Human & Veterinary Medicine
International Journal of the Bioflux Society HVM

OPEN ACCESS
Research Article

The behavior of circulating matrix Gla protein, matrix metalloproteinase-9 and nitrotyrosine in patients with varicose veins

[1]Ciprian N. Silaghi, [2]Gabriel Olteanu, [1]Alexandra M. Crăciun

[1] Department of Medical Biochemistry, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania; [2] Department of Surgery, "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania.

Abstract. Objectives: The main objective was to assess the interplay between circulating matrix Gla protein (MGP) - marker for vascular calcification, matrix metalloproteinase-9 (MMP-9) - marker for extracellular matrix remodeling and nitrotyrosine (NT) - marker for oxidative stress in patients with varicose veins (VV). Moreover, we wanted to investigate the behavior of these parameters before and after a stressful event (surgical removal of VV from lower limbs) and to find out if there is a contribution of MGP originating from superficial veins of the lower limb to the total pool of circulating MGP. Material and method: The pilot cohort study was accomplished on patients with VV (n=29) before and after a stressful event (surgical removal of VV from inferior limbs) and a group of age-gender matched apparently healthy volunteers (n=29). Plasma levels of tMGP, MMP-9 and NT were assayed with commercially available immunoassay kits. Results: Differences between patients with VV and age-sex matched healthy subjects were reflected only by higher levels of MMP-9 [82 (19-159) ng/mL versus 36 (2-108) ng/mL, $p<0.05$]; and not by circulating tMGP or NT levels. When patients before removal of VV were compared to patients after surgery, only tMGP was found significantly decreased [65 (32-97) µg/L versus 40 (17-95) µg/L, $p<0.05$]. We also found a correlation between tMGP and MMP-9 in patients with VV ($r = 0.37$, $p<0.05$). We did not find any correlation of NT with tMGP or MMP-9 and no significant differences in plasma NT levels in any pairwise comparisons. Conclusion: Higher circulating levels of MMP-9 could differentiate between healthy individuals and patients with chronic venous insufficiency. Consequently, oxidative stress assessed by NT did not affect circulating levels of tMGP or MMP-9 after surgical removal of VV. The constitutive decrease in plasma level of tMGP could be considered the contribution of MGP from superficial veins of the inferior limb to the total pool of circulating MGP.

Key Words: matrix Gla protein, matrix metalloproteinase-9, nitrotyrosine, varicose veins.

Corresponding Authors: G. Olteanu, e-mail: olteanu@umfcluj.ro

Introduction

Varicose veins (VV) are the most common manifestation of chronic venous insufficiency affecting the superficial veins of the lower extremity. The prevalence of VV in the general population is between 15-50%, thus being an important cause of morbidity (Woodside et al 2003). Aging, female gender, pregnancy, obesity, and family history have been identified as risk factors for developing VV (Carpentier et al 2004), whereas the pathogenesis and progression of the disease remain unclear.

Matrix Gla protein (MGP), a major local ectopic calcification inhibitor synthesized by vascular smooth muscle cells (VSMCs) and chondrocytes, was considered playing a key role in both physiological and pathological calcification processes (Schurgers et al 2008). This vitamin K-dependent extracellular protein could be found in different forms, depending on its two post-translational modifications (carboxylation and phosphorylation): carboxilated, uncarboxylated, phosphorylated, desphosphorylated, or combinations thereof. The only available kit is an immunoassay for MGP [designated by us as total MGP (tMGP) because it can not discriminate between the conformations mentioned above]. Serum MGP levels depend on protein synthesis and secretion, and also on the amount of MGP accumulated at the calcification areas. The contribution of each tissue (bone, cartilage, arterial and venous wall) to the circulating pool of MGP is yet unknown.

In respect to chronic venous insufficiency, the only study published on MGP has shown an increased MGP expression in VV and an association between over-expression of MGP transcripts and increased local MGP levels (Cario-Toumaniantz et al 2007). The study showed that carboxylated MGP was more abundant in the media of non-VV, and uncarboxylated MGP was detected abundantly in the media of the VV. VSMCs from VV showed an increased proliferation rate and enhanced matrix mineralization. Because MGP inhibits the proliferation and mineralization of VSMCs, the study concluded that high MGP expression in VV may have a contribution to vein wall remodeling. Hence, there is no published data on the behavior of circulating MGP in patients with VV.

