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Serum total matrix Gla protein: Reference interval in healthy adults and variations in patients with vascular and osteoarticular diseases

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ABSTRACT

Background: Matrix Gla protein (MGP) species are inhibitors of ectopic calcification in vascular diseases (VD) and osteoarticular diseases (OD). Among the MGP assays, we aimed to establish the reference interval for serum total MGP (tMGP) in healthy adults, the variation in patients with VD and OD and the associations with common cardiovascular risk factors.

Methods: We enrolled n = 124 healthy subjects and n = 95 patients with VD and OD in a small cross-sectional study. Serum high sensitivity C-reactive protein (hs-CRP), tMGP, glucose and lipid profile was measured.

Results: We established the reference interval for tMGP as 6–108 μg/L in healthy adults, the population under 40 having higher tMGP levels than those over 40 (61 ± 28, 51 ± 22 μg/L, $p < 0.05$). In healthy participants, tMGP was associated with smoking ($\beta = 0.303$, $p = 0.001$), age under 40 ($\beta = -0.201$, $p = 0.032$) and marginally with hs-CRP ($\beta = -0.165$, $p = 0.08$). In multivariate regression models, the association between smoking and tMGP was preserved even after adjusting for age under 40 and hs-CRP ($\beta = 0.267$, $p = 0.005$). The healthy population over 40 had lower tMGP levels than patients with OD and VD (51 ± 22, 90 ± 26, 106 ± 30 μg/L, $p < 0.001$).

Conclusions: Higher tMGP levels could identify patients with VD and OD, being also associated with smoking in healthy adults.

1. Introduction

Ectopic calcifications were subject of many studies in recent decades, but the risk factors and line of events that trigger this condition, as well as the mechanisms that inhibit the onset of the pathology are still elusive [1]. Vascular calcification is an independent risk factor for cardiovascular diseases, closely associated with age, hyperlipidemia and smoking [2], but also with hypertension (HT) [3], diabetes mellitus (DM) [4], stroke [5] and coronary artery disease (CAD) [2]. Atherosclerosis and vascular calcifications were also associated with low bone mineral density (BMD) [6], which is a paradox encountered in osteoporosis, since the bone is devoid of calcium which is instead deposited in the arteries. Furthermore, patients with rheumatoid arthritis (RA) are prone to develop atherosclerosis on a chronic inflammation background [7].

Matrix Gla protein (MGP), a validated local calcification inhibitor, is secreted by vascular smooth muscle cells (VSMCs) and chondrocytes into the bloodstream [8]. Following two posttranslational modifications (carboxylation and phosphorylation), different MGP conformations are born. With respect to the reference interval of the various MGP species found within the adult population, there are only three cross-sectional studies published to date on: uncarboxylated MGP (ucMGP) [9], total uncarboxylated MGP (t-ucMGP) [10], desphosphorylated MGP (dpMGP) [11], total desphosphorylated MGP (t-dpMGP), desphosphorylated-carboxylated MGP (dp-cMGP) or desphosphorylated-uncarboxylated (dp-ucMGP) [10]. There is also an enzyme-linked immunosorbent assays (ELISA) kit available for MGP, which was designated as total MGP (tMGP) because it has no capacity to distinguish between carboxylated/uncarboxylated and phosphorylated/

Abbreviations: βst, standardized beta-regression coefficient; MGP, matrix Gla protein; BMD, bone mineral density; NA, not analyzed; BMI, body mass index; NS, not significant; CAD, coronary artery disease; OD, osteoarticular disease; CV, coefficient of variation; OP, osteopenia and osteoporosis; DBP, diastolic blood pressure; RA, rheumatoid arthritis; DM, diabetes mellitus; SBP, systolic blood pressure; dpMGP, desphosphorylated MGP; SD, standard deviation; dp-cMGP, desphosphorylated-carboxylated MGP; t-dpMGP, total desphosphorylated MGP; dp-ucMGP, desphosphorylated-uncarboxylated MGP; t-ucMGP, total uncarboxylated MGP; ELISA, enzyme-linked immunosorbent assay; tMGP, total matrix Gla protein; HDL-C, high density lipoprotein cholesterol; ucMGP, uncarboxylated MGP; hs-CRP, high-sensitivity C-reactive protein; VD, vascular disease; HT, hypertension; VSMCs, vascular smooth muscle cells; LDL-C, low density lipoprotein cholesterol

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desphosphorylated species, or combinations thereof [12]. However, we are not aware of studies that have established the reference intervals for tMGP.

