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Association of kidney function and uncarboxylated matrix Gla protein: Data from the Heart and Soul Study

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Abstract

Background. Vascular calcification is highly prevalent in persons with chronic kidney disease (CKD) and predicts cardiovascular disease (CVD) events. Matrix Gla protein (MGP) is a potent inhibitor of vascular calcification, and lower levels of its precursor—uncarboxylated MGP (ucMGP)—are associated with vascular calcification and atherosclerosis. Whether mild to moderate decrements in kidney function are associated with lower serum ucMGP is unknown.

Methods. In a cross-sectional study among 842 outpatients with stable CVD, estimated glomerular filtration rate (eGFR), serum cystatin-C and urine albumin-to-creatinine ratio (ACR) were measured and serum ucMGP levels were determined by ELISA. Multivariate linear regression evaluated the association of each kidney function measure with serum ucMGP levels.

Results. The mean eGFR was 76 ± 23 mL/min/1.73 m^2, and 186 subjects (22%) had moderate CKD (eGFR <60 mL/min/1.73 m^2). The mean ± SD ucMGP level was 3289 ± 1177 nM. In unadjusted analysis, each 10 mL/min/1.73 m^2 lower eGFR was associated with 101 nM lower ucMGP level. This association was only minimally attenuated in final multivariate models wherein each 10 mL/min/1.73 m^2 lower eGFR was associated with 79 nM lower ucMGP (95% confidence interval [CI]; 44 to 115; $P < 0.001$) after adjustment for age, sex, race, body mass index, blood pressure, smoking, hypertension, diabetes; and serum albumin, calcium, phosphorus, and fetuin-A levels. Similarly, in models adjusted for identical covariates, each 0.1 mg/L higher cystatin-C was associated with 39 nM lower ucMGP (95% CI 23 to 55; $P < 0.001$). In contrast, no significant association was observed between ACR and ucMGP in either unadjusted or adjusted analyses (adjusted $P = 0.17$). All associations were similar among subjects with or without diabetes (P-values for interaction > 0.50).

Conclusions. Among outpatients with stable CVD, a reduced glomerular filtration rate is associated with a decreased serum ucMGP level. In contrast, ACR is not associated with ucMGP levels. Whether ucMGP is a useful marker of vascular calcification and CVD event risk in persons with CKD deserves future study.

Keywords: atherosclerosis; chronic kidney disease; matrix Gla protein; vascular calcification

Introduction

Chronic kidney disease (CKD) is a strong risk factor for cardiovascular disease (CVD) mortality [1–4] and affects ∼13% of the US population [5]. At each stage of CKD, the risk of CVD mortality is several times higher than the risk of progression to end-stage renal disease (ESRD) [1,6,7]. Despite intensive investigation, the mechanisms responsible for this strong association remain unknown [8]. One candidate mechanism may be accelerated vascular calcification, which is highly prevalent in CKD and independently predicts CVD events [9–17]. Recent research

has demonstrated that vascular calcification is an actively regulated process and therefore may be modifiable [18,19]. Thus, understanding mechanisms of vascular calcification will provide novel insights into this disease process and may ultimately identify new therapeutic targets to prevent CVD among persons with CKD.

Matrix γ-carboxyglutamate (Gla) protein (MGP) is a potent inhibitor of vascular calcification [20]. MGP knockout mice develop severe aortic calcification and die at ∼6 weeks of age due to spontaneous aortic rupture [21]. Vascular calcification has been described in human Keutel syndrome, which is caused by MGP gene loss-of-function mutations [22]. MGP exerts its effects on vascular calcification directly, through inhibition of calcium crystal formation in conjunction with other calcification inhibitors such as fetuin-A, and indirectly, by influencing transcription factors that inhibit vascular cell differentiation to an osteoblast-like phenotype [23–25].

