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ORIGINAL ARTICLE

From varices to venous ulceration: the story of chronic venous disease described by metalloproteinases

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Key words
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Abstract

Chronic venous disease (CVD) and its most frightening complication, chronic venous ulceration (CVU), represent an important socioeconomic burden in the western world. Metalloproteinases have been identified in the pathogenesis of several vascular diseases such as venous problems. The aim of this study was to evaluate a broad range of metalloproteinases, such as matrix metalloproteinases (MMPs), ADAMs (a disintegrin and metalloproteinases) and ADAMTSs (a disintegrin and metalloproteinases with thrombospondin motifs) and their inhibitors, tissue inhibitor of metalloproteinases (TIMPs) and a related protein, neutrophil gelatinase-associated lipocalin (NGAL), in patients with CVD in order to correlate their serum levels with each stage of the disease. We performed a multicenter open-label study that comprised the enrolment of 541 patients with CVD of clinical stages C1–C6, (178 males, 363 females; mean age 57·29, median age 53·72, age range 29–81); 29 subjects without CVD were included in this study (9 males and 20 females; mean age 54·44, median age 50, age range 28–84) as the control group. Enzyme-linked immunosorbent assay (ELISA) was performed for measuring serum levels of proteases and related proteins. The study found that the serum elevation of MMP-2, ADAMTS-1 and ADAMTS-7 appeared to be correlated with the initial stages of CVD, whereas the serum elevation of MMP-1, MMP-8, MMP-9, NGAL, ADAM-10, ADAM-17 and ADAMTS-4 was particularly involved in skin change complications. This study showed that each stage of CVD may be described by particular patterns of metalloproteinases, and this may have therapeutic implications in discovering new targets and new drugs for the treatment of CVD.

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Introduction

Chronic venous disease (CVD) is a very common problem affecting the Western adult population, with a prevalence of <10% among individuals younger than 30 years for both genders and with a prevalence up to 77% in individuals aged ≥70 years (1,2).

According to clinical classes (C) of the Clinical-Aetiology-Anatomy-Pathophysiology (CEAP) classification (3), the spectrum of CVD ranges from various types of varices (telangiectasia, reticular veins and varicose veins) (classes C1–C2) to leg oedema (class C3) and serious skin changes, including hyperpigmentation, eczema, lipodermatosclerosis (class C4) and venous skin ulceration (classes C5–C6). Classes C3–C6 indicate a more advanced form of CVD called chronic venous insufficiency (CVI) (1).

A further sign of CVD, corona phlebectatica (fan-shaped intradermal telangiectases on the medial or lateral aspects of the foot), has been poorly investigated as it is not yet included in CEAP classification, although it appears to correlate with the clinical severity in the progression of CVD (4,5).

The pathogenesis of CVD encloses several theories focusing on endothelial dysfunction and extracellular matrix (ECM) unbalance. It has been particularly suggested that the balance between matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) plays a crucial role in all the clinical manifestations of CVD. Furthermore, MMP-1, MMP-2, MMP-8, MMP-9, TIMP-1, TIMP-2 and neutrophil gelatinase-associated lipocalin (NGAL), which is directly involved in the regulation of MMPs activity, appear to be directly associated with venous disease (6–14).

There are also further members of different metalloproteinases families, such as ADAMs (a disintegrin and metalloproteinases) and ADAMTSs (a disintegrin and metalloproteinases with Thrombospondin motifs), that were investigated for the cardiac and the arterial side of cardiovascular disease (ADAM-10, ADAM 12, ADAM-17, ADAMTS-1, ADAMTS-4, ADAMTS-5, ADAMTS-7) (15,16), but they have not yet been evaluated in venous disease.

The aim of this study is to describe the entire pattern of clinical manifestations of CVD related to serum levels of some members of the MMP, TIMP, ADAM and ADAMTS families and NGAL.

