Ciprian N. Silaghi, Daniela Fodor and Alexandra M. Crăciun*

Circulating matrix Gla protein: a potential tool to identify minor carotid stenosis with calcification in a risk population

Abstract

Background: Carotid calcification is an independent marker for future ischemic events, which are more frequently encountered in postmenopausal women as the prevalence of type 2 diabetes mellitus (T2DM) and hypertension (HT) increases. Matrix Gla protein (MGP) is a major inhibitor of vascular calcification. Here, we report on the prospect of serum MGP to become an identifying tool for minor carotid stenosis (minCAS) with calcification in a risk population.

Methods: Based on carotid ultrasound examination, out of 72 enrolled postmenopausal women, 33 had minCAS with carotid calcification (minCAS group) and 39 were without minCAS and carotid calcification (non-minCAS group). Serum total MGP, high-sensitivity C-reactive protein (hs-CRP), bone mineral density (BMD) and carotid intima-media thickness (CIMT) were determined.

Results: We found significantly elevated serum MGP levels in the minCAS compared to the non-minCAS group ($p<0.05$). MGP was independently associated with hs-CRP (unstandardized β-regression coefficient=2.6; 95% CI 0.007–5.3; p=0.049) and CIMT (β=−611.3; 95% CI −1172.6–−49.9; p=0.034) within the minCAS group, but not with BMD. Furthermore, significantly higher MGP levels were determined in two minCAS subgroups (one with HT or T2DM and second with both diseases) compared to a non-minCAS subgroup with HT or T2DM ($p<0.05$ and $p<0.01$, respectively). A threshold of 87.9 μg/L serum MGP (area under the receiver operating characteristic=0.72±0.06; 95% CI 0.60–0.84; p=0.001) may identify minCAS with calcification in postmenopausal women with 63% precision.

Conclusions: Higher circulating MGP levels could help identify minCAS with calcification in a relatively homogenous risk population (i.e., postmenopausal women), regardless of underlying cardiovascular risk factors.

Keywords: calcification; carotid stenosis; C-reactive protein; intima-media thickness; matrix Gla protein.

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Introduction

Vascular calcification has captured the interest of researchers due to a growing body of literature regarding its association with stroke, myocardial infarction and mortality [1]. Nowadays, it is recognized that a major mechanism by which vascular calcification arises is the loss of the calcification inhibitors [2].

One of the key proteins involved in ectopic calcification process is matrix Gla protein (MGP), an extracellular calcification inhibitor, mainly secreted by vascular smooth muscle cells and chondrocytes [3, 4]. MGP is present in the media and intima of calcified arteries, in atherosclerotic plaque and other tissues [5–7], where a vitamin K-dependent γ-carboxylation is a prerequisite for its activation in order to perform calcium binding and inhibition of bone morphogenetic protein-2 activity [8, 9].

Depending on its two post-translational modifications (carboxylation and phosphorylation), different conformational enzyme-linked immunosorbent assays (ELISA) have been developed: competitive ELISA for desphosphorylated MGP (dpMGP) [10] and uncarboxylated MGP (ucMGP) [5] or sandwich ELISA for desphosphorylated-carboxylated MGP and desphosphorylated-uncarboxylated MGP (dp-ucMGP) [11]. Circulating dpMGP was found to be high in severe atherosclerosis [10], type 2 diabetes mellitus (T2DM) and ischemic heart disease [12], or low and even unmodified in various cardiovascular diseases [13]. Low serum ucMGP levels in patients with angioplasty or aortic stenosis [14] and a positive correlation with total arterial calcium score were demonstrated [15]. Therefore, ucMGP was designated as a

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biomarker of prevalent arterial calcification [14], uninfluenced by systemic vitamin K status [16]. The homogenous dp-ucMGP conformation was elevated in patients with chronic kidney disease [11], calcified valvular aortic stenosis [17] and positively correlated with the aortic calcification score [11] and long-term mortality [17], thus allowing consideration of a robust marker for vascular vitamin K status, disease itself or a predictor of cardiovascular outcomes.

Only a small amount literature has been published on MGP assessment in carotid atherosclerosis. In fact, neither carotid calcification scores [15], nor carotid intima-media thickness (CIMT) [18] were associated with circulating ucMGP. The increase of CIMT is frequently used as a surrogate marker for atherosclerosis in populations with cardiovascular risk [19]. Also C-reactive protein (CRP), a low-grade inflammation marker, has been elusively associated with dpMGP in patients with T2DM and ischemic heart disease [12].