The activation of MGP occurs by post-translational carboxylation, a vitamin K-dependent process that is inhibited by warfarin [20]. Treatment of rats with warfarin results in vascular calcification [26] and diets enriched with vitamin K ameliorate the effect [27]. Recent studies have demonstrated that lower serum levels of uncarboxylated MGP (ucMGP)—the precursor to the active carboxylated MGP protein—are associated with vascular calcification and atherosclerosis [28,29]. In addition, persons with ESRD have lower ucMGP levels as compared to healthy controls [28–30]. It is not known, however, whether persons with less severe decrements in kidney function also have lower ucMGP levels compared to persons with normal kidney function. If so, ucMPG may be a novel factor linking CKD and vascular calcification. To that end, we evaluated the association of several measures of kidney function with serum ucMGP levels among 842 outpatients with stable CVD who had a range of kidney function from normal to moderate CKD. We hypothesized that more advanced CKD would be associated with lower serum ucMGP levels.

Subjects and methods

Study participants

The Heart and Soul Study is an observational study designed to investigate the influence of psychosocial factors on progression of CVD [31,32]. Briefly, participants were recruited from outpatient clinics in the San Francisco Bay Area if they met one of the following inclusion criteria: (i) history of myocardial infarction; (ii) angiographic evidence of >50% stenosis in one or more coronary vessels; (iii) evidence of exercise-induced ischaemia by treadmill or nuclear testing; (iv) history of coronary revascularization or (v) documented diagnosis of coronary artery disease by an internist or cardiologist. Participants were excluded if they were not able to walk one block, had a myocardial infarction within the past 6 months or were likely to move out of the area within 3 years. The study protocol was approved by the Institutional Review Boards of participating institutions and all participants provided written informed consent.

Between September 2000 and December 2002, a total of 1024 participants enrolled and underwent a daylong baseline study appointment that included a medical history interview, a physical examination and a comprehensive health status questionnaire. Fasting (12 h) serum samples were obtained and frozen at −70°C. Subjects for whom frozen serum was not available for ucMGP measurement ($n = 182$, 18%) were excluded from this analysis, resulting in a final sample size of 842 subjects for this study. Kidney function was similar among subjects with and without ucMGP measurements (Table 1).

Table 1. Comparison of kidney function in persons with or without available uncarboxylated matrix Gla protein (ucMGP) measurements

	Available ucMGP measurements ($N = 842$)	No ucMGP measurements ($N = 182$)	*P*-value
MDRD eGFR			
Mean (SD) (mL/min/1.73 m^2)	76 (23)	75 (24)	0.51
Cystatin-C			
Mean (SD) (mg/L)	1.19 (0.51)	1.24 (0.77)	0.27
Urine albumin-to-creatinine			
Median (IQR) (mg/g)	10 (6–20)	9 (6–17)	0.08

Kidney function

Serum creatinine was determined by the Jaffe reaction and was combined with age, sex and race to estimate glomerular filtration rate (eGFR) by the four-variable Modification of Diet in Renal Disease Study equation [33]. Serum cystatin-C concentrations were measured using a BNII nephelometer (Dade Behring, Inc., Deerfield, IL, USA) with a particle-enhanced immunonephelometric assay (N Latex Cystatin-C, Dade Behring, Inc.) [34]. The assay range was 0.195–7.330 mg/L; the intra-assay coefficient of variation was <2.8%, and the inter-assay coefficient of variation was <3.1%. Urine albumin and creatinine were measured by nephelometry and the Jaffe method, respectively, and urine albumin-to-creatinine ratios (mg albumin/g creatinine) were calculated.

Uncarboxylated matrix Gla protein

ucMGP was measured by competitive enzyme linked immunosorbent assay (ELISA) at the VitaK BV, Maastricht, The Netherlands) as previously described [28]. In brief, anti-ucMGP (VitaK BV, Maastricht, The Netherlands) was coupled to a microtitre plate via polyclonal rabbit anti-mouse IgG (Dako, Heeverlee, Belgium). After stringent washing, 5 μL of serum sample or standard was mixed with tracer (biotinylated peptide consisting of residues 35–54 in human MGP), transferred to the microtitre plate and incubated overnight at 4°C. After washing, the plate was incubated with streptavidine peroxidase (Zymed, Breda, The Netherlands) and stained with TMB (KPL, Gennep, The Netherlands) after washing. The process was stopped by adding H_2SO_4, and the plate was read at 450 nm. The lower limit of detection was 98 nM, and the intraassay coefficient of variation was 6% and the inter-assay coefficient was 11.4%.