Key Messages

- chronic venous disease (CVD) and its complications, such as chronic venous ulceration (CVU), is a very important problem in the Western adult population
- metalloproteinases, such as matrix metalloproteinases (MMPs), ADAMs (a disintegrin and metalloproteinases) and ADAMTSs (a disintegrin and metalloproteinases with Thrombospondin motifs) and their inhibitors, tissue inhibitor of metalloproteinases (TIMPs), and a related protein, neutrophil gelatinase-associated lipocalin (NGAL), are involved in vascular disease
- this study evaluated serum concentrations of some proteases, and related molecules, in patients with CVD and identified several patterns of metalloproteinases according to each stage of CVD
- MMP-2, ADAMTS-1 and ADAMTS-7 serum elevation appears to be correlated with the initial stages of CVD, while MMP-1, MMP-8, MMP-9, NGAL, ADAM-10, ADAM-17 and ADAMTS-4 serum elevation was particularly involved in skin change complications

Materials and methods

Study design

We performed a multicenter open-label study between 1 January 2013 and 30 June 2015. This study was approved by the Investigational Review Board (IRB) of the Interuniversity Center of Phlebolymphology (CIFL) International Research and Educational Program in Clinical and Experimental Biotechnology in accordance with the Declaration of Helsinki and the Guideline for Good Clinical Practice. Before the beginning of the study, all participants provided written informed consent.

Population

Inclusion criteria

Patients with CVD, of both genders, older than 18 years, belonging to classes C1–C6 of the CEAP classification (3) were included.

Exclusion criteria

Patients with cancer, hepatic failure, infectious or autoimmune diseases, arthritis, arterial aneurysms, hernias, previous or active venous thromboembolism, nephritis, peripheral artery disease, fibrosis and other diseases associated with increased levels of MMPs were excluded from the study. Patients were treated with corticosteroids or cytostatic drugs.

On inclusion in the study, blood samples were collected from venipuncture in order to evaluate serum levels of MMP-1, MMP-2, MMP-8, MMP-9, TIMP-1, TIMP-2, NGAL, ADAM-10, ADAM 12, ADAM-17, ADAMTS-1, ADAMTS-4, ADAMTS-5 and ADAMTS-7.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were used in this study. The blood samples were collected and allowed to clot before centrifugation. After centrifugation, serum was removed and stored at −20°C. An enzyme-linked immunosorbent assay (ELISA) was performed to measure serum levels using commercially available kits (USCNK, USCN Life Science, Wuhan, China) following the manufacturer's instructions. Thereafter, 100 µl of the standards or the samples were added to each well and incubated for 2 hours at 37°C. The plates were then washed and incubated for 1 hour at 37°C with 100 µl/well of prepared detection reagent A. The plates were then washed and incubated with 100 µl/well of detection reagent B. After half an hour, at 37°C, the plates

Table 1 Demographics

	Group A							Group B
	Total	C1	C2	C3	C4	C5	C6	Total
Males	178 (32·90%)	23 (13·86%)	47 (27·01%)	63 (54·78%)	23 (52·27%)	10 (52·63%)	12 (52·17%)	9 (31.03%)
Females	363 (67·10%)	143 (86·14%)	127 (72·99%)	52 (45·22%)	21 (47·73%)	9 (47·37%)	11 (47·83%)	20 (68·97%)
Mean age	57·29	46·50	55	56·18	57·56	65	63·5	54·44
Median age	53·72	44	54·33	50	54·5	62	57·5	50
Age range	29–81	29–58	32–72	40–68	42–73	49–76	44–81	28–84
BMI (mean)	27·86	26·58	27·09	27·65	28·17	28·23	29·5	26·92
Smoke	182 (33·64%)	53 (31·92%)	58 (33·33%)	37 (32·17%)	17 (38·63%)	8 (42·10%)	9 (39·13%)	9 (31·03%)
Diabetes mellitus	62 (11·46%)	16 (9·64%)	19 (10·91%)	11 (9·57%)	7 (15·9%)	4 (21·05%)	5 (21·74%)	3 (10·34%)
Lipid disorders	84 (15·52%)	20 (12·05%)	24 (13·79%)	17 (14·78%)	10 (22·73%)	6 (31·58%)	7 (30·44%)	4 (13·79%)
Hypertension	75 (13·86%)	20 (12·05%)	17 (9·77%)	17 (14·78%)	9 (20·45%)	5 (26·31%)	7 (30·44%)	3 (10·34%)
Corona phlebectatica	135 (24·95%)	14 (8·43%)	23 (13·22%)	48 (41·74%)	23 (52·27%)	12 (63·15%)	15 (65·22%)	0
Total	541 (100%)	166 (30·68%)	174 (32·17%)	115 (21·26%)	44 (8·13%)	19 (3·51%)	23 (4·25%)	29 (100%)