There is a myriad of divergent outcomes in patients with vascular disease (VD) on the relation between MGP species and common cardiovascular risk factors [age, male gender, blood pressure, body mass index (BMI), smoking, HT, DM, glucose, cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), tryglicerides, high-sensitivity C-reactive protein (hs-CRP)]. Several cross-sectional studies conducted on patients with VD have reported mounting evidence about the influence of hs-CRP [12–15], age [12,13,16] and blood pressure [12] on the circulating MGP species. For instance hs-CRP, a confirmed biomarker of inflammation, was reported as being positively correlated with dp-ucMGP [13] and tMGP [12], negatively associated with t-dpMGP [14] or uncorrelated with t-ucMGP [15]. On the other hand, age was found positively associated with both dp-ucMGP and dp-cMGP in patients with aortic stenosis [13] or tMGP in postmenopausal women [12], but was also associated with circulating dp-ucMGP levels in patients with DM [16]. There was no association between MGP species and the common cardiovascular risk factors or BMD in patients with osteoarticular diseases (OD) [10,11].

Conversely, few studies have been concentrated towards the association of MGP with cardiovascular risk factors in the apparently healthy population. Of the transversal studies published to date, only age and smoking have been reported to be related to MGP. Age was thereby negatively associated with ucMGP [9], positively with dpMGP, dp-cMGP and dp-ucMGP [10,11], but no association was found with t-ucMGP and t-dpMGP [10]. A study of Dalmeijer et al. [17] established a negative association between smoking and dp-ucMGP, but did not find any association with t-ucMGP and dp-cMGP in healthy women.

To our knowledge on researches related to MGP species, there are only three cross-sectional studies focused on tMGP in patients with VD [12,18,19] and none concerning the healthy population or the patients with OD.

Therefore our approach considered the missing data per se on tMGP from literature and proposed three main objectives. First, we aimed to establish the reference interval of circulating tMGP in the healthy population. Then we wanted to define the association of tMGP with common cardiovascular risk factors (i.e. age, smoking, inflammation, BMI, male gender, lipid profile, fasting glucose) but also to investigate the relation between tMGP and t-ucMGP (a proven marker of cardiovascular calcifications) in the healthy population. Finally, we intended to assess the variation of tMGP levels by comparing the healthy population to patients with VD and OD.

2. Material and method

2.1. Subjects and study design

In our small cross-sectional case-control study, a group of apparently healthy participants (n = 124) and a group of patients (n = 95) were consecutively enrolled. The healthy population was enrolled voluntarily, only adults over 18 years being included with no medical history of HT, CAD, DM, chronic kidney disease, inflammatory diseases, RA, osteopenia and osteoporosis. The patients' group consisted of individuals diagnosed with VD (n = 72) and OD (n = 23) who have been referred to the University County Hospital. Patients with VD [i.e. CAD (n = 26), stroke (n = 6), HT (n = 30) or DM (n = 10)] were recruited from the Outpatient department of the Second Internal Medicine Department and patients with OD [i.e. OP (n = 13) or RA (n = 10)] were enrolled from the Rheumatology Department. Based on the assessment of total hip T-score [20], ten out of thirteen patients from the OP group had osteopenia (T-score < -1 to > -2.5) and three had osteoporosis (T-score ≤ -2.5). The two categories were considered as OP subgroup (n = 13) due to the low number of patients with osteoporosis. The presence of OD in patients with VD, along with CAD, stroke or DM in patients with OD was considered exclusion criteria. The healthy participants and the patients did not receive treatment with anticoagulants, vitamin D antagonists, bisphosphonates or corticosteroid drugs in the last two years.

All the patients and the healthy participants gave their informed consent. Our study design was consistent with the declaration of Helsinki and approved by the Medical Ethics Committee of the University.

Demographics and the medical history of the participants were recorded. BMI was calculated as the ratio between weight and square of the height (kg/m^2). Smoking was defined as current smokers or smoking history for at least two years in the last five years. Absence or cessation of smoking habit in the past five years was defined as non-smoking. Total hip T-score was calculated as the standard deviation (SD) from the mean BMD of a healthy young adult. Blood pressure was measured with a sphygmomanometer placed on the right arm, after 5 min of rest. The average blood pressure was recorded after two measurements taken 20 min apart. HT was defined as systolic blood pressure $\geq$ 140 mmHg and diastolic blood pressure $\geq$ 90 mmHg, or treatment with antihypertensive medication. We defined CAD as angina pectoris with consistent response to sublingual nitrates, or characteristic ischemic modification on electrocardiogram in the past five years. A history of cerebral ischemic event in the last year was recorded as stroke. We considered DM as fasting plasma glucose level $\geq$ 7 mmol/L [21] or therapy with oral anti-diabetic medication. Also, RA was defined according to the revised criteria of the American Rheumatism Association [22].

2.2. BMD assessment

BMD was assessed in the hip area by dual-energy X-ray absorptiometry (Lunar Prodigy Advance, GE Lunar, Madison, WI, USA) and the total hip T-score was recorded for all patients (VD and OD group, respectively). Each BMD measurement was performed by two experienced technicians and the intra-observer coefficient of variation (CV) was 1.1%.