Other participant characteristics and laboratory tests

Age, sex, race/ethnicity and smoking status were determined by questionnaire. Alcohol use was measured by use of the AUDIT-C questionnaire [35], with a score of ≥4 used to define regular alcohol use. Weight and height were measured at the baseline study physical examination. Body mass index (BMI) was calculated (weight [kg]/height [meters]^2). Medical history of hypertension, diabetes, myocardial infarction, angioplasty, coronary bypass and heart failure was all determined by questionnaire. Fasting blood was drawn for the measurement of albumin, calcium, phosphorus, glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride and C-reactive protein concentrations using standard clinical chemistry analysers. Serum fetuin-A was measured with a BNII nephelometric assay as described in detail elsewhere [36].

Statistical analysis

We categorized participants by tertiles of serum ucMGP levels and compared baseline characteristics across tertiles using analysis of variance (ANOVA) or the Kruskal–Wallis test for continuous variables and the chi-squared test or Fisher's Exact test for categorical variables. Next, we evaluated the association of each measure of kidney function with serum ucMGP levels using linear regression analysis. Sequential models were developed. Model 1 was unadjusted; model 2 adjusted for age, sex and race and model 3 adjusted for age, sex, race and all variables that were associated with ucMGP levels in bivariate analysis ($P < 0.05$). Urine albumin-to-creatinine was positively skewed and was therefore log-transformed to approximate a normal distribution. Urine albumin-to-creatinine results are

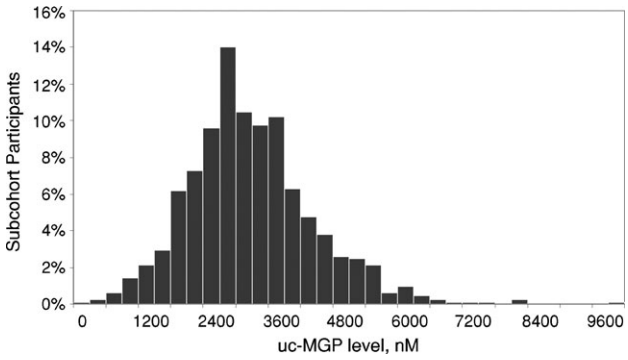

Fig. 1. Distribution of serum uncarboxylated matrix Gla protein among 842 participants with coronary artery disease.

provided as 'per doubling'. Last, we created a multiplicative interaction term (ucMGP × diabetes) to determine whether or not observed associations were similar among subjects with or without diabetes. This candidate effect modifier was selected *a priori* on the basis of previously published results [36,37]. All analyses were performed using Stata Statistical Software, version 9.2 (College Station, TX, USA).

Results

Among the 842 study participants, the mean age was 67 ± 11 years, 81% were male, 40% were non-white and 26% had diabetes mellitus. The mean eGFR was 76 ± 23 mL/min/1.73 m^2, and 186 subjects (22%) had moderate CKD (eGFR < 60 mL/min/1.73 m^2) [38]. The mean cystatin-C level was 1.19 ± 0.51 mg/L. The median albumin-to-creatinine ratio was 10 mg/g (interquartile range 6–20 mg/g), and 130 subjects (19%) had microalbuminuria (urine albumin-to-creatinine ≥ 30 mg/g) [38]. The mean ± SD ucMGP level was 3289 ± 1177 nM, and its distribution was approximately normal among the study participants (Figure 1). As compared to subjects in the lowest ucMGP tertile, those with higher ucMGP levels were younger, were more likely to drink alcohol and smoke, had higher BMI and were more frequently diabetic (Table 2). Prior CVD history was similar across ucMGP groups. Subjects with higher ucMGP also had higher diastolic blood pressure and serum albumin, calcium, phosphorus, fetuin-A, glucose, total cholesterol, and triglyceride levels. The relation of ucMGP with other candidate determinants of vascular calcification is shown in Table 3. Direct correlations were observed between ucMGP with calcium, phosphorus, and fetuin-A and were of modest strength, with the strongest of these observed with fetuin-A ($r = 0.27$, $P < 0.01$).