It was demonstrated that oxidative stress increases in VV, leading to accumulation of reactive oxygen species (ROS) due to leukocyte trapping in the vessel wall and subsequent activation (Krzyściak et al 2011). The reaction between two free radicals, nitric oxide and superoxide, leads to the formation of peroxynitrite which reacts with the phenolic ring of tyrosine and forms nitrotyrosine (NT). Serum accumulation of NT was considered a reflection of the oxidant/antioxidant imbalance (Sies 1991) being the footprint of the interaction between nitric oxide and ROS. A loss of nitric oxide bioavailability with increased ROS production was considered characteristic for endothelial dysfunction (Guzik et al 2002) which is a possible mechanism in VV development (Carrasco et al 2009).

The human matrix metalloproteinases family are either secreted or membrane-bound enzymes, distinguished by their Zn^{2+} binding capacity to carry out the proteolytic activity and also by their capability to degrade extracellular matrix (EM) (Shin et al 2007). Among them, matrix metalloproteinase-9 (MMP-9) is an enzyme involved in EM remodeling by digesting denatured collagens (Allan at al 1995). It was demonstrated that MMP-9 is localized in the endothelial cells, VSMCs and adventitial microvessels in both normal veins and VV (Woodside et al 2003) with increased expression and activity in thrombophlebitic VV (Kowalewski et al 2004). Furthermore, serum levels of pro-MMP-9 were found elevated in blood stasis due to venous insufficiency, providing evidence of polymorphonuclear activation in the VV walls in response to postural stasis (Jacob et al 2002).

The complex interplay between vascular wall remodeling, oxidative stress and matrix mineralization reflected on circulating level was not investigated before. Only a study was published on circulating NT in patients with VV (Condezo-Hoyos et al 2013) but the serum markers for EM remodeling (e.g., MMP-9) and matrix mineralization (e.g., MGP) were not assessed. If arterial calcifications have received more attention, there is still no published data on the behavior of circulating tMGP in patients with VV.

The main objective of our study was to determine the variations in circulating tMGP, MMP-9 and NT levels and also to evaluate the relationship between them in patients with VV compared to controls. Moreover, we wanted to investigate the behavior of these parameters before and after a stressful event (surgical removal of VV from inferior limbs) and to find out if there is a contribution of MGP originating from the veins of inferior limbs to the total circulating pool of MGP.

Material and method

The study was conducted on a control group (n=29) and a patients group (n=29). The mean age was 47±10 years and gender distribution was 9 males and 20 females in both groups. The patients group included patients diagnosed with VV, admitted at the 2nd Vascular Surgery Clinic, University County Hospital Cluj-Napoca, for crossectomy, great saphenous vein stripping and phlebectomies (Muller's method). Patients with history of vitamin K antagonist treatment were excluded from the study. The control group consisted of age-sex matched apparently healthy volunteers with no history of VV, diabetes mellitus or coronary artery disease. Also, subjects diagnosed with chronic kidney disease, inflammatory or osteoarticular diseases were excluded from the study. Smokers were defined as current habit or smoking history for at least two years in the past five years. Prior to enrollment, informed consent was obtained from all participant and demographics and medical history were recorded. All data will remain confidential and will not be used outward the study. The study design was approved by the Medical Ethics Committee of our University and was in accordance with the declaration of Helsinki.

We obtained venous blood samples after approximately 12 hours of fasting from both control and patients group before surgical intervention and 4-5 days after surgery. Venous blood samples were collected in sodium citrated tubes and were centrifuged in maximum 1 hour. We preserved the obtained citrated plasma at -80°C until assaying.

Plasma levels of MMP-9 were determined with a commercially available sandwich ELISA kit (IBL International GMBH, Hamburg, Germany) on an Organon 230S reader (Organon Teknika, Oss, the Netherlands), following the manufacturers protocol. The detection limit of the assay was 0.05 ng/ml and our intra-assay CV was 7.4%.

Plasma levels of NT were assayed with a commercially available sandwich ELISA kit (Abnova, Jhongli, Taiwan) on an Organon 230S reader (Organon Teknika, Oss, the Netherlands). The detection limit of the assay was 2 nM and our intra-assay CV was 2.2 %.

Plasma tMGP levels were assessed with a sandwich 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 detection range for tMGP in plasma was 39-2500 pg/ml. The sensitivity of the assay and our intraday CV was 20 ng/L and 6.1%, respectively.

Variables were expressed as median with minimum and maximum value between parentheses. For comparisons between patients with varicose veins before surgery and patients after surgery, the Wilcoxon test was used. The chi-square and Fischer's exact tests were used for categorical variables to test the homogeneity of proportions. Mann-Whitney U test was computed to assess differences between controls and patients with VV, between genders or smoking/non-smoking subjects. For bivariate correlation analysis the Spearman's Rho correlation coefficient (r) was performed. The reported p-values are based on two-tailed tests and $p<0.05$ was considered statistically significant. Statistical analysis was completed with SPPS version 15.0 for Windows (SPSS inc., Chicago, Il, USA).