The presence of calcified carotid atherosclerotic plaque could independently predict vascular ischemic events [20], the risk for stroke or non-stroke vascular death being relatively high (5.7%–24.0%) even in patients with minor carotid stenosis (minCAS) [21]. Moreover, the cardiovascular risk is greater in postmenopausal women than in men as the prevalence of T2DM, obesity or hypertension (HT) becomes higher [22], with the highest risk among women with low bone mineral density (BMD) [23]. Considering carotid calcification an independent marker for stenosis and ischemic events [24], new tools should help identify individuals with calcified minCAS as early as possible.

In this context, the present study aims to investigate the relationship between serum MGP (designated as total MGP because the undiscriminating capacity between different MGP conformations) and minCAS with calcification. We also examined the interplay between serum MGP, inflammation (CRP) and atherosclerosis (CIMT), presuming that serum MGP levels would be independently associated with CIMT and CRP. Even if osteoporosis has no influence on serum MGP [10, 13], we wanted to exclude the potential confounding effect of low BMD in our population. The main objective was to establish whether circulating total MGP could differentiate between the presence and absence of minCAS with calcification in a relatively homogenous risk population.

Materials and methods

Subjects and study design

Over a 6-month period, we recruited 72 consecutive Caucasian postmenopausal women who had been referred to our Internal Medicine outpatient clinic for health-screening tests, due to the presence of at least one cardiovascular risk factor (HT, T2DM, hyperlipidemia, overweight/obesity or smoking). All participants underwent carotid ultrasound examination and also BMD assessment. The presence or absence of minCAS with calcification was considered inclusion criterion. Enrolled women had been in menopause for at least 2 years. Patients with heart failure, moderate or major carotid stenosis [25], severe HT (taking three or more different antihypertensive drugs), history of ischemic events, intravascular stents, renal and rheumatoid diseases, hysterectomy, insidious malignancies and ongoing infections were excluded. Enrolled subjects had not received therapy with anticoagulants, vitamin D, calcium, calcium antagonists, bisphosphonates, corticosteroids or hormone replacement drugs for the past 2 years. Our cross-sectional study design complied with the declaration of Helsinki and was approved by the Medical Ethics Committee of our University. Informed consent was obtained from all subjects.

Among postmenopausal women, n=39 were without minCAS and free of carotid calcification (designated as the non-minCAS group) and n=33 had minCAS with at least one calcified carotid deposit (designated as the minCAS group). Blood pressure was measured using a sphygmomanometer on the right arm, after the subject had been seated for at least 5 min. Two measurements were performed at 20 min interval and their average was recorded. Body mass index (BMI) was calculated dividing the weight in kilograms by the square of the height in meters (kg/m^2). HT was defined as systemic arterial blood pressure ≥140/90 mm Hg or treatment with antihypertensive drugs. T2DM was defined either as self-reported, therapy with oral glucose lowering drugs, or fasting glucose level ≥7 mmol/L [26]. Smoking habit was defined as more than 2 years of smoking history in the past 5 years or current smoker.

Carotid ultrasound assessment

Bilateral measurements of CIMT in the common carotid artery (CCA) were recorded by B-mode imaging (Aloka Prosound alpha 10, Tokyo, Japan) with a 7.5–13 MHz linear transducer. The left and right CCA were scanned longitudinally with the patient in the supine position and the image focused on the posterior (far) wall of the artery. Consequently, CIMT was assessed as the distance (mm) between the two parallel echoic lines (lumen-intima interface and media-adventitia interface). Three measurements were conducted for each patient, proximally to the bifurcation of the CCA: at 10, 15 and 20 mm, respectively. The average value of the three measurements was recorded as intima-media thickness (IMT) of the left or right CCA. Additionally, the mean CIMT was registered as the average of the six measurements (three in each CCA). If an atherosclerotic plaque (defined as a focal widening with protrusion into the lumen) was present in the specified area, the measurement was performed outside the edges of the plaque. The intra-observer coefficient of variation (CV) for mean CIMT was 7.6%, consistent with the literature [27].

Carotid calcification was defined as a bright echogenic deposit with acoustic shadowing, present either in the common or the first 15 mm of the internal or external carotid artery walls. The minCAS was defined as the presence of atherosclerotic plaque accompanied by a luminal reduction of <50% of the internal carotid artery and a peak systolic velocity <125 cm/s [25].