In unadjusted analysis, each 10 mL/min/1.73 m^2 lower eGFR was associated with 101 nM lower ucMGP levels (Table 4 and Figure 2A). This association was minimally attenuated and remained strongly statistically significant with adjustment for age, sex and race as well as in the final multivariate model. eGFR in isolation accounted for ∼4% of the variance in ucMGP levels, whereas the multivariable model collectively accounted for 20% of its variance. Results were similar when cystatin-C was evaluated as the measure of kidney function (Table 4 and Figure 2B). In the final adjusted model, each 0.1 mg/L increase in cystatin-C was associated with a 39 nM lower ucMGP level. In contrast, we observed no significant association between the albumin-to-creatinine ratio and serum ucMGP levels in either unadjusted or multivariable adjusted models (Table 4 and Figure 2C). All associations were similar among subjects with or without diabetes (P-values for interaction >0.50).

Discussion

We found that mild to moderate decrements in glomerular filtration rate were associated with lower serum ucMGP levels in persons with stable CVD. In contrast, urine albumin-to-creatinine levels were not associated with serum ucMGP levels. Prior studies have demonstrated that subjects with atherosclerosis and cardiac valve calcification have lower ucMGP levels than healthy controls. Thus, serum ucMGP may ultimately prove useful as a marker of the presence and severity of vascular calcification in persons with CKD.

Although our cross-sectional study results cannot determine the direction of association between kidney function and ucMGP, we believe it is unlikely that decreased kidney clearance directly leads to lower ucMGP levels in this study. Like creatinine, most substances accumulate in serum, rather than decline, as a direct result of decreased GFR, and MGP is nearly absent from the urine of normal individuals [39], because it complexes in blood with larger molecules [25]. Furthermore, while MGP may be extracted from the circulation by the kidney, as shown by simultaneous renal vein and artery sampling, the rate of extraction is independent of GFR across mild to moderate stages of CKD [39].

We propose three potential pathways that may account for the association between decreased GFR and lower serum ucMGP. Firstly, metabolic or genetic abnormalities associated with kidney dysfunction may suppress ucMGP production. For example, vitamin D deficiency is highly prevalent in CKD [40] and suppresses ucMGP production [41]. Transforming growth factor beta (TGF-β) is up-regulated in certain forms of CKD [42] and down-regulates vascular smooth muscle cell MGP transcription [41]. Polymorphisms in the promoter region of the MGP gene, which alter affinity for the transcriptional factor complex AP-1, influence MGP expression in humans [43]. Compared to healthy individuals, these same polymorphisms that associate with lower MGP expression are more prevalent among those on dialysis [44].

Secondly, predicated on the development of severe vascular calcification in MGP knockout mice, it is possible that low ucMGP may be causally related to the development of vascular calcification. Associated haemodynamic changes and vascular stiffness, in turn, may contribute to kidney dysfunction.

Finally, it is possible that reduced kidney function may directly lead to vascular calcification, which, when abundant, may decrease serum ucMGP because ucMGP has affinity for hydroxyapatite deposited within the vasculature [26,45]. Prior studies have demonstrated that serum MGP levels decline when arteries calcify in animal models and that lower ucMGP levels are associated with vascular calcification and atherosclerosis in select human populations [26,28,29].