C1–C6, clinical stages.

were washed 5 times, and 90 µl/well of substrate solution was added. After 15–25 minutes at 37°C, the reaction was stopped with 50 ml/well 2 M H_2SO_4, and the absorbance was read at 450 nm with a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Statistical analysis was performed using GraphPad Prism 2·01 (GraphPad Software, San Diego, CA, USA).

Results

During the study period, 541 patients with CVD, clinical stages C1–C6, were enrolled (178 males, 363 females; mean age 57·29, median age 53·72, age range 29–81), and they represented Group A.

Finally, 29 subjects without CVD were included in this study (9 males and 20 females; mean age 54·44, median age 50, age range 28–84) as the control group (Group B).

Complete demographic characteristics of both groups, and for each clinical stage, are shown in Table 1.

Figures 1 and 2 report the analysis of the levels of MMP-1, MMP-2, MMP-8, MMP-9, TIMP-1, TIMP-2, NGAL, ADAM-10, ADAM 12, ADAM-17, ADAMTS-1, ADAMTS-4, ADAMTS-5 and ADAMTS-7 during all the stages of CVD and in healthy subjects.

In the mild forms of CVD (C1–C2), we found an initial significant increase of MMP-2, ADAMTS-1 and ADAMTS-7 and an initial decrease of ADAMTS-5, TIMP-1 and TIMP-2 with respect to healthy controls.

Notably, C1–C2 patients with corona phlebectatica [14 (8·43%) for C1 patients and 23 (13·22%) for C2 patients] had higher values of the aforementioned metalloproteinases (MMP-2, ADAMTS-1 and ADAMTS-7) and of MMP-9 and NGAL and lower values of ADAMTS-5, TIMP-1 and TIMP-2 with respect to patients in the same group without corona phlebectatica. The proteases levels of patients with corona phlebectatica determined, in the C1–C2 groups, the increase of the upper range limit of the aforementioned MMPs, even coinciding with the levels of C3 patients (Figures 1, 2; Table 2).

The C3 patients, with moderate disease and with the initial stage of CVI, had similar protease values as the C1–C2 groups, except for a slightly higher value of ADAM-10, ADAMTS-4, MMP-9 and NGAL that indicates a more intense inflammatory response with respect to the C1–C2 patients (Figures 1, 2; Table 2).

For more advanced stages of CVI (C4–C6), with skin changes, we found higher values of MMP-1, MMP-8, ADAM-10, ADAM 17, MMP-9, NGAL and a very significant increase of ADAMTS-4, which reached the maximum level in the ulceration stage (C6) (Figures 1, 2; Table 2).

Among patients with chronic venous ulceration (CVU), we identified a subgroup of five patients (three males and two females) with hard-to-heal and long-lasting ulcers, with an important elevation of MMP-1, MMP-8 ADAM-17 and ADAMTS-4 and with a marked decrease of ADAMTS-5, TIMP-1 and TIMP-2 (Table 2).

ADAM-12 does not appear to be involved in any stage of CVD, and its values were always similar to the healthy control group.

ADAMTS-1 and ADAMTS-7 were generically elevated in all stages of CVD with respect to the control group, but there was no significant difference between the stages of CVD.

ADAMTS-5, TIMP-1 and TIMP-2 decreased progressively during the worsening of CVD.

Table 2 shows a glance of the full spectrum of clinical manifestations of CVD and their relationship with the proteases investigated.