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BMD measurements

BMD was assessed in three sites (L2-L4 in the lumbar region, femoral neck and total hip) by dual-energy X-ray absorptiometry (Lunar Prodigy Advance, GE Lunar, Madison, WI, USA). BMD values (g/cm^2), Z-score [the standard deviation (SD) from the mean BMD of the age-sex matched population] and T-score (the SD from the mean BMD of a healthy young adult) were recorded. The intra-observer CV for BMD measurement was 1.1%.

Based on the total hip T-score [28], n=38 postmenopausal women had normal bone mass (T-score ≥–1), n=30 had osteopenia (T-score <–1 to >–2.5) and n=4 had osteoporosis (T-score ≤–2.5). Due to the low number of patients with osteoporosis, the last two categories were considered as one (osteopenia and osteoporosis group, n=34).

Biochemical assays

Blood was collected by venipuncture after a 10-hour fasting, then centrifuged at 1000 *g* for 10 min and stored at –80°C until assaying.

Total cholesterol, high-density lipoprotein cholesterol (HDL-C), triglycerides and glucose were measured using enzymatic methods with an automated chemistry analyzer (Cobas Mira Plus, Roche Diagnostic, Basel, Switzerland). The intraday CV for each parameter was <5%. Low-density lipoprotein cholesterol (LDL-C) was calculated by Friedewald's formula [total cholesterol – (HDL-C + triglycerides/5)].

Serum high-sensitivity CRP (hs-CRP) was determined by an immunoturbidimetric assay (CRP U-hs, Diasys Diagnostic System, Holzheim, Germany) with an auto-chemistry analyzer (CS-T240, Dirui, Changchun, China). The lower detection limit was 0.3 mg/L and the intraday CV was 1.8%.

Serum MGP was measured with an ELISA kit (USCN Life Science Inc., Wuhan, China) using Organon Reader 230S (Organon Teknika, Oss, The Netherlands), in accordance with the manufacturer's instruction. The sensitivity of the assay and our intraday CV was 20 ng/L and 6.5%, respectively.

Statistical analysis

According to distribution (Kolmogorov-Smirnov test), variables were expressed as mean±SD or median (minimum to maximum value). Comparison between two groups was performed with Student's t-, Mann-Whitney U or χ^2-tests. Also the effect size (Cohen's *d*) for MGP, hs-CRP and mean CIMT was computed to assess the magnitude of differences between the minCAS and non-minCAS groups. Correlations were reported as Spearman's rho coefficient (r). To assess the relationship between MGP, CIMT and hs-CRP, univariate regression analyses were followed by multivariate linear regression analysis, with MGP appointed as a dependent variable. Unstandardized β-regression coefficient (β) with 95% CI and coefficient of determination (r^2) were reported. Kruskal-Wallis test was used for comparison between three subgroups (designated by the presence of HT or T2DM) and pairwise comparisons were adjusted for multiplicity with a Shaffer correction. The receiver operating characteristic (ROC) for MGP was constructed to assess the area under the ROC curve and the threshold value for discriminating between minCAS and non-minCAS subjects (an area of 1 represents an ideal and of 0.5, an insignificant test). Statistical analysis was completed with the SPPS software v.15.0 (SPSS inc., Chicago, IL, USA). Based on the two-tailed tests, p<0.05 was considered statistically significant.

Results

Among postmenopausal women, the prevalence of minCAS with calcification was 46%. We found significantly elevated serum MGP, hs-CRP and CIMT values in the minCAS compared to the non-minCAS group, and also a large size effect: Cohen's *d* was 1.61 for mean CIMT, 0.83 for MGP and 0.52 for hs-CRP. On the contrary, serum MGP, hs-CRP or CIMT were not significantly modified when women on different medication were compared and no correlation of these parameters with drug therapy was noticed. The characteristics of the study population are summarized in Table 1.

As expected, mean CIMT was strongly correlated with both IMT of the right CCA (r=0.94; p<0.001) and left CCA (r=0.95; p<0.001) in postmenopausal women. Due to these strong correlations, only mean CIMT was considered for further analyses. Correlations of MGP, hs-CRP and mean CIMT are presented in Table 2.

In order to evaluate the influence of low BMD on circulating MGP, postmenopausal women were stratified in two groups: normal bone mass (n=38) vs. osteopenia and osteoporosis (n=34). We did not find a significant difference in serum MGP (88±28 vs. 94±35; p=0.48), hs-CRP (5.6±3.3 vs. 6.1±3.6; p=0.63) or mean CIMT (0.94±0.22 vs. 0.96±0.24; p=0.64) between the two groups. Also MGP, hs-CRP and CIMT were not correlated with BMD or Z-scores in neither of groups.