Table 2. Baseline measures by uncarboxylated matrix Gla protein (ucMGP) tertiles

Characteristics	ucMGP (nM) Tertile 1 <2766 ($n = 282$)	Tertile 2 2766–3650 ($n = 280$)	Tertile 3 >3650 ($n = 280$)	*P*-value
Demographics				
Age (year)	71 (63–79)	68 (60–76)	64 (56–71)	<0.001
Male sex, no. (%)	239 (85)	227 (81)	219 (78)	0.14
Race				0.56
Caucasian, no. (%)	180 (64)	176 (60)	159 (57)	
African American, no. (%)	42 (15)	47 (17)	47 (17)	
Other, no. (%)	60 (21)	65 (23)	73 (26)	
Regular alcohol use, no. (%)	70 (25)	76 (27)	94 (34)	0.06
Regular tobacco use, no. (%)	44 (16)	61 (22)	66 (24)	0.05
Body mass index (kg/m^2)[a]	27 (24–30)	27 (25–31)	29 (26–33)	<0.001
Medical history				
Hypertension, no. (%)	193 (69)	211 (75)	200 (72)	0.26
Diabetes, no. (%)	57 (20)	74 (27)	91 (33)	0.01
Myocardial infarction, no. (%)	159 (57)	141 (51)	147 (53)	0.33
Angioplasty, no. (%)	105 (38)	108 (39)	115 (41)	0.64
Coronary bypass, no. (%)	107 (38)	108 (39)	91 (33)	0.30
Heart failure, no. (%)	45 (16)	54 (19)	52 (19)	0.57
Medication use				
Anticoagulant/thrombolytic, no. (%)	20 (7)	20 (7)	23 (8)	0.85
Measurements				
SBP (mmHg)	132 (22)	135 (22)	133 (20)	0.10
DBP (mmHg)	72 (10)	75 (12)	76 (11)	<0.001
Albumin (g/dL)[a]	3.8 (3.6–4.0)	3.9 (3.7–4.1)	4.0 (3.8–4.2)	<0.001
Calcium (mg/dL)	9.4 (0.5)	9.5 (0.5)	9.6 (0.5)	<0.001
Phosphorus (mg/dL)[a]	3.6 (3.2–3.9)	3.7 (3.3–4.0)	3.7 (3.3–4.1)	0.02
Fetuin-A (g/L)[a]	0.60 (0.52–0.68)	0.64 (0.57–0.73)	0.68 (0.60–0.79)	<0.001
Glucose (fasting) (mg/dL)[a]	104 (95–117)	108 (99–123)	111 (101–137)	<0.001
Cholesterol (mg/dL)[a]	163 (143–186)	169 (145–191)	181 (157–210)	<0.001
HDL (mg/dL)	47 (14)	45 (14)	46 (14)	0.34
Triglycerides (mg/dL)[a]	88 (63–136)	111 (79–157)	130 (87–207)	<0.001
CRP (mcg/dL)[a]	2.27 (1.00–4.92)	2.21 (0.84–5.43)	2.32 (1.01–4.97)	0.95

CRP, C-reactive protein; DBP, diastolic blood pressure; HDL, high-density lipoprotein cholesterol; SBP, systolic blood pressure.
Data are presented as mean (SD) unless otherwise specified.
[a]Median (intra-quartile range).

Table 3. Correlation of serum uncarboxylated matrix Gla protein (ucMGP), fetuin-A, calcium and phosphorus

	ucMGP	Fetuin-A	Calcium	Phosphorus
ucMGP	1			
Fetuin-A	0.2716	1		
Calcium	0.1874	0.1631	1	
Phosphorus	0.1021	0.0904	0.1591	1

Pearson correlation coefficients showing levels of correlation between measured mediators of calcification ($P < 0.01$ each measure).

Future studies with repeated measures of ucMGP, kidney function and vascular calcification are required to differentiate between these possibilities but it is our hypothesis that this final possibility is most likely. Two important post-translational modifications of matrix Gla protein exist: (1) a carboxylation step which is dependent on vitamin K and inhibited by warfarin, and (2) phosphorylation of serine residues within the protein [46]. Carboxylation has been shown to be necessary for MGP's ability to inhibit the 'pro-osteoblastic' transcription factor BMP-2 [23,24] and is also necessary for binding to a circulating fetuin-A complex, which may be important to solubilize circulating calcium crystals [25]. Phosphorylated ucMGP likely retains its affinity for calcium and it has been shown to colocalize in the vessel walls of calcified arteries [45], potentially depleting serum levels in persons with vascular calcification. The present analysis uses an assay that detects both the phosphorylated and un-phosphorylated forms of ucMGP, so competing influences of (1) upregulated MGP transcription in response to vascular stress and (2) the absorptive forces of already calcified vessels will likely dictate serum ucMGP levels (Figure 3). It is likely that the latter factor will be dominant in the setting of vascular calcification, as most MGP produced is in a phosphorylated state [46]. New assays that can distinguish the phosphorylation status of ucMGP are being produced and may provide important insights into future research.