Discussion

CVD is a common condition in Western countries that has an important socioeconomic impact because of its high prevalence in the general population (1,2). CVD is a progressive disease that is well and simply described, in its clinical component (C classes), by the CEAP classification (3). Mild forms of CVD are represented by the first two classes: teleangectesias (class C1), that is, the confluence of dilated intradermal venules less than 1 mm in calibre, and reticular veins (class C1) that are dilated, usually tortuous, bluish subdermal veins, usually 1 mm to less than 3 mm in diameter and varicose veins (class C2) that are subcutaneous, dilated and tortuous veins, 3 mm in diameter or larger and may involve saphenous veins, their tributaries or the non-saphenous superficial veins of lower limbs (1). Moderate

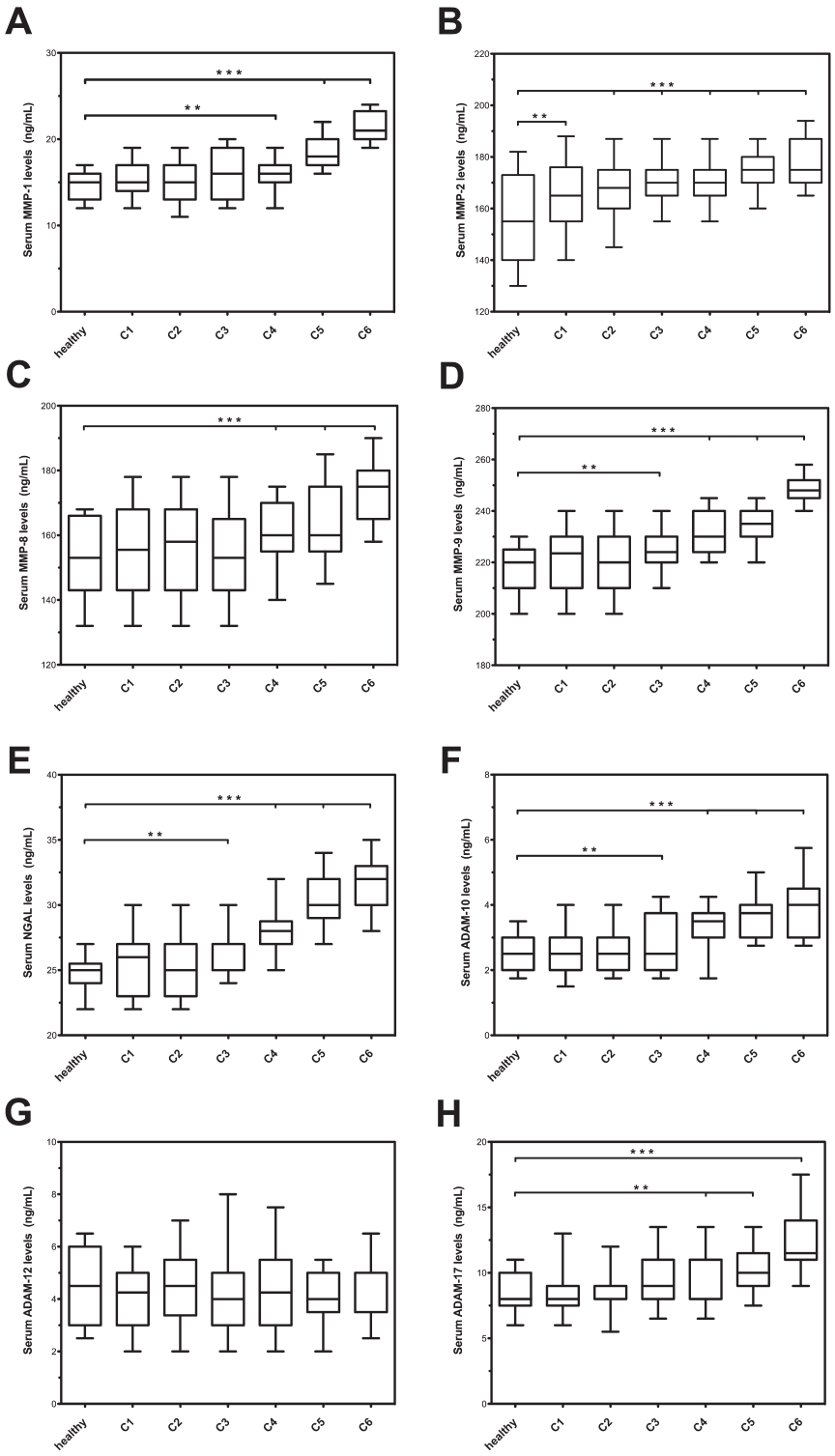

Figure 1 Serum levels of MMP-1 (matrix metalloproteinases), MMP-2, MMP-8, MMP-9, NGAL (neutrophil gelatinase-associated lipocalin), ADAM-10 (a disintegrin and metalloproteinases), ADAM-12, ADAM-17. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

to severe forms of CVD refer to CVI, which may include oedema (class C3), skin changes (class C4) such as eczema or pigmentation (C4a) and lipodermatosclerosis or atrophie blanche (white atrophy) (C4b) and, finally, healed venous ulcers (class C5) or active venous ulceration (class C6) (1).