In respect to minCAS group, MGP was independently associated only with mean CIMT and hs-CRP, as depicted in Figure 1. There was no association between MGP, hs-CRP and mean CIMT within the non-minCAS group.

Next, we performed a multivariate linear regression analysis with MGP pointed as dependent variable, and both mean CIMT and hs-CRP as independent variables. Only the association between MGP and mean CIMT (β=–568.9; 95% CI –1108.1 to –29.8; r^2=0.117; p=0.039) was preserved.

To emphasize the differences between the presence and absence of minCAS with calcification, we compared three subgroups, designated by the presence of HT or T2DM, as follows: non-minCAS with HT or T2DM (n=23), minCAS with HT or T2DM (n=28) and minCAS with both HT and T2DM (n=10). Because only n=5 of non-minCAS postmenopausal women had both HT and T2DM, they were not considered for statistical analysis. Even if a

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	All (n=72)	non-minCAS (n=39)	minCAS (n=33)	p-Value
Anthropometric and clinical characteristics				
Age, years	64±10	61±9	67±9	<0.05
Years since menopause	16±10	13±9	20±10	<0.05
BMI, kg/m^2	29±5	28±5	30±5	0.17
Smokers, n (%)	24 (33)	10 (26)	14 (42)	0.13
HT, n (%)	44 (61)	21 (54)	23 (70)	0.16
T2DM, n (%)	22 (31)	7 (18)	15 (45.5)	<0.05
Both HT and T2DM, n (%)	15 (21)	5 (13)	10 (30)	0.07
Osteopenia and osteoporosis, n (%)	34 (47)	15 (38)	19 (57.5)	0.16
SBP, mm Hg	140±21	131±18	150±19	<0.001
DBP, mm Hg	81±11	79±10	85±10	<0.05
Medication				
Angiotensin-converting enzyme inhibitors, n (%)	23 (32)	10 (26)	13 (39)	0.21
Angiotensin II type I receptor blockers, n (%)	5 (7)	2 (5)	3 (9)	0.65
β-blockers, n (%)	18 (25)	8 (21)	10 (30)	0.33
Statins, n (%)	13 (18)	6 (15)	7 (21)	0.52
Fibrates, n (%)	4 (6)	2 (5)	2 (6)	0.86
Glucose lowering drugs, n (%)	17 (24)	7 (18)	10 (30)	0.21
Imaging measurements				
IMT of the right CCA, mm	0.95±0.23	0.82±0.18	1.11±0.19	<0.001
IMT of the left CCA, mm	0.96±0.25	0.83±0.2	1.11±0.21	<0.001
Mean CIMT, mm	0.95±0.23	0.82±0.18	1.11±0.18	<0.001
Lumbar spine BMD, g/cm^2	1.02±0.19	1.02±0.19	1.01±0.19	0.82
Lumbar spine Z-score	−0.22±1.32	−0.22±1.36	−0.21±1.29	0.99
Femural neck BMD, g/cm^2	0.86±0.15	0.88±0.15	0.84±0.15	0.34
Femural neck Z-score	−0.01±0.92	0.02±0.95	−0.03±0.91	0.82
Total hip BMD, g/cm^2	0.51 (−2.6–1.4)	0.61 (−2.6–1.4)	0.34 (−0.7–1.2)	0.19
Total hip Z-score	0.32±1.05	0.39±1.19	0.22±0.85	0.52
Total hip T-score	−0.64±1.14	−0.44±1.12	−0.88±1.15	0.12
Biochemical analyses				
Total cholesterol, mmol/L	5.46±1.15	5.39±1.00	5.54±1.31	0.59
HDL-C, mmol/L	1.36±0.34	1.29±0.27	1.43±0.41	0.11
LDL-C, mmol/L	3.44±1.18	3.41±1.05	3.46±1.35	0.86
Triglycerides, mmol/L	1.40±0.67	1.39±0.56	1.40±0.80	0.96
Fasting glucose, mmol/L	5.96±1.47	6.04±1.64	5.86±1.26	0.61
hs-CRP, mg/L	5.85±3.46	5.04±2.93	6.80±3.83	<0.05
MGP, μg/L	91±31	80±28	104±30	<0.05

