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Research Article

Circulating matrix Gla protein in patients with vascular pathology

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Abstract. Objectives: The main purpose of our study was to investigate the difference of serum total MGP (tMGP) levels, assessed with a commercially available kit, by comparing healthy subjects to patients with vascular diseases (VD). Furthermore, we evaluated the association of tMGP with various cardiovascular risk factors [age, body mass index (BMI), smoking, inflammation - by means of high sensitive C reactive protein (hs-CRP)] in different VD populations. Material and method: This pilot case-control study has included a group of apparently healthy subjects (n=49) and a population with VD (n=72), stratified in subgroups with hypertension (HT), stroke, diabetes mellitus (DM) or coronary artery disease (CAD). Common clinical measurements and laboratory analyses were completed. Serum levels of tMGP and also hs-CRP were assayed in all participants using commercially available immunoassay kits. Results: We observed a positive association between tMGP and smoking ($r=0.332$, $p<0.05$) within the healthy population and a negative correlation between tMGP and age ($r=-0.25$, $p<0.05$) in patients with VD. No significant differences in tMGP or hs-CRP levels were noticed between VD subgroups, and also no correlations between tMGP and hs-CRP were found in any of the groups or subgroups. The main finding was that healthy population had significantly lower levels ($p < 0.001$) of tMGP (51±22 µg/L) and hs-CRP (3.5±0.8 mg/L) than VD population (106±30 µg/L and 6.3±3.9 mg/L, respectively). Also, significantly lower levels of tMGP in healthy subjects compared to patients with CAD ($p<0.001$), stroke ($p<0.01$), HT ($p<0.001$) or DM ($p<0.001$) were observed. Conclusion: Circulating tMGP levels were considerably lower in healthy subjects than patients with VD or in any of its subgroups. Therefore, with further validation, serum tMGP levels could be used as a reasonable screening instrument for discriminating between healthy subjects and patients with VD.

Key Words: vascular calcifications, matrix Gla protein, hs-CRP.

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Introduction

Mineral deposition in vascular walls was considered a passive process and a consequence of excess circulating calcium and phosphates. Among different studies, Schurgers et al (2007) has noticed similarities between development of ectopic calcification and bone formation, but the risk factors, trigger events or inhibitory mechanisms are still restrained (Abedin et al 2004). It was established that vascular calcification is an independent risk factor for vascular diseases (VD), closely associated with hypertension (HT) (Kalra & Shanahan 2012), diabetes mellitus (DM) (Jeffcoate et al 2009), stroke (Lee & Oh 2010) and coronary artery disease (CAD) (Rennenberg et al 2010), but also with age, hyperlipidemia and smoking (Rennenberg et al 2010). VD is considered a leading cause of mortality worldwide, commonly diagnosed in adult patients.

Matrix Gla protein (MGP), a vitamin K dependent protein, exerts a strong inhibitory effect on vascular calcification. Secreted by vascular smooth muscle cells and activated through γ-glutamyl carboxylation, it was demonstrated to prevent vascular deposition and crystallization of calcium (Proudfoot & Shanahan 2006).

Various conformational combinations of MGP can be assayed using ELISA (enzyme-linked immunosorbent assay) techniques: competitive ELISA for desphosphorylated MGP (dpMGP) (Braam et al 2000) and uncarboxylated MGP (ucMGP) (Schurgers et al 2005a) and also sandwich-ELISA for desphosphorylated-carboxylated MGP (dp-cMGP) or desphosphorylated-uncarboxylated MGP (dp-ucMGP) (Schurgers et al 2010). Several studies were established an association of these conformations with VD: low serum ucMGP was considered a potential indicator for active calcifications (Hermans et al 2007), being associated with atherosclerosis (Cranenburg et al 2008), while high dp-ucMGP levels were correlated with mortality in a population with aortic stenosis (Ueland et al 2010). On the contrary, few studies have been focused towards healthy population to investigate the relationship between risk factors for VD and serum MGP levels. In this respect, only age was found negatively correlated with circulating levels of ucMGP (Cranenburg et al 2008) or, on the contrary, a positive association with dpMGP was observed (Schurgers et al 2005b).