A further sign, that is corona phlebectatica, is not actually included in the CEAP classification, but it is thought to be an early sign of advanced venous disease (4,5).

Several studies have described defects in the regulation of the composition of the ECM and subsequent wall remodelling in varicose veins, leading to decreased elasticity and increased distensibility of the vessel wall (17,18). Thus, a genetic defect in the regulation of the composition of the ECM might be involved in the pathogenesis of varicose veins (2). Such a genetic defect and ECM alterations should also affect different connective tissues as seen in

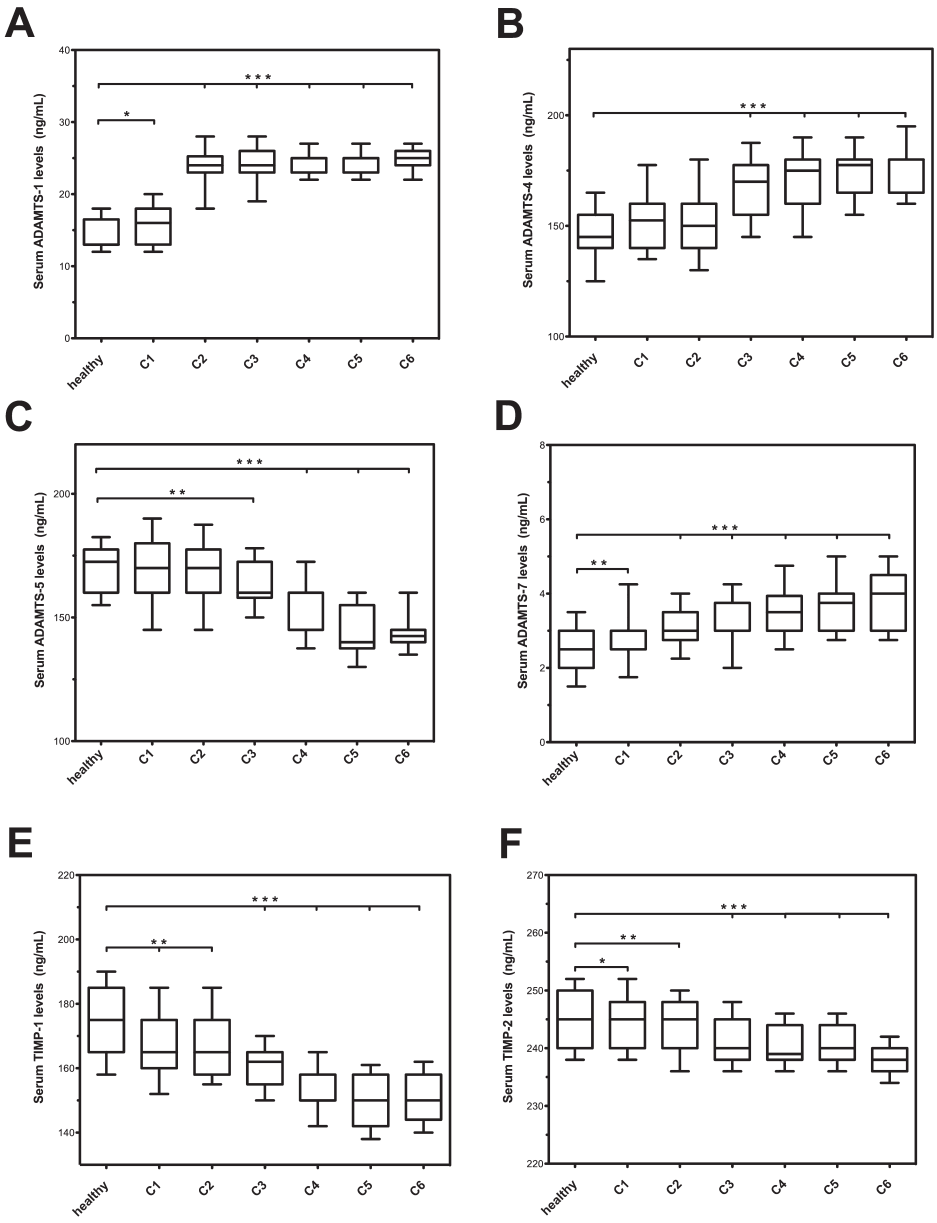

Figure 2 Serum levels of a disintegrin and metalloproteinases with thrombospondin motifs (ADAMTS)-1, ADAMTS-4, ADAMTS-5, ADAMTS-7, tissue inhibitor of metalloproteinases (TIMP)-1, TIMP-2. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

several documented experiences patients with concomitant CVD (6,19,20).

The ECM provides a structural framework and plays an essential role in the structure and function of vessel walls (21) as well as in the metabolism and homeostasis of skin tissues (22).

In fact, metalloproteinases function not only as regulators of ECM composition and structural integrity but also as important mediators in the control of cellular interactions and response to their environment in conditions that promote tissue turnover and wound healing. In this context, metalloproteinases are involved in the functional regulation of several ECM molecules such as growth factors and their receptors, cytokines, chemokines, adhesion receptors and a variety of related enzymes (23).

There are three main families of metalloproteinases that are involved in cardiovascular disease: MMPs ADAMs and ADAMTSs; and TIMPs regulate their activities (8,15,16).