Table 1 Study population characteristics (n=72).
Data are presented as mean±standard deviation, median (minimum to maximum) or number and percentage n (%), as appropriate. The p-values correspond to differences between the minCAS and the non-minCAS groups. BMD, bone mineral density; BMI, body mass index; CCA, common carotid artery; CIMT, carotid intima-media thickness; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; HT, hypertension; IMT, intima-media thickness; LDL-C, low-density lipoprotein cholesterol; MGP, matrix Gla protein; minCAS, minor carotid stenosis with carotid calcification; non-minCAS, without minor carotid stenosis and free of carotid calcification; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus.

similar ascending trend was noticed for serum MGP, hs-CRP and mean CIMT, as depicted in Figure 2, only MGP and mean CIMT were significantly elevated in the minCAS subgroups with HT or T2DM and both diseases, compared to the non-minCAS subgroup with HT or T2DM.

Finally, using a ROC curve depicted in Figure 3, we established an optimum threshold value of 87.9 μg/L for serum MGP (area under the ROC=0.72±0.06; 95% CI 0.60–0.84; p=0.001) that may discriminate between minCAS and non-minCAS subjects with 73% sensitivity and 64% specificity. Also the positive and negative predictive values for this threshold were 63% and 73.5%, respectively.

Discussion

The present study was designed to investigate the relationship between circulating MGP and minCAS with calcification in postmenopausal women due to their high

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	MGP	Mean CIMT	hs-CRP
minCAS with calcification	0.38[b]	0.64[c]	NS
SBP	0.38[b]	0.52[c]	0.35[b]
DBP	0.27[a]	NS	NS
Smoking	NS	NS	NS
T2DM	NS	0.31[b]	NS
Age	NS	0.45[c]	NS
Years since menopause	NS	0.44[c]	NS
BMI	NS	NS	0.32[b]

Table 2 Correlations of MGP, mean CIMT and hs-CRP in study population (n=72).
Values represent r (Spearman's Rho correlation coefficient) with [a]p<0.05, [b]p<0.01, [c]p<0.001 and NS, not significant. BMI, body mass index; CIMT, carotid intima-media thickness; DBP, diastolic blood pressure; hs-CRP, high-sensitivity C-reactive protein; MGP, matrix Gla protein; minCAS, minor carotid stenosis; SBP, systolic blood pressure; T2DM, type 2 diabetes mellitus.

cardiovascular risk [22, 23]. The higher prevalence of calcified plaque in our study population (46% vs. 32% found by Prabhakaran et al.) [20] could be related either to the higher CIMT or the relative homogeneity of our group. As evidence, the mean CIMT of minCAS group was above the upper cut-off value reported for pathologically increased CIMT (≥0.90–1.0 mm) [29, 30] and the serum hs-CRP level was higher than the conventional cut-off value of 3 mg/L for high cardiovascular risk [31].

To our knowledge, this is the first study that demonstrates significantly higher serum total MGP levels (unable to discriminate between carboxylated/uncarboxylated or phosphorylated/desphosphorylated conformations) in the minCAS compared to the non-minCAS subjects, within a relatively homogenous risk population. As atherosclerotic plaque evolves concurrently with the calcification process, ucMGP (inactive) becomes more abundant around intimal calcium deposits emphasizing a poor vascular vitamin K status [5]. Therefore, local MGP expression is enhanced by a feedback mechanism as carotid atherosclerosis develops and the high total MGP level found in the minCAS group is probably the reflection of a greater release into the circulation due to its local abundance but poor carboxylation.

Significantly elevated MGP and mean CIMT were noticed in the minCAS subgroup with HT or T2DM compared to the non-minCAS subgroup with HT or T2DM, with the highest levels exhibited by the minCAS subgroup with both diseases. This additive effect on serum MGP is congruent with recent findings in which dpMGP [12] or ucMGP [32] were assessed. Hence, we may state that high serum total MGP levels can distinguish between the presence and absence of minCAS with calcification in postmenopausal women, regardless of underlying risk factors (e.g., HT and T2DM). Even if medial calcium deposits (related to diabetes mellitus) occur independently of intimal calcifications (associated with atherosclerosis), vascular smooth muscle cells may be related to both processes [33], either by their migration and proliferation, or by osteogenic metaplasia which induces extracellular matrix mineralization [34]. The synthesis and secretion of MGP by smooth muscle cells is triggered by the presence of extracellular calcium [35]. By contrast, lower MGP mRNA levels were found in the vascular tissue of diabetic limbs, implying an increase predisposition to vascular calcification for diabetic patients [36]. It is yet unknown to which extent each tissue contributes to the total serum MGP pool. Prolonged contact with high glucose levels or macro- and microvascular complications of T2DM may upregulate MGP synthesis in tissues other than vascular, thus providing additional contribution to its circulating pool. For instance,