Results

The laboratory measurements of the age-gender matched controls and patients with VV before and after surgery are summarized in Table 1. The mean age was 47±10 years and gender distribution was 9 males and 20 females in both groups. There was no significant difference in terms of demographics or medical history between patient and control groups except for a lower body mass index (BMI) in the control group ($p<0.001$). We found significantly lower serum tMGP and MMP-9 levels in patients after surgery compared to controls ($p<0.05$ and $p<0.001$, respectively). Only MMP-9 was significantly decreased in patients before surgery compared to controls ($p<0.001$). Circulating tMGP levels decreased with 15% after surgery compared to patients before surgery. Serum NT levels did not differ significantly between groups in all pairwise comparisons.

Table 1. Laboratory measurements in controls and patients before and after surgery

	Control group (n=29)	Patient group		p (patients)
		Before surgery (n=29)	After surgery (n=29)	
Measurements				
tMGP, µg/L	59 (18-154)	65 (32-97)	40 (17-95)	<0.05
NT, nM/L	2143.5 (1680-2883)	2469.5 (1782-2961)	2430 (1654-3058)	NS
MMP-9, ng/mL	82 (19-159)	36 (2-108)	29 (4-92)	NS

Data are presented as median with minimum and maximum value between parentheses. The p-values for comparisons between patients before and after surgery are given. Abbreviations: NS - not significant.

Table 2. Correlations of circulating tMGP, MMP-9 and NT

	Controls					Patients with VV				
	Smoking	**HT**	**tMGP**	**MMP9**	**NT**	**Smoking**	**HT**	**tMGP**	**MMP9**	**NT**
tMGP	NS	NS		NS	NS	0.28*	-0.31*		0.37*	NS
MMP9	0.61**	-0.47*	NS		NS	NS	-0.41*	0.37*		NS
NT	NS	NS	NS	NS		-0.43*	NS	NS	NS	

Values represent r (Spearman's Rho correlation coefficient) with *p<0.05, and ** p<0.001. Abbreviations: HT - hypertension.

Regarding the differences in serum tMGP, MMP-9 and NT levels between smokers and non-smokers in the control group, only circulating MMP-9 was higher in smokers than non-smokers [133 (95-159) ng/mL versus 67 (19-153) ng/mL, p=0.001]. Besides a marginal statistical variation of tMGP levels in patients with VV [82 (19-154) µg/L in smokers versus 50 (27-116) µg/L in non-smokers, p=0.056], the other two circulating parameters were not significantly modified. In addition, we did not find significant gender differences for serum tMGP, MMP-9 and NT levels in the two groups.

Even if BMI was higher in patients compared to controls, there was no correlation with serum tMGP, MMP-9 or NT levels in any group. Moreover, these circulating parameters were not correlated with age and gender neither in patients nor in controls. The strong association between MMP-9 and smoking found in the control group (r=0.61, p<0.001) had become marginal in patient group (r=0.26, p=0.052) and was reported as not significant. There was no correlation between tMGP and NT in any group. Correlations of tMGP, MMP-9 and NT are presented in Table 2.

Discussion

The etiology and mechanisms that lead to structural changes in VV are not entirely understood, although recent studies suggest an imbalance between VSMCs multiplication and EM organization (Badier-Commander et al 2000). Degradation of EM is required to permit relocation of cells throughout remodeling (Raffetto et al 2008). EM surrounding VSMCs was degraded by MMP-9 along with other matrix metalloproteinases (Borden & Heller 1997).

Our study showed a decrease of circulating MMP-9 levels in patients with VV before and after surgery compared to the control group. A study demonstrating less MMP-9 activity in VV then normal veins (Woodside et al 2003) could support our finding, suggesting a disruption in the EM remodeling of VV, possibly due to differences in MMP activation/inhibition or other factors related to enzyme activity. Conversely, a study reporting variation in MMP-9 expression when comparing normal veins with VV (Parra et al 1998) found no immunohistochemical differences.