The association of CKD with ucMGP has implications for prior and future studies evaluating ucMGP as a marker of vascular calcification or CVD events. Because there was no adjustment for severity of CKD in prior studies, it is possible that the association of ucMGP with vascular calcification reported previously [28] may reflect residual confounding by kidney disease. On the basis of our results, future studies evaluating the association of ucMGP with vascular calcification should adjust for the severity of CKD to determine

Table 4. Association of kidney function with the uncarboxylated matrix Gla protein (ucMGP)

	Coefficient of regression (ß)	95% Confidence interval	*P*-value	R^2
Change in ucMGP per 10 mL/min/1.73 m^2 decrease in estimated glomerular filtration rate				
Unadjusted	−101	−135 to −67	<0.001	0.039
Adjusted for age, sex and race	−71	−107 to −35	<0.001	0.073
Multivariable adjusted[a]	−79	−115 to −44	<0.001	0.197
Change in ucMGP per 0.1 mg/L increase in cystatin-C				
Unadjusted	−47	−62 to −32	<0.001	0.041
Adjusted for age, sex and race	−38	−53 to −23	<0.001	0.082
Multivariable adjusted[a]	−39	−55 to −23	<0.001	0.200
Change in ucMGP per doubling of urine albumin/creatinine[b]				
Unadjusted	−26	−71 to 20	0.27	0.002
Adjusted for age, sex and race	−23	−68 to 21	0.30	0.068
Multivariable adjusted[a]	−33	−82 to 15	0.17	0.173

[a]Adjusted for age, sex, race, body mass index (BMI), blood pressure, albumin, smoking status, calcium, phosphorus, hypertension, diabetes and fetuin-A.
[b]Data were obtained after natural log-transformation; Beta from naturally log-transformed data was multiplied by 0.693 so as to represent the predicted change in albumin/creatinine per doubling of ucMGP.

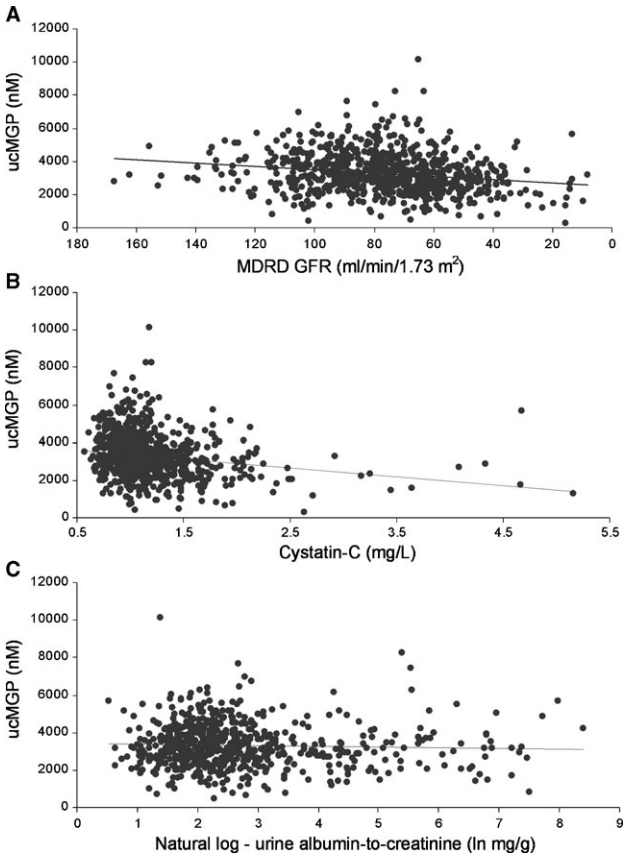

Fig. 2. Association of MDRD eGFR (2A), cystatin-C (2B) and urine albumin-to-creatinine ratio (2C) with serum ucMGP.

if the associations remain. Furthermore, a number of other novel regulators of vascular calcification have been identified, including fetuin-A [47], fibroblast growth factor 23 [48] and pyrophosphate [49]. Because these factors likely work in concert, future studies evaluating the associations of ucMPG with vascular or valvular calcification may benefit from the measurement of other regulators simultaneously to determine their independent and potential additive effects. Because several of these novel inhibitors appear not only to influence vascular calcification, but may also influence insulin resistance [50–52], future studies should also evaluate whether associations are similar among persons with or without diabetes.