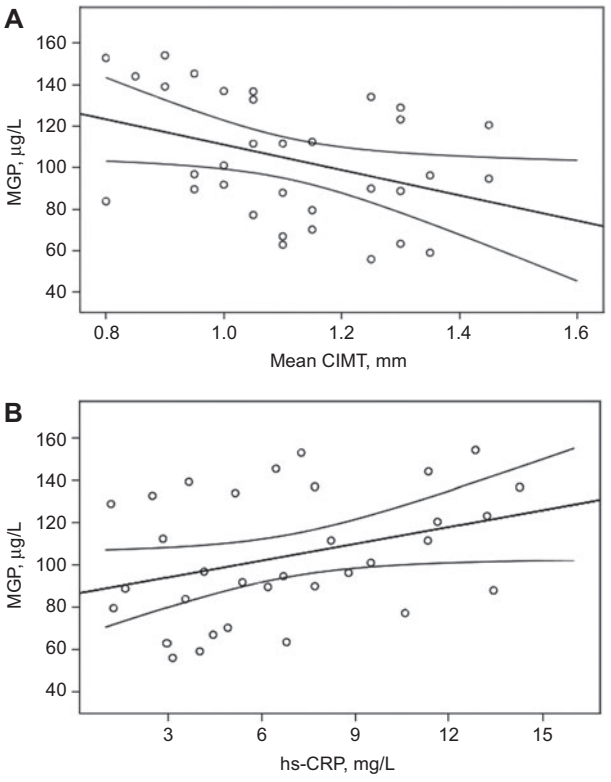

Figure 1 Independent associations of serum MGP within the minCAS group (n=33).
(A) negative linear association between MGP and mean CIMT (β=−611.3; 95% CI −1172.6 to −49.9; r^2=0.137; p=0.034);
(B) positive linear association between MGP and hs-CRP (β=2.6; 95% CI 0.007–5.3; r^2=0.119; p=0.049). Unstandardized β-regression coefficient (β) with 95% confidence interval (95% CI) and coefficient of determination (r^2) are reported. CIMT, carotid intima-media thickness; hs-CRP, high-sensitivity C-reactive protein; MGP, matrix Gla protein; minCAS, minor carotid stenosis.

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the human epidermal growth factor can cause an extensive reduction of MGP expression in rat kidney cells [37], but its receptor is desensitized by high glucose levels [38],