Low grade inflammation was demonstrated to have an important role in atherogenesis, as shown by Ross (1999). The assessment of high sensitive C reactive protein (hs-CRP), a certified marker of inflammation, was fundamental in classifying the population with low, medium or high risk for CAD (Pearson et al 2003).

Several studies had concentrated on the association between hs-CRP and conformations of MGP in patients with VD. Thus, a direct association with dp-ucMGP (Ueland et al 2010), negative association with dpMGP (Thomsen et al 2010) or no association of ucMGP with hs-CRP (Parker et al 2010) was found. Currently, there is only one available commercial kit to assay MGP which does not distinguish between various MGP conformational combinations, therefore we designated it as total MGP (tMGP). As far as we know, besides our researches, only one study concerning the tMGP behavior in VD population was published (Albu et al 2013).

Therefore, the main purpose of this study was to investigate the difference of serum total MGP (tMGP) levels, assessed with a commercially available kit, by comparing healthy subjects to patients with vascular diseases (VD). Furthermore, we evaluated the association of tMGP with various cardiovascular risk factors [age, body mass index (BMI), smoking, inflammation - by means of high sensitive C reactive protein (hs-CRP)] in general VD population, but also in subgroups (HT, stroke, DM, CAD). In conclusion, we intended to assess if circulating tMGP could be used as marker for discriminating among healthy individuals and VD patients.

Material and method

Study design and subjects

In this pilot case-control study, we have enrolled consecutively apparently healthy subjects (n=49) and a group of patients with VD (n=72). The healthy subjects were enrolled voluntarily, directed from the general practitioners during the Screening Program of National Health. Only individuals over 40 years were included, without a medical history of VD, chronic kidney disease, osteopenia or osteoporosis, rheumatoid arthritis or inflammatory diseases. The patients group has included individuals diagnosed with VD and admitted in Cluj County Hospital. None of the patients had medical history of osteopenia /osteoporosis or rheumatoid arthritis. The subgroups of patients with VD were enrolled fom the outpatient clinic of Internal Medicine Department: HT (n=30), CAD (n=26), DM (n=10) and stroke (n=6). The group of healthy subjects, but also patients with VD had no history of medication with vitamin K antagonists, anticoagulants, corticosteroid drugs or bisphosphonates in the past 2 years.

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Measurements

BMI was computed as the proportion between weight and height squared (kg/m2). Current smokers or history of smoking for at least 2 years in the past 5 years were defined as smokers. Blood pressure was quantified after resting 5 minutes by a sphygmomanometer positioned on the right arm.

We have collected venous blood after fasting eight hours. Following 10 minutes centrifugation at 3,000 rpm, serum was deposited at –80° C until analysis.

Enzymatic or end-point methods for the assessment of glucose, cholesterol, triglycerides and HDL-colesterol were carried out on automated equipment (Cobas Mira Plus, Roche). The intra-assay coefficient of variation (CV) for these parameters was below 5%. LDL-colesterol was calculated using Friedewald`s formula [total cholesterol - (HDL-colesterol + triglycerides/5)].

For the measurement of serum hs-CRP, we used an immunoturbidimetric assay (CRP U-hs, Diasys) on a automated biochemistry analyzer (CS-T240, Dirui). We obtained an intra-assay CV of 6.5 %.

We used a sandwich ELISA kit to assess tMGP (USCN Life Science) on the Organon 230S (Organon Teknika) plate reader. An intra-assay CV of 5.8 % was obtained.