2.3. Laboratory measurements

Blood was collected by venipuncture after 8 h of fasting. After centrifugation at 3000 rpm for 10 min, the serum was stored at -80 °C prior to analysis.

Enzymatic methods were performed on an automated chemistry analyzer (Cobas Mira Plus, Roche Diagnostic, Basel, Switzerland) to measure serum levels of glucose, cholesterol, HDL-C and triglycerides. The intra-assay CV for each parameter was < 5%. LDL-C was assessed by Friedewald's formula [total cholesterol - (HDL-colesterol + triglycerides/5)].

An immunoturbidimetric assay (CRP U-hs, Diasys Diagnostic System, Holzheim, Germany) was used for serum hs-CRP assessment on CS-T240 analyzer (Dirui, Changchun, China). Our intra-assay CV for hs-CRP was 6.5%.

A sandwich ELISA kit was used to measure serum tMGP (USCN Life Science Inc., Wuhan, China) with a microtitre plate reader Organon 230S (Organon Teknika, Oss, the Netherlands). The detection range for tMGP was 39–2500 pg/mL. The assay sensitivity was 20 ng/L and our intra-assay CV was 5.8%.

Also, t-ucMGP was assessed by competitive ELISA as described by Cranenburg et al. [10] in n = 47 randomly selected healthy subjects. Intra- and inter-assay CV were 8.9% and 11.4%, respectively and the lowest limit of detection was 98 nM.

2.4. Statistical analysis

Distribution of continuous variables was assessed by Kolmogorov-Smirnov test. Variables with Gaussian distribution were expressed as

mean ± standard deviation (SD), while the non-Gaussian variables as median with minimum and maximum values between parentheses. Differences of tMGP between two groups with Gaussian distribution were evaluated with the student *t*-test. The Cohen's *d* (effect size) for tMGP was calculated to assess the extent of variation between healthy subjects, VD and OD patients. For non-Gaussian variables, the Mann-Whitney *U* test was used, whereas for categorical variables, Chi-square or Fischer's exact tests were performed. Comparisons of tMGP levels in healthy subjects stratified by decades of age or between patients with CAD, stroke, HT, DM, OP and RA were assessed by One-Way ANOVA followed by Scheffe's post-hoc test. Linear univariate regression was performed to determine the clinical and biochemical correlates of MGP. Subsequently, the independent association between smoking and MGP was assessed in a crude model and in multivariate regression analysis after adjusting for significant or marginal correlates. Standardized beta-regression coefficients (βst) are presented to allow a better comparison between independent associations. Statistical analysis was conducted using SPSS software v.22.0 (SPSS inc., Chicago, Il, USA). Statistical significance was based on two-tailed tests at p values < 0.05.

3. Results

3.1. Baseline characteristics and reference intervals in the healthy population

The apparently healthy subjects (n = 124) were divided in two age subgroups: under 40 (n = 64) and over 40 (n = 60), with their characteristics summarized in Table 1. The group under 40 was found to have higher levels of circulating tMGP ($p < 0.05$), but significantly lower levels of hs-CRP ($p < 0.001$) than subjects over 40. Also, elevated tMGP levels were noticed in smoking compared to non-smoking subjects (69 ± 27 μg/L versus 52 ± 24 μg/L, $p < 0.01$).

The Gaussian distribution of healthy population allowed us to use the common formula (mean ± 1.96 × SD) to define the reference interval for tMGP. Therefore, we have established the reference interval as 6–108 μg/L. There was no statistically significant difference between genders.

Even if a descending trend with age stratified by decades was observed in serum levels of tMGP, with no statistical significance, we considered appropriate to establish the reference interval for each decade of age for future comparisons with similar populations (Fig. 1).

3.2. Smoking is independently associated with tMGP in the healthy population

Because we did not find significant differences of tMGP levels in the healthy population stratified by decades of age, but only between participants under 40 and over 40, we considered useful to introduce age as a categorical variable, defined as age over 40. As a first step in assessing the relation between tMGP and cardiovascular risk factors, univariate regression analysis was performed to determine which potential risk factor can explain the variation of MGP levels in healthy adults (Table 2). Current smoking and age under 40 were significantly associated with higher tMGP concentrations, but lower hs-CRP levels had a marginal association with higher levels of circulating tMGP. Table 2 also shows three multivariate regression models, in which smoking habit was tested as a most significant independent variable associated with tMGP. In all models, smoking remained the most influential variable, keeping its significance in unadjusted model, but also in two adjusted models with significant or marginal correlates (age over 40 and hs-CRP as independent variables). The adjusted models predicted 11% of the variance in tMGP.

Also, an interesting finding was the absence of association between tMGP (72 ± 32 μg/L) and t-ucMGP (3455 ± 685 nM) in n = 47 randomly selected healthy individuals.