MMPs are a group of several multi-domain zinc-dependent enzymes that are involved in the remodelling of several components of ECM (24). In the vasculature, MMPs influence the migration, proliferation and apoptosis of vascular smooth muscle cells, endothelial cells and inflammatory cells, thereby affecting intima formation, atherosclerosis, aneurysms, varicose veins and its complications, such as venous ulceration and post-thrombotic syndrome (6–14,25,26). Various MMPs patterns have been identified to indicate several stages within the disease. MMP-2, MMP-9 and NGAL (which is a protein belonging to the lipocalin family, with the ability to positively

Table 2 Synopsis – CVD clinical manifestations and proteases

	Healthy subjects (controls)	C1 patients	C2 patients	C3 patients	C4 patients	C5 patients	C6 patients	Subgroup of C1–C2 patients with corona flebectatica	Subgroup of C6 patients with hard-to-heal ulcers
MMP-1	+	+	+	+	++	++	+++	+	++++++
MMP-2	+	++	+++	+++	+++	+++	+++	+++	+++
MMP-8	+	+	+	+	++	++	+++	+	++++++
MMP-9	+	+	+	++	+++	+++	++++	++	+++++
NGAL	+	+	+	++	+++	+++	++++	++	+++++
ADAM-10	+	+	+	++	+++	+++	++++	++	+++++
ADAM-12	+	+	+	+	+	+	+	+	+
ADAM-17	+	+	+	++	+++	+++	++++	++	++++++
ADAMTS-1	+	++	+++	+++	+++	+++	+++	+++	+++
ADAMTS-4	+	+	+	+++	++++	+++++	+++++	+++	+++++++
ADAMTS-5	+++++	++++	+++	++	++	++	+	++	+
ADAMTS-7	+	++	+++	+++	+++	+++	+++	+++	+++
TIMP-1	+++++	++++	+++	++	++	++	+	++	+
TIMP-2	+++++	++++	+++	++	++	++	+	++	+

ADAMTS, a disintegrin and metalloproteinases with thrombospondin motifs; C1–C6, clinical stages; NGAL, neutrophil gelatinase-associated lipocalin; MMP, matrix metalloproteinases; TIMP, tissue inhibitor of metalloproteinases.

modulate the activities of MMP-9) appear to be mostly involved in the acute phase of vascular disease (such as aneurysm rupture and pulmonary embolism) (27,28), while MMP-1 and MMP-8 appear to be primarily involved with chronic or irreversible complications of vascular disease (such as difficult-to-heal or infected venous ulcers and post-thrombotic syndrome) (13,14,26).