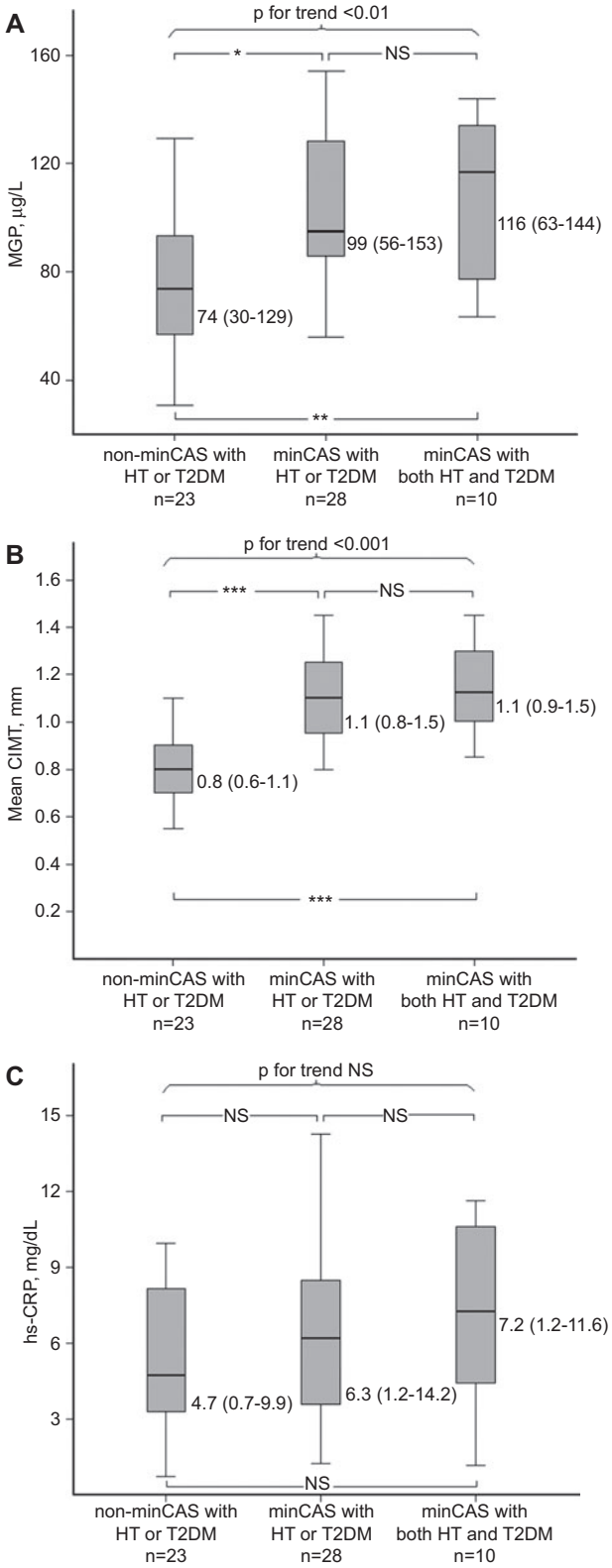

Figure 2 Comparison of serum MGP (A), mean CIMT (B) and serum hs-CRP (C) between three subgroups, designated by the presence of HT or T2DM.

Median is depicted as horizontal bar within boxes. Bottom and top of the box are 25th and 75th percentiles; error bars represent 95% confidence interval. Data are expressed as median (minimum-maximum values); * p<0.05; ** p<0.01; *** p<0.001; NS, not significant. CIMT, carotid intima-media thickness; hs-CRP, high-sensitivity C-reactive protein; HT, hypertension; MGP, matrix Gla protein; minCAS, minor carotid stenosis with carotid calcification; non-minCAS, without minor carotid stenosis and free of carotid calcification; T2DM, type 2 diabetes mellitus.

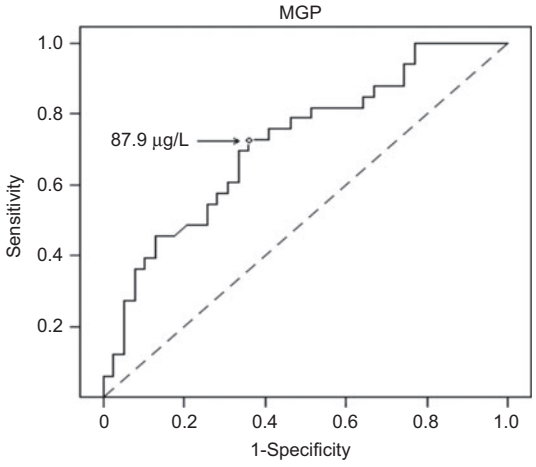

Figure 3 The receiver operating characteristic (ROC) curve for serum MGP.

The dotted reference line represents the area of 0.5 for an insignificant test (area of 1 indicates an ideal test). The area under the ROC curve was 0.72±0.06 (95% CI 0.60–0.84; p=0.001). Arrow indicates the 87.9 µg/L optimum threshold of serum MGP which discriminates between minCAS and non-minCAS group with 73% sensitivity and 64% specificity.

supporting the hypothesis of a transcriptional upregulation of MGP in diabetes by tissues other than vascular.

Insignificant differences in mean CIMT, hs-CRP and MGP levels were noticed between normal bone mass and osteopenia and osteoporosis group. Even if vascular calcification appeared to be associated with osteoporosis [39], dpMGP levels of osteoporotic patients were generally close to the population reference values [10, 13]. In line with previous findings, we confirmed that total MGP was not influenced by low BMD, although postmenopausal women were mainly osteopenic.

Our hypothesis, that serum MGP would be independently associated with CIMT and CRP, was validated by the present study. MGP was also positively correlated with the presence of minCAS with calcification, SBP and DBP. First, we found that higher MGP levels were independently predicted by the lower mean CIMT in the minCAS

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group. Earlier studies conducted in children on dialysis or renal transplantation [18, 40] failed to establish a correlation between CIMT and circulating ucMGP. CIMT reflects vascular remodeling by intimal hyperplasia and fibrocellular hypertrophy in shear and tensile wall stress [41], extracellular calcifications and matrix protein glycosylation in T2DM [42], or medial hypertrophy in HT [43]. In our minCAS group, free of manifest renal diseases, the increase of CIMT will lead to a diminished release of MGP into the bloodstream. This could be explained either by capturing MGP in the artery wall due to its calcium binding [14, 44], or by a constitutive decrease of total MGP due to osteogenic metaplasia of vascular smooth muscle cells [34].