Table 1
Characteristics of the healthy population.

	All healthy participants (n = 124)	Healthy participants stratified by age		p
		< 40 years (n = 64)	> 40 years (n = 60)	
Demographics				
Age, years	35 (18–74)	28 ± 5	54 ± 9	< 0.001
Gender, m/f	47/77	26/43	21/34	NS
BMI, kg/m^2	24 ± 3	23 ± 3	25 ± 3	< 0.01
Medical history				
Smokers, n (%)	37 (30)	26 (41)	11 (18)	< 0.01
HT, n (%)	0 (0)	0 (0)	0 (0)	NS
CAD, n (%)	0 (0)	0 (0)	0 (0)	NS
Stroke, n (%)	0 (0)	0 (0)	0 (0)	NS
DM, n (%)	0 (0)	0 (0)	0 (0)	NS
Osteoarticular disease, n (%)	0 (0)	0 (0)	0 (0)	NS
Inflammatory disease, n (%)	0 (0)	0 (0)	0 (0)	NS
Chronic kidney disease, n (%)	0 (0)	0 (0)	0 (0)	NS
Biochemical measurements				
Glucose, mmol/L	4.9 ± 0.7	4.8 ± 0.7	5.1 ± 0.7	< 0.01
Cholesterol, mmol/L	5.2 ± 1.0	4.9 ± 1.0	5.5 ± 1.0	< 0.01
HDL-C, mmol/L	1.3 (0.6–2.1)	1.3 (0.8–2.0)	1.3 ± 0.3	NS
LDL-C, mmol/L	3.2 ± 0.8	3.0 ± 0.8	3.4 ± 0.9	< 0.01
Triglycerides, mmol/L	1.1 (0.5–3.2)	0.9 (0.5–3.2)	1.5 ± 0.6	< 0.05
hs-CRP, mg/L	2.9 ± 0.8	2.5 ± 0.6	3.5 ± 0.8	< 0.001
tMGP, μg/L	57 ± 26	61 ± 28	51 ± 22	< 0.05

Depending on distribution, the data are presented as mean ± SD, median (minimum - maximum value) or number (percentage), as appropriate. The p-values for differences between groups < 40 years and > 40 years are given. Abbreviations: m/f, males/females; BMI, body mass index; HT, hypertension; CAD, coronary artery disease; DM, diabetes mellitus; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; tMGP, total matrix Gla protein; NS, not significant.

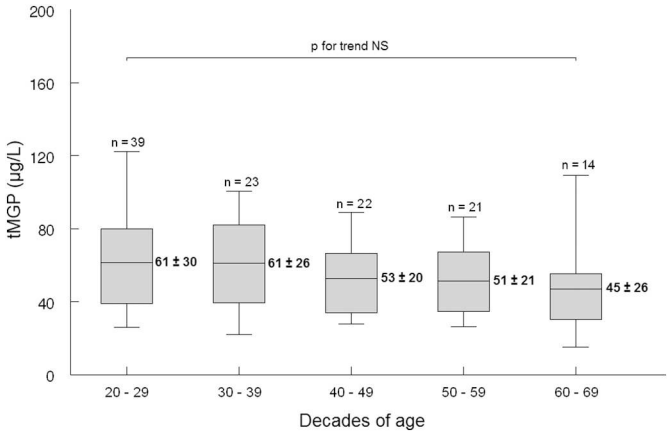

Fig. 1. Serum levels of tMGP in healthy subjects stratified by decades of age. Data are presented as means ± SD and whiskers represent standard error. Subjects over 70 (n = 3) and between 18 and 19 years of age (n = 2) were excluded due to their low number. Abbreviations: tMGP, total matrix Gla protein; NS, not significant.

3.3. Higher tMGP levels in patients with VD versus OD

With regard to the patients population (n = 95), their characteristics are summarized in Table 3. Patients with VD had higher levels of tMGP compared to patients with OD ($p < 0.05$), but no significant

Table 2
Association of cardiovascular risk factors with tMGP in the healthy population.

	Univariable analysis		Multivariable analysis					
			Model 1		Model 2		Model 3	
	β_{st}	p	β_{st}	p	β_{st}	p	β_{st}	p
Smoking	0.303	0.001	0.303	0.001	0.269	0.005	0.267	0.005
Age over 40	−0.201	0.032			−0.132	0.157	−0.117	0.326
hs-CRP	−0.165	0.08					−0.024	0.842
Male	0.017	0.856						
BMI	0.018	0.852						
Glucose	−0.013	0.889						
Cholesterol	−0.074	0.436						
HDL-C	0.020	0.832						
LDL-C	−0.098	0.297						
Triglycerides	0.006	0.946						

Standardized coefficients are presented to allow a better comparison between independent associations. MGP was pointed as the dependent variable in each univariate analysis and multivariate models. Model 1- unadjusted (smoking as predictor); model 2- adjusted with age over 40; model 3- adjusted with age over 40 and hs-CRP. Abbreviations: β_{st}, standardized beta-regression coefficient; hs-CRP, high-sensitivity C-reactive protein; BMI, body mass index; HDL-C, high density lipoprotein cholesterol. LDL-C, low density lipoprotein cholesterol.

