Gender, M/F	5/5	CKD <sup>2</sup>	10 (100)

Data are given either as absolute values  $\pm$  SD or as numbers with percentage of total between parentheses. CAD = Coronary artery disease; CKD = chronic kidney disease.

<sup>1</sup> All 52 hemodialysis patients were by definition in CKD stage 5.

<sup>2</sup> Seven patients in the calciphylaxis group were dialysis patients (CKD stage 5: 6 hemodialysis, 1 peritoneal dialysis), 3 patients were renal transplant recipients (CKD stage 3–4).

patients as well as in dialysis and calciphylaxis patients. Moreover, ucMGP levels in the dialysis and calciphylaxis patients did not overlap with those in the reference population. Clearly, serum ucMGP is a marker capable of discriminating between healthy subjects and patients with end-stage renal disease.

Dialysis patients have a 60–80% 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 was demonstrated by using synthetic MGP-related 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. Additionally, ucMGP was not measurable in serum of a patient with Keutel syndrome – a functional knockout-human of MGP – further demonstrating the specificity of this assay for ucMGP. Finally, 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 ucMGP assay show that in patient populations known for cardiovascular calcification serum ucMGP levels are significantly lower as compared to an apparently healthy reference population. The accumulation of ucMGP 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 in calcified arteries may be different from that in healthy ones with increased ucMGP levels in calcified tissues and consequently less ucMGP secretion in the circulation. 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 sandwich-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 multislice computed tomography (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 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 fraction could have potential to serve as a noninvasive biochemical marker for cardiovascular calcification and possibly contribute to the identification of patients requiring radiological assessment of cardiovascular calcification. Obviously, diagnostic applications of 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 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 serum MGP levels in these patients were within normal range. A principal difference with our newly developed ucMGP assay is that the commercial assay does not discriminate between carboxylated and uncarboxylated MGP.

In conclusion, we have developed a robust assay for circulating ucMGP allowing the assessment of the circu-

lating 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 ucMGP and could be discriminated from the healthy reference population. Further studies are necessary to determine the exact correlation between ucMGP levels and the extent of vascular calcification as well as the predictive value of ucMGP measurements.

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