Secondly, we found that circulating MGP and hs-CRP were positively associated within the minCAS patients, even if hs-CRP was insignificantly modified in subgroups with HT or T2DM, or both diseases. The relationship between CRP and MGP was inconsistent, either positive [17], negative [12], or no association [32] being reported. Beside its hepatic synthesis as a response to inflammation, CRP is also produced in the atherosclerotic lesion and vascular endothelium [45, 46]. It is noteworthy that MGP restrains the effects of bone morphogenetic protein-2, thus limiting endothelial inflammation [47]. The CRP concentration found by Abe et al. [48] in carotid atherosclerotic plaques with mild and severe calcification was slightly lower than the systemic level, suggesting that vascular release of CRP does not have a significant contribution on its circulating level. Accordingly, after the adjustment with CIMT, the association between MGP and hs-CRP was not preserved in our minCAS group. Either the relationship between MGP and CIMT is stronger and driven by CIMT at a certain extent of calcified carotid stenosis, or the circulating CRP level does not entirely reflect the complex interplay between inflammation and vascular calcification. The increase of CIMT is disjunctive: up to 1.0–1.1 mm, there is a positive linear association between the inner and outer diameter of the carotid artery (as an adaptive response to atherosclerosis); above these values the decrease of the inner diameter (as a proxy for stenosis) is accompanied by a more rapid increase of CIMT [49]. Our minCAS group had the mean CIMT of 1.11±0.18 mm and the highest hs-CRP level. Therefore, the release of MGP into circulation may be halted by the increasing vascular inflammation, which creates a local barrier, implying that above certain mean CIMT value a negative association with serum MGP occurs.

Taken together, our results provide additional insights into the physiopathology of vascular calcification. Although a commercial ELISA kit has been used, the outcomes need to be interpreted with caution. Serum MGP may be recommended as an additional marker for calcified minCAS in a risk population and could be extended when carotid ultrasound is technically limited (e.g., ulcerations, wounds or extensive surgical scars in the carotid area, tortuosity, presence of significant contralateral disease, and stenosis in the last segment of the carotid artery) [50].

The strengths of our study are in the availability of ultrasound and MGP assay, the relatively homogenous study population, the parallel assessment of CIMT and hs-CRP, and the exclusion of low BMD influence on circulating MGP level. The present study also has several limitations. Due to the cross-sectional design, we could not establish a cause-effect relationship neither between MGP and calcified minCAS, nor between MGP, CIMT and hs-CRP. The total MGP assay does not distinguish between carboxylated/uncarboxylated or phosphorylated/desphosphorylated conformations. However, considering the area under the ROC, the assay had a fair capacity in discriminating between minCAS and non-minCAS subjects with a precision of 63% (positive predictive value). The 64% specificity for the threshold of 87.9 μg/L serum MGP could be augmented to the detriment of 73% test sensitivity by increasing the threshold value. In the future we intend to compare specificity of this kit with different conformational MGP assays (i.e., ucMGP and dp-ucMGP) when they become commercially available. Furthermore, non-minCAS postmenopausal women could have calcifications in other vascular areas. In order to minimize this bias, the study population was relatively homogenous and stratified either by the presence of calcified minCAS or pathological risk factors (HT and T2DM), rather than using expensive and irradiating techniques (computer tomography or magnetic resonance imaging). Our outcomes cannot be extrapolated to other populations, thus further investigation in larger groups with different degrees of carotid stenosis should be considered.

In conclusion, we found higher serum MGP levels in the minCAS compared to the non-minCAS group. Although MGP was independently associated with both mean CIMT and hs-CRP in the minCAS group, the relationship with mean CIMT is stronger, implying that higher serum MGP levels are independently predicted by lower mean CIMT values. The threshold of 87.9 μg/L serum MGP may help discriminate between the presence and absence of calcified minCAS in postmenopausal women with 63% precision. If our outcomes are further validated, circulating MGP might be used as a simple and available tool for identification of calcified carotid stenosis in a risk population.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
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