Table 3
Characteristics of the patients with VD and OD.

	All patients n = 95	VD (n = 72)					OD (n = 23)			p
		CAD n = 26	Stroke n = 6	HT n = 30	DM n = 10	all VD n = 72	OP n = 13	RA n = 10	All OD n = 23	
Demographics										
Age, years	63 ± 11	63 ± 9	60 ± 12	63 ± 11	55 ± 6	62 ± 10	66 ± 13	63 ± 17	65 ± 15	NS
Gender, m/f	21/74	7/19	1/5	6/24	2/8	16/56	4/9	1/9	5/18	NS
BMI, kg/m^2	29 ± 6	28 ± 4	32 ± 7	30 ± 6	32 ± 9	29 ± 6	28 ± 6	25 ± 5	27 ± 7	NS
Medical history										
Smokers, n (%)	24(25)	5(19)	1(17)	7(23)	6(60)	19(26)	4(31)	1(10)	5(22)	NS
CAD, n (%)	29(31)	26(100)	3(50)	0(0)	0(0)	29(40)	0(0)	0(0)	0(0)	< 0.001
Stroke, n (%)	6(6)	0(0)	6(100)	0(0)	0(0)	6(8)	0(0)	0(0)	0(0)	NS
HT, n (%)	58(61)	14(54)	6(100)	30(100)	0(0)	50(69)	5(38)	3(30)	8(35)	< 0.01
DM, n (%)	13(14)	0(0)	3(50)	0(0)	10(100)	13(18)	0(0)	0(0)	0(0)	< 0.05
OP, n (%)	13(14)	0(0)	0(0)	0(0)	0(0)	0(0)	13(100)	0(0)	13(57)	< 0.001
RA, n (%)	10(11)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	10(100)	10(43)	< 0.001
Measurements										
SBP, mmHg	142 ± 25	140 ± 26	157 ± 27	151 ± 23	128 ± 21	144 ± 25	139 ± 24	131 ± 20	136 ± 22	NS
DBP, mmHg	83 ± 12	80 ± 12	94 ± 14	87 ± 10	77 ± 14	84 ± 12	81 ± 8	77 ± 12	79 ± 10	NS
Total hip T-score	−0.6 ± 1.3	−0.5 ± 1.3	−0.6 ± 0.9	−0.1 ± 1.2	−0.6 ± 0.7	−0.3 ± 1.2	−2.1 ± 0.7	−1.3 ± 1.3	−1.8 ± 1	< 0.001
Glucose, mmol/L	5.7(4.3–14.4)	6.2 ± 1.9	8.2 ± 4.1	6.4 ± 1.9	6.6 ± 3.1	5.8(4.3–14.4)	5.5 ± 0.6	5.5 ± 0.7	5.5 ± 0.6	0.05
Chol, mmol/L	5.5 ± 1.5	6.1 ± 1.6	4.3 ± 0.9	5.5 ± 1.6	5.5 ± 1.3	5.6 ± 1.6	5.5 ± 1.3	4.8 ± 0.8	5.2 ± 1.2	NS
HDL-C, mmol/L	1.2(0.8–2.8)	1.3 ± 0.4	1.3 ± 0.6	1.4 ± 0.3	1.2 ± 0.2	1.3 ± 0.3	1.4 ± 0.6	1.3 ± 0.3	1.4 ± 0.5	NS
LDL-C, mmolL	3.5 ± 1.4	4.2 ± 1.6	2.2 ± 0.8	3.6 ± 1.6	3.7 ± 0.9	3.7 ± 1.4	3.2 ± 1.6	2.8 ± 1.1	3.0 ± 1.3	NS
TG, mmol/L	1.5(0.3–6.9)	1.6 ± 0.8	1.4 ± 0.6	1.8 ± 1.5	1.7 ± 0.9	1.7 ± 1.1	1.7 ± 0.6	1.5 ± 1.1	1.6 ± 0.8	NS
hs-CRP, mg/L	6.3 ± 4.1	6.8 ± 3.9	8.6 ± 5.3	5.4 ± 3.6	5.8 ± 4.2	6.3 ± 3.9	6.6 ± 5.1	6.5 ± 4.4	6.6 ± 4.7	NS
tMGP, μg/L	102 ± 29	107 ± 30	108 ± 34	105 ± 28	105 ± 33	106 ± 30	77 ± 21	106 ± 22	90 ± 26	< 0.05