Statistical analysis

We used Kolmogorov-Smirnov test to assess the distribution of continuous variables. Depending on distribution, variables were stated as mean ± standard deviation (SD) or median with minimum and maximum values. Differences between variables with normal distribution were assessed with student T test. We performed Chi-square test or Fischer's test for categorical variables. Differences in tMGP or hs-CRP levels between subgroups were assessed using One-Way ANOVA with post-hoc Scheffe's test. Pearson's coefficient was reported for correlations of tMGP or hs-CRP with different variables. We used SPSS version 15.0 for statistical analysis and significance was founded on two-tailed tests with p values < 0.05.

Results

The features of healthy population (n = 49) are depicted in Table 1. Within this population, we evaluated the association of tMGP with different characteristics and biochemical measurements. We have noticed only the correlation between tMGP and smoking ($r = 0.332$, $p < 0.05$).

Table 1. Features of healthy population

Healthy population (n=49)	
Characteristics	
Age, years	54 ± 9
Gender, male/female	15/34
BMI, kg/m²	25 ± 3
Smokers, n (%)	8 (16)
Biochemical analyzes	
Glucose, mmol/L	5.1 ± 0.7
Cholesterol, mmol/L	5.5 ± 1.0
HDL-colesterol, mmol/L	1.3 ± 0.3
LDL-colesterol, mmol/L	3.4 ± 0.9
Triglycerides, mmol/L	1.5 ± 0.6
hs-CRP, mg/L	3.5 ± 0.8
tMGP, µg/L	51 ± 22

Data are expresses as mean ± standard deviation or number (percentage), depending on distribution. Abbreviations: BMI = body mass index; hs-CRP = high-sensitivity C-reactive protein; tMGP = total matrix Gla protein.

In terms of population with VD (n=72), the characteristics are detailed in Table 2.

Table 2. Characteristics of population with vascular diseases

	Population with vascular diseases (n = 72)				
	All (n=72)	**HT (n=30)**	**CAD (n=26)**	**DM (n=10)**	**Stroke (n=6)**
Demographics					
Age, years	62±10	63±11	63±9	55±6	60±12
Gender, male/female	16/56	6/24	7/19	2/8	1/5
BMI, kg/m²	29±6	30±6	28±4	32±9	32±7
Smokers, n (%)	19(26)	7(23)	5(19)	6(60)	1(17)
Measurements					
DBP, mmHg	84±12	87±10	80±12	77±14	94±14
SBP, mmHg	144±25	151±23	140±26	128±21	157±27
Glucose, mmol/L	5.8(4.3-14.4)	6.4±1.9	6.2±1.9	6.6±3.1	8.2±4.1
Cholesterol, mmol/L	5.6±1.6	5.5±1.6	6.1±1.6	5.5±1.3	4.3±0.9
HDL-colesterol, mmol/L	1.3±0.3	1.4±0.3	1.3±0.4	1.2±0.2	1.3±0.6
LDL-colesterol, mmolL	3.7±1.4	3.6±1.6	4.2±1.6	3.7±0.9	2.2±0.8
Triglycerides mmol/L	1.7±1.1	1.8±1.5	1.6±0.8	1.7±0.9	1.4±0.6
hs-CRP, mg/L	6.3±3.9	5.4±3.6	6.8±3.9	5.8±4.2	8.6±5.3
tMGP, µg/L	106±30	105±28	107±30	105±33	108±34

Data are expresses as mean ± standard deviation or number (percentage), depending on distribution. Abbreviations: BMI = body mass index; hs-CRP = high-sensitivity C-reactive protein; tMGP = total matrix Gla protein.