Within the patient group, circulating tMGP was significantly decreased after surgery, whereas serum level of MMP-9 has remained relatively unchanged. Furthermore, serum tMGP and MMP-9 behaved differently when comparing patients and control group: both were higher in controls versus patients after surgery, but only MMP-9 was significantly lower in terms of differences between controls and patients before surgery. VSMCs are involved in EM remodeling which is altered in VV, explaining why MMP-9 levels are declined in patients with VV compared to controls. Even if lower levels of tMGP were found in patients with VV before surgery compared to the control group, statistical significance was not reached. Furthermore, no correlation between MMP-9 and tMGP in controls was found. Therefore, the EM alteration in patients with VV before surgery is reflected only by MMP-9 and not by circulating tMGP levels, since calcification of superficial veins of inferior limbs is rare (de Godoy & Batigália 2011).

Patients after surgery had significantly lower levels of tMGP than patients before surgery and control group, respectively. Even if MMP-9 was also decreased, statistical significance was not reached. MMP-9 is localized in VSMCs (Woodside et al 2003) which also represent the site of MGP synthesis (Schurgers et al 2008). It seems possible that these findings could be the consequence of the surgical removal of VV, resulting in a decrease of venous tissue and therefore of VSMCs. This explanation was strengthened by the relationship found between tMGP and MMP-9 in patients with VV. The constitutive decrease in tMGP was 15%, suggesting that it can be the contribution of MGP from inferior limb veins to the total pool of circulating MGP. A study reporting low constitutive MGP expression in patients after percutaneous coronary intervention compared to a healthy control group (Schurgers et al 2005) could support our hypothesis of constitutive decrease.

No significant differences regarding NT levels between controls and patients before and after surgery were found. These findings are supported by a recent study conducted on patients in early stages of chronic venous insufficiency, in which NT was not included in a global index of oxidative stress in chronic venous insufficiency (Condezo-Hoyos et al 2013). Subsequently,

we found no correlations between NT and MMP-9 or MGP, inferring that oxidative stress did not have a contribution to the decrease of tMGP and MMP-9 in patients with VV.

The strengths of our study were the comparison of tMGP, MMP-9 and NT levels between age-sex matched healthy subjects and patient population, but also the evaluation of these parameters behavior before and after surgery.

Drawbacks of the present study were the number of subjects, the transversal design and the fact that patients with VV were not stratified according to the clinical, etiologic, anatomic, and pathophysiologic data (CEAP classification) of chronic venous disease (Eklöf et al 2004). We also did not assess the possible influence of medication (statines, flavonoides) or stasis of the compression stockings on tMGP levels. This information would have offered a better insight and a more accurate assessment of tMGP in patients with VV, leading to an advanced evaluation of tMGP status. Longitudinal studies exploring the behavior of tMGP in chronic venous insufficiency are needed in order to determine if there is a specific role of MGP in EM remodeling. Therefore, the results of this study warrant further investigations and should be interpreted with caution. Furthermore, future work conducted on larger cohorts and VV patients distributed based on the CEAP classification are recommended.

Conclusion

The differences between patients with VV before surgery and controls are reflected only by higher levels of MMP-9 and not by circulating tMGP levels. Higher circulating levels of MMP-9 (a proxy for EM remodeling) might differentiate between controls and patients with chronic venous insufficiency. Also, oxidative stress (using NT as marker) did not affect the circulating levels of tMGP or MMP-9 after surgical removal of VV. The substantial lower tMGP after venous stripping compared to levels before surgery, along with the correlation with MMP-9 found in patients group are evocative for low constitutive MGP. Thus, it is noteworthy that MGP originating from veins had a contribution to the total pool of circulating MGP.

References

Allan JA, Docherty AJ, Barker PJ, Huskisson NS, Reynolds JJ, Murphy G. Binding of gelatinases A and B to type-I collagen and other matrix components. Biochem J 1995;309:299-306.

Badier-Commander C, Verbeuren T, Lebard C, Michel JB, Jacob MP. Increased TIMP/MMP ratio in varicose veins: a possible explanation for extracellular matrix accumulation. J Pathol 2000;192(1):105-12.

Borden P, Heller RA. Transcriptional control of matrix metalloproteinases and the tissue inhibitors of matrix metalloproteinases. Crit Rev Eukaryot Gene Exp 1997;7:159-78.

Cario-Toumaniantz C, Boularan C, Schurgers LJ, Heymann MF, Le Cunff M, Léger J, Loirand G, et al. Identification of differentially expressed genes in human varicose veins: involvement of matrix gla protein in extracellular matrix remodeling. J Vasc Res 2007;44:444-59.

Carpentier PH, Maricq HR, Biro C, Pancot-Makinen CO, Franco A. Prevalence, risk factors, and clinical patterns of chronic venous disorders of lower limbs: a population-based study in France. J Vasc Surg 2004;40:650-9.