Data are presented as mean ± SD, median (minimum-maximum value) or number (percentage), appropriate to their distribution. The p-values for differences between VD and OD groups are given. Abbreviations: VD, vascular disease; OD, osteoarticular disease; m/f, males/females; BMI, body mass index; CAD, coronary artery disease; HT, hypertension; DM, diabetes mellitus; OP, osteopenia and osteoporosis; RA, rheumatoid arthritis; SBP, systolic blood pressure; DBP, diastolic blood pressure; Col, cholesterol; HDL-C, high-density; LDL-C, low density lipoprotein cholesterol; TG, triglycerides; hs-CRP, high-sensitivity C-reactive protein; tMGP, total matrix Gla protein.

difference in tMGP levels was found between the subgroups of VD patients with CAD, stroke, HT and DM. However, patients with OP had lower levels of tMGP than patients with RA ($p < 0.05$).

In univariate regression analysis, tMGP was only associated with hs-CRP in all patients (β st = 0.225, p = 0.036), with age in patients with VD (β st = −0.251, p = 0.033), but no association was found in patients with OD with any of the cardiovascular risk factors [age, male gender, systolic blood pressure (SBP), diastolic blood pressure (DBP), BMI, smoking, HT, DM, glucose, cholesterol, HDL-C, LDL-C, tryglicerides, hs-CRP] or with total hip T-score (data not shown). Accordingly, there was no need to build multivariate regression models in patients with VD and OD.

3.4. Lower tMGP levels in the healthy population compared to patients with VD and OD

Further, we continued to analyze the differences between the healthy population and patients. Due to the fact that subjects under 40 (n = 64) had a lower average age than patients, only subjects over 40 (n = 60) were considered for the following analysis. Subjects over 40 years of age had significantly lower levels of tMGP compared to patients with OD ($p < 0.001$) and VD ($p < 0.001$), as illustrated in Fig. 2. We noticed a very large size effect for tMGP when we compared healthy subjects to OD patients (Cohen's $d = 1.62$) or healthy subjects to VD patients (Cohen's $d = 2.09$).

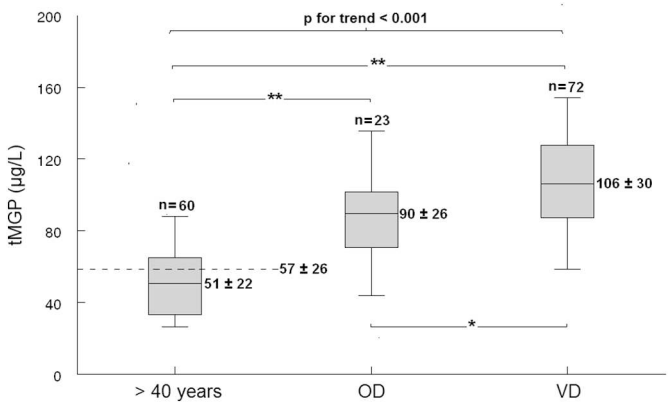

Fig. 2. Comparison of tMGP levels between healthy subjects over 40 and patients with OD and VD.
Data are mean ± SD; whiskers represent standard error; dotted line is mean ± SD of all healthy subjects; * $p < 0.05$; ** $p < 0.001$. Abbreviations: tMGP, total matrix Gla protein; OD, patients with osteoarticular disease; VD, patients with vascular disease.

Finally, lower levels of tMGP were observed in healthy subjects over 40 compared to patients with CAD ($p < 0.001$), stroke ($p < 0.01$), HT ($p < 0.001$), DM ($p < 0.001$) and RA ($p < 0.001$). Healthy subjects over 40 had also lower levels of tMGP than patients with OP, but statistical significance was not reached.

4. Discussion

We achieved our goals to investigate the variation of tMGP levels between the healthy population and the patients with VD or OD, to assess the associations of tMGP with common cardiovascular risk factors and to establish the reference interval for circulating tMGP in healthy adults. The study was the first to establish the reference interval for tMGP as 6–108 µg/L and also the reference values per decade of age in healthy individuals (Fig. 1). For a clear overview of the existing MGP species and to see if other studies are in line with our findings, we summarized the data in Table 4.