The strengths of this study include the measurement of ucMGP rather than total MGP. The association of lower serum ucMGP with atherosclerosis or vascular calcification has been consistent [28,29] whereas the association of total serum MGP with vascular calcification has provided conflicting results [53–56]. Additional strengths include the relatively large study population, availability of several markers of kidney function and other molecules involved in regulation of vascular calcification and measurement of multiple potential confounding variables.

The manuscript also has important limitations. Firstly, all participants had prevalent CVD and most were older men. Results may differ in younger persons, women and persons without CVD. Dietary vitamin K intake was not measured and warfarin use was infrequent in our study sample. Frozen serum was not available for ucMGP measurement among 18% of Heart and Soul participants. However, kidney function was similar in subjects with and without available ucMGP data. The study participants had moderate CKD, at most, and our results may not generalize to persons with advanced stage CKD. However, prior studies have demonstrated that ucMGP are also lower in persons with ESRD compared to healthy controls [28–30]. We cannot exclude that the observed associations may reflect residual confounding by unmeasured factors. However, to explain the strength of associations observed here, any such factor would have to be strongly linked with both moderate CKD and serum ucMGP levels.

In conclusion, mild to moderate decrements in glomerular filtration rate are strongly associated with lower serum ucMGP levels in out-patients with stable CVD. Future studies should evaluate whether serum ucMGP may account for

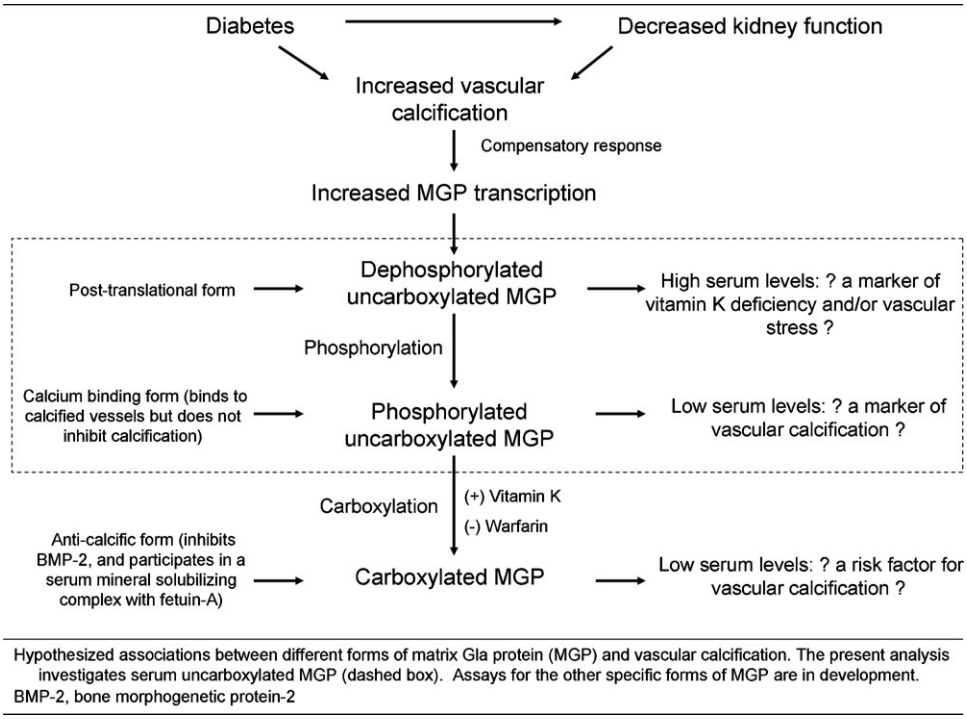

Fig. 3. Different forms of the matrix Gla protein and their hypothesized association with vascular calcification.

some of the association of CKD with vascular calcification and whether serum ucMGP levels may identify individuals at increased risk of future CVD events.

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Conflict of interest statement. None declared.

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