Carrasco OF, Ranero A, Hong E, Vidrio H. Endothelial function impairment in chronic venous insufficiency: effect of some cardiovascular protectant agents. Angiology 2009;60: 763-71.

Condezo-Hoyos L, Rubio M, Arribas SM, España-Caparrós G, Rodríguez-Rodríguez P, Mujica-Pacheco E, at al. A plasma oxidative stress global index in early stages of chronic venous insufficiency. J Vasc Surg 2013;57:205-13.

de Godoy JM, Batigália F. Spontaneous elimination of superficial vein calcified. Cent Eur J Med 2011;6:233.

Eklöf B, Rutherford RB, Bergan JJ, Carpentier PH, Gloviczki P, Kistner RL, et al. Revision of the CEAP classification for chronic venous disorders: consensus statement. J Vasc Surg 2004;40(6):1248-52.

Guzik TJ, West NE, Pillai R, Taggart DP, Channon KM. Nitric oxide modulates superoxide release and peroxynitrite formation in human blood vessels. Hypertension 2002;39:1088-94.

Jacob MP, Cazaubon M, Scemama A, Prié D, Blanchet F, Guillin MC, et al. Plasma matrix metalloproteinase-9 as a marker of blood stasis in varicose veins. Circulation 2002;106:535-8.

Kowalewski R, Sobolewski K, Wolanska M, Gacko M. Matrix metalloproteinases in the vein wall. Int Angiol 2004;23:164-9.

Krzyściak W, Kózka M. Generation of reactive oxygen species by a sufficient, insufficient and varicose vein wall. Acta Biochim Pol 2011;58:89-94.

Parra JR, Cambria RA, Hower CD, Dassow MS, Freischlag JA, Seabrook GR, et al. Tissue inhibitor of metalloproteinase-1 is increased in the saphenofemoral junction of patients with varices in the leg. J Vasc Surg 1998;28:669-75.

Raffetto JD, Qiao X, Koledova VV, Khalil RA. Prolonged increases in vein wall tension increase matrix metalloproteinases and decrease constriction in rat vena cava: Potential implications in varicose veins. J Vasc Surg 2008;48:447-56.

Schurgers LJ, Cranenburg EC, Vermeer C. Matrix Gla-protein: the calcification inhibitor in need of vitamin K. Thromb Haemost 2008;100:593-603.

Schurgers LJ, Teunissen KJ, Knapen MH, Kwaijtaal M, van Diest R, Appels A, et al. Novel conformation-specific antibodies against matrix gamma-carboxyglutamic acid (Gla) protein: undercarboxylated matrix Gla protein as marker for vascular calcification. Arterioscler Thromb Vasc Biol 2005;25:1629-33.

Shin Y, Yoon SH, Choe EY, Cho SH, Woo CH, Rho JY, et al. PMA-induced up-regulation of MMP-9 is regulated by a PKCalpha-NF-kappaB cascade in human lung epithelial cells. Exp Mol Med 2007;39:97-105.

Sies H. Oxidative stress: from basic research to clinical application. Am J Med 1991;91:31S-38S.

Woodside KJ, Hu M, Burke A, Murakami M, Pounds LL, Killewich LA, et al. Morphologic characteristics of varicose veins: possible role of metalloproteinases. J Vasc Surg 2003;38:162-9.

Authors

•Ciprian N. Silaghi, Department of Medical Biochemistry, "Iuliu Haţieganu", University of Medicine and Pharmacy, 6th Pasteur Street, Cluj-Napoca, Romania, EU, e-mail: silaghiciprian@yahoo.com

•Gabriel Olteanu, Department of Surgery, 2nd Surgery Clinic, "Iuliu Haţieganu" University of Medicine and Pharmacy, 3-5th Clinicilor Street, Cluj-Napoca, Romania, EU, e-mail: golteanu@umfcluj.ro

•Alexandra M. Crăciun, Department of Medical Biochemistry, "Iuliu Haţieganu" University of Medicine and Pharmacy, 6th Pasteur Street, Cluj-Napoca, Romania, EU, e-mail: acraciun@umfcluj.ro

Citation	Silaghi CN, Olteanu G, Crăciun AM. The behavior of circulating matrix Gla protein, matrix metalloproteinase-9 and nitrotyrosine in patients with varicose veins. HVM Bioflux 2015;7(2):65-69.
Editor	Stefan C. Vesa
Received	5 February 2015
Accepted	17 February 2015
Published Online	17 February 2015
Funding	None reported
Conflicts/ Competing Interests	None reported