In our study, we observed that tMGP decreases in healthy individuals over 40 compared to those under 40, finding supported by the negative association noticed between tMGP and age over 40. We decided to choose the limit of 40 because evidence has shown very low coronary calcium scores in asymptomatic adults under 40 compared to the population over 40 [23]. Therefore, the influence of calcification on MGP was minimized in healthy adults under 40. Our finding was in agreement with Cranenburg et al. [9] who have also found lower levels in the healthy population over 40 and a significant decreasing trend with age, but for serum ucMGP. Although it was no longer significant, the decreasing trend of tMGP was maintained in our healthy population when stratifying subjects by decades of age (Fig. 1). A possible explanation might be that ectopic calcifications are more common with aging and the increased local requirement for MGP is circumscribed near the calcification sites in the arterial wall, resulting in a poorer escape of MGP into the bloodstream. Therefore, the level of circulating tMGP declines in elderly healthy subjects if there is no overt VD or OD associated. It should be noted that when enrolling healthy subjects in our study we did not assess arteries and joint calcifications through imaging techniques. For this reason, our elder subjects may have a certain degree of vascular or osteoarticular calcification, but with no clinical manifestations. In the healthy population, it is noteworthy that tMGP resembles well ucMGP in terms of age influence on both MGP species (see Table 4).

On the issue of the relation between tMGP and cardiovascular risk factors in the healthy population, besides the independent association with age under 40, we also found a marginal association with low-grade inflammation (hs-CRP used as proxy) and a positive association with smoking. It was demonstrated that subjects over 40 had higher levels of hs-CRP associated with arterial stiffness related to arterial aging [24]. Therefore, at certain level, the local inflammatory barrier constituted in the arterial wall could halt the luminal secretion of MGP by VSMCs, leading to lower levels of circulating MGP. This assumption could explain the marginal negative association found between tMGP and hs-CRP. However, in multivariate regression models, after adjusting for age over 40 and hs-CRP, only the association between smoking and tMGP preserved its significance. Our finding appeared to echo the correlation between tMGP and smoking foreshadowed in a previous pilot study [19]. It is a controversial stance, because other study found a negative association of smoking with dp-ucMGP and no association with t-ucMGP and dp-cMGP, but was limited to healthy women [17]. The existing data entitle us to support the following deduction: smokers could have a greater local need for protection against ectopic calcification than non-smokers, reflected by the higher circulating levels of tMGP found in smokers. Thus, if we encompass the previous findings [17,19] and our study, we could state that smoking is independently associated only with tMGP.

Surprisingly, we did not find an association between tMGP and t-ucMGP in the healthy population. The commercial ELISA kit used to assay tMGP probably has detected the native MGP or its fragments (N-terminal, C-terminal or middle structural domains), but did not discriminate between other MGP species (ucMGP, t-ucMGP, dpMGP, t-dpMGP, dp-cMGP and dp-ucMGP). In this respect, the modifications in tMGP levels may not be driven by t-ucMGP, which represents an unknown fraction of the total detectable MGP. As shown in Table 4, tMGP resembles well dp-ucMGP in terms of variation in patients with CAD. In a newly published systematic review, vitamin K supplementation was associated with decreasing dp-ucMGP levels and vascular calcification [25]. Therefore it would be desirable in the future to study the dynamics of tMGP along with dp-ucMGP in patients with VD.

Regarding the comparison between the healthy population and patients, we observed that patients with VD had higher levels of tMGP than patients with OD, but both populations (VD and OD) had higher tMGP levels than healthy individuals over 40 (Fig. 2). The proportion in which vascular or osteoarticular MGP contributes to its total circulating pool is yet unknown. The level of serum MGP depends on its synthesis and secretion by VSMCs and the binding rate to the calcified areas within the vascular walls [26]. A plausible explanation of our finding would be that in patients with VD the presence of chondrocyte-like VSMCs and their proximity to the vascular lumen may have a larger contribution to the circulating pool of MGP compared to the bone (situated at distance from the vascular lumen) or cartilage (an avascular tissue). The smaller contribution of bone to the circulating pool of MGP is strengthen by the lack of association between tMGP and BMD in patients with VD and OD and was also in line with another study conducted in postmenopausal women with carotid calcifications, where increased circulating levels of tMGP were not influenced by BMD [12]. Due to fewer calcifications in apparently healthy individuals compared to patients with VD and OD, the local need for MGP is probably smaller, reflected as such by lower circulating MGP levels.

Low-grade inflammation contributes to the development of atherosclerosis, creating a milieu for ectopic calcifications [27]. The higher local inflammation, reflected by higher hs-CRP found in our patients with VD and OD (approximately two fold higher than healthy population), will probably exceed the local barrier (instituted by lower hs-CRP values) and will lead to an increased permeability of the arterial wall and consequently to higher MGP levels escaping into circulation. The positive association found between tMGP and hs-CRP in overall patients (VD and OD) strengthens this theory. The hypothesis previously issued according to which circulating tMGP will increase in patients with VD and OD when a certain level of inflammation is exceeded, requires confirmation by further studies.