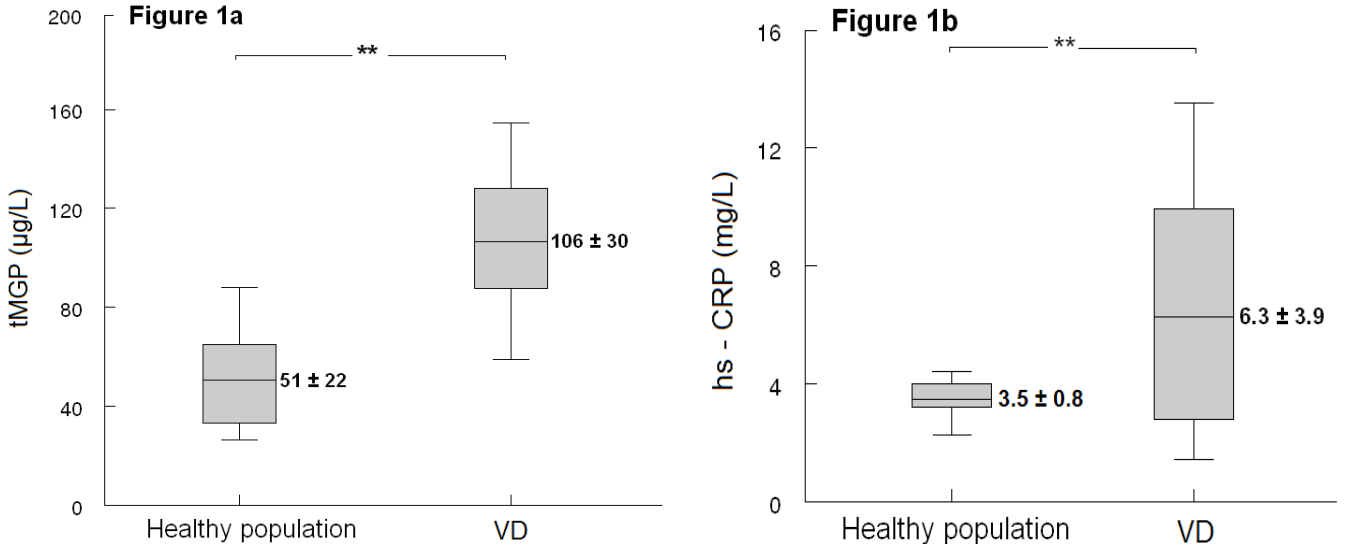

Figure 1a: Diferrences of tMGP and Figure 1b: diferrences of hs-CRP in healthy subjects versus patients with vascular disease. Data are mean ± standard deviation; whiskers correspond to standard error; ** stand for $p < 0.001$. Abbreviations: tMGP = total matrix Gla protein; hs-CRP = high sensitivity- C reactive protein; VD = patients with vascular disease.

We did not notice significant difference in terms of circulating tMGP and hs-CRP between the subgroups of patients with HT, CAD, DM or stroke.

Next, we analyzed the correlations between levels of tMGP and hs-CRP, but also their correlation with risk factors (variables of demographics, medical history and measurements) in patients with VD. We found a relative weak correlation between tMGP and age ($r = -0.25$, $p < 0.05$) or between BMI and hs-CRP ($r = 0.32$, $p < 0.05$), but no correlation between hs-CRP and tMGP. This lack of association between hs-CRP and tMGP was also observed within HT, CAD, DM or stroke subgroups. We found only an association between hs-CRP and triglycerides ($r = 0.66$, $p < 0.05$) within DM group.

Subsequently, we ensued in investigating the differences in hs-CRP and tMGP levels between healthy and VD populations. Significantly lower levels of hs-CRP and tMGP were found in healthy subjects compared to VD population ($p < 0.001$), as depicted in Figure 1a, respectively Figure 1b.

Eventually, significantly lower tMGP was found in healthy subjects compared to subgroups with HT ($p < 0.001$), CAD ($p < 0.001$), DM ($p < 0.001$) or stroke ($p < 0.01$).