The ADAMs are a family of transmembrane and secreted proteins that have functions in cell adhesion and the proteolytic processing of the ectodomains of diverse cell surface receptors and signalling molecules, and they have been identified in many species. ADAMs have been linked functionally in many biological processes as well as in some cardiovascular conditions (15). In fact, ADAM-12 is expressed above all in cardiomyocytes and fibroblasts, and its inhibition prevents cardiac hypertrophy (29). It has also been identified as an important mediator of vascular smooth muscle cells (VSMCs) hypertrophy and appears to be directly involved in hypertension vascular diseases (30,31). Both ADAM-10 and -17 appear to be involved in immune and inflammatory responses by regulating pro-tumour necrosis factor (TNF)-α activation and other related molecules and cytokines (15).

The ADAMTS members descend from the ADAM family of proteases; they have diverse functions and major roles, including the maturation of proproteins such as procollagen and extracellular matrix remodelling during morphogenesis. ADAMTS deficiencies lead to a variety of congenital anomalies, inherited connective tissue disorders, a haemostatic defect (thrombotic thrombocytopenic purpura) and infertility, and they also appear to be involved in vascular disease. In fact, in this family, we can find that ADAMTS-1, the founding member of the ADAMTS family, is an angiogenic factor that is induced by inflammatory mediators such as lipopolysaccharide and tumour necrosis factor alpha. In atherosclerotic lesions, it is expressed by smooth muscle cells (32,33).

ADAMTS-1 and ADAMTS-4 protein and mRNA expressions were significantly higher in thoracic aortic aneurysms and dissection tissues than in healthy aortic tissues (34)

ADAMTS-5 has been suggested to be even protective for the vascular wall as it is depleted in atherosclerotic aortas, regulating vascular proteoglycan catabolism and altering lipoprotein retention (35).

ADAMTS-7 is known to play an important role in vascular wall homeostasis as it contributes directly to neointima formation by mediating vascular smooth muscle cell migration (36,37), and it may also represent a novel therapeutic target for atherosclerosis and for preventing postangioplasty restenosis (38).

The TIMPs are tissue-specific, endogenous inhibitors of metalloproteinases, including MMPs as well as the closely related ADAMs and ADAMTS (39), and the most studied are represented by TIMP-1 and TIMP-2 (26), which are also involved in several vascular diseases (26,40).

In healthy tissues, TIMPs appear to spare the ECM from degradation, and only the following injury/infection TIMPs appear to indirectly control ECM deposition. However, there is still much to be determined about their roles and functions (39).

This is the first study that considered and analysed a wide range of metalloproteinases (MMPs, ADAMs and ADAMTs), their inhibitors (TIMPs) and NGAL in the context of CVD and its full range of clinical manifestations.

The study found that the elevation of MMP-2, ADAMTS-1 and ADAMTS-7 appears to be correlated with the initial stages of CVD and may represent the start of the disease; aADAM-10, ADAM-17, ADAMTS-4, MMP-9 and NGAL appear to be particularly active in the inflammatory progression of the disease towards the onset of overt CVI and remained elevated during the more severe clinical manifestations, including CVU.

MMP-1, MMP-8, ADAM-17 and ADAMTS-4 appear to be primarily involved with chronic or irreversible complications of vascular disease and delayed wound healing.

ADAM-12 appears not to be involved in venous disease as there was no difference between patients with CVD in all stages and healthy subjects.

ADAMTS-1 and ADAMTS-7 were found to be generically elevated in all stages of CVD with respect to the healthy subjects.

ADAMTS-5, TIMP-1 and TIMP-2 were negatively associated with the progression of the disease and decreased progressively during the worsening of CVD.

In this study, patients with corona phlebectatica were found to have a marked increase of the inflammatory MMPs (ADAM-10, ADAM-17, ADAMTS-4 and MMP-9) and NGAL, even in the first stages of CVD, and this appears to justify the concern that corona phlebectatica may be a good predictor of the advanced forms of CVD with skin changes (4,5,41).

This study clearly showed that each stage of CVD may be described by particular patterns of metalloproteinases, and this may be useful for identifying new therapeutic targets in order to prevent or to better treat clinical manifestations related to CVD in the near future.

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