Table 4
Overview of the MGP assays.

	ucMGP	t-ucMGP	dpMGP	t-dpMGP[a]	tMGP[a]	dp-ucMGP	dp-cMGP
Bibliography							
Reference	Cranenburg et al., J Vasc Res 2008	Cranenburg et al., Thromb Haemost 2010	Schurgers et al., Clin Chim Acta 2005	Cranenburg et al., Thromb Haemost 2010	This study	Cranenburg et al., Thromb Haemost 2010	Cranenburg et al., Thromb Haemost 2010
Type of study	Cross-sectional	Cross-sectional	Cross-sectional	Cross-sectional	Cross-sectional	Cross-sectional	Cross-sectional
Assay summary							
Type of assay	Mono-atb ELISA	Mono-atb ELISA	Mono-atb ELISA	Mono-atb ELISA	Mono-atb ELISA	Dual-atb ELISA	Dual-atb ELISA
Type of sample	Serum or plasma	Serum or plasma	Serum or plasma	Serum or plasma	Serum or plasma	Plasma	Plasma
Healthy population							
Reference values	475 ± 91[b]	4704 ± 1053	11.2 ± 3.5	14 ± 3	57 ± 26	447 ± 188	1763 ± 478
Measurement units	nM	nM	nM	nM	μg/L	pM	pM
Age interval	25–80	25–80	20–80	25–80	18–75	25–80	25–80
Number of subjects	165	75	121	75	124	75	75
Increasing age	↓	↔	↑	↔	↓	↑	↑
Patients with:							
CAD or AVD	↓	↓	↓	↑	↑	↑	↑
DM	NA	NA	NA	NA	↑	NA	NA
HT	NA	NA	NA	NA	↑	NA	NA
OD	NA	NA	↓	NA	↑	NA	NA
RD	NA	↓	NA	↔	NA	↑	↔
ESRD	↓	↓	↓	↔	NA	↑	↑

Abbreviations: MGP, matrix Gla protein; ucMGP, uncarboxylated MGP; t-ucMGP, total uncarboxylated MGP; dpMGP, desphosphorylated MGP; t-dpMGP, total desphosphorylated MGP; tMGP, total MGP; dp-ucMGP, desphosphorylated-uncarboxylated MGP; dp-cMGP, desphosphorylated-carboxylated MGP; atb, antibody; ELISA, enzyme-linked immunosorbent assay; CAD, coronary artery disease; AVD, aortic valve disease; DM, diabetes mellitus; HT, hypertension; OD, osteoarticular disease; RD, rheumatic disease; ESRD, end-stage renal disease; ↑increased; ↓decreased; ↔ unmodified; NA, not analyzed. Observations: The assay for t-ucMGP is a modified version of ucMGP assay. The dp-ucMGP is also commercially available as a chemiluminescence assay, but currently there is no data on reference values. The cMGP conformation is not yet developed, but was estimated by dividing t-ucMGP by the osteocalcin ratio [2]. For pMGP there is no assay available at this moment.

[a] Commercial kits.

[b] Reference values were calculated as the average of ucMGP obtained by Craneburg et al. [8] in the three age groups: under 40, between 40 and 60 and over 60, respectively.

The present study needs to be addressed in terms of strengths and limitations. The strengths were the assessment of tMGP in a relatively homogenous apparently healthy population, the first established reference interval in healthy adults by using an available ELISA kit, the first identification of an independent association between smoking and tMGP levels in healthy individuals and the parallel evaluation of the tMGP dynamics in the healthy population and patients with VD and OD. Although it was a small cross-sectional study, we complied with CLSI guidelines recommendation to determine the reference interval in at least 120 individuals [28].

However, there were several limitations of the study. We did not assess the extent of vascular or osteoarticular calcification by performing whole body imaging techniques, since the irradiation would be considerable and rather expensive. We could not establish a cause-effect relationship between tMGP and smoking due to the cross-sectional design of the study. Even if a commercial ELISA kit was used which could not discriminate between native MGP, fragments or different conformations of the protein, we recommend caution in interpreting the findings.

To modify the overall clinical approach, as currently recommended by the imaging protocols, future prospective studies in larger populations are warranted to establish a clear screening algorithm in which serum modification of different MGP species will be introduced in order to achieve an early identification of patients with ectopic calcifications.

5. Conclusions

We found that serum levels of tMGP were significantly higher in patients with VD and OD compared to the healthy population. The study has established the reference interval for tMGP as 6–108 μg/L, but no association with t-ucMGP was found in the healthy population. Healthy participants under 40 had higher levels of tMGP than subjects over 40. Furthermore, in the apparently healthy population, serum tMGP levels were increased in smokers versus non-smokers, smoking habit being independently associated with circulating tMGP. Also, higher tMGP levels were found in patients with VD and OD compared to the healthy population over 40 years of age. These data lend support to a selective future strategy in terms of choosing among various MGP species, tMGP being suitable to discriminate between the healthy population and patients with VD and OD despite of the associated cardiovascular risk factors.

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Competing interests

The funding organization played no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

Declaration of interest

None.

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