Discussion

The purpose of the study was to investigate the difference between apparently healthy subjects and patients with VD. We

enrolled asymptomatic healthy subject over 40 years because evidence indicated that individuals over 40 years had significantly higher calcium scores found in coronary arteries than asymptomatic subjects under 40 years (Nasir et al 2004). In this pilot study, the lack of association between age and tMGP in healthy population was in disagreement with Cranenburg's study (Cranenburg et al 2008) which has noticed a declining trend with age, but for circulating ucMGP. The explanation could lie, on one hand, in the difference between the average age of healthy populations: our average was 54±9 years and in Cranenburg's study was a 49±16 years. Secondly, we assessed the concentration of tMGP and not ucMGP. These two forms may not have a correlation between them, but this assumption was not yet demonstrated.

To our knowledge, this study was the first to demonstrate a positive association between smoking and circulating tMGP in healthy subjects. In healthy women, Dalmeijer et al (2013) did not find any relationship between ucMGP or dp-cMGP and smoking, but still has established an inverse correlation with dp-ucMGP. Smokers could have a superior vascular need for protection against calcification compared to non-smokers. Probably smoking may cause an increase of MGP expression in lung, which is not reflected by an increase of its concentration in blood. This hypothesis could be supported by an increase of MGP mRNA expression found in lungs, despite the fact that concentration of MGP in lung tissue was decreased (Fraser & Price 1998).

The correlation between circulating tMGP and smoking was not preserved in patients with VD, in which tMGP decreases with age. The explanation could lie in the fact that our VD population had a higher average age than healthy subjects, leading to a decrease of serum MGP and, subsequently, to a smaller concentration in the vascular wall of smokers with VD. In this way, the correlation between smoking and tMGP found among apparently healthy subjects was not preserved in smokers with VD. This is just a hypothesis, because the contribution rate of vascular MGP to the total pool of circulating MGP is yet unknown. The only evidence is that serum tMGP level is depending on the production and secretion of vascular smooth muscle cells, but also to its binding rate to calcified sites within vascular walls (Schurgers et al 2008).

In the group of healthy subjects, as well as in patients with VD or in subgroups with HT, stroke, DM or CAD, there was no association between tMGP and hs-CRP. Studies were divergent regarding this association: a direct association with dp-ucMGP (Ueland et al 2010), negative association with dpMGP (Thomsen et al 2010) or no association of ucMGP with hs-CRP (Parker et al 2010) was reported. It was already established that low-grade inflammation has a contribution in the evolution of atherosclerosis, generating an environment for vascular calcifications (Ross 1999). The hs-CRP levels in VD patients were almost two times higher than healthy population, leading to greater permeability in vascular cells and thus, an increase in circulating tMGP. The decreased levels of hs-CRP and tMGP observed in healthy individuals compared to VD population also strengthen this theory. Therefore, it is notable that serum tMGP will increase with inflammation in VD population.

One important finding was that increased levels of circulating tMGP found in VD population might discriminate between patients with VD and healthy subjects. Also, these significantly higher serum MGP levels were also noticed in subgroups with HT, stroke CAD or DM. A possible reason might be that vascular calcification is more common in a population with VD and the requirements for tMGP around the calcification areas will increase. Hence, the circulating tMGP increases in subjects if there is an overt VD associated.

One of the strong points of the study was the evaluation of serum tMGP in a reasonably homogenous healthy group (over 40 years) by means of an available commercial kit. We assessed in parallel the association with cardiovascular risk factors, but also we performed a categorization of patients with VD by different pathology (HT, CAD, DM and stroke).

There are also some drawbacks of this pilot research: we could not ascertain a cause-effect association between tMGP and risk factors for VD (age, smoking and inflammation) because of the cross-sectional design and also we did not evaluate the amount of vascular calcification by using electron-beam computed tomography or multislice computed tomography.

Conclusion

In conclusion, circulating tMGP was positively associated with smoking in apparently healthy subjects. In patients with VD this association was not preserved, but tMGP decreases with age. Moreover, circulating tMGP levels were considerably lower in healthy subjects than population with VD or in any of its subgroups. Accordingly, with further validation, serum tMGP levels could be used as a reasonable screening instrument for discriminating between healthy subjects and patients with VD.

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