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Review Article

Matrix metalloproteinase activity and glycosaminoglycans in chronic venous disease: the linkage among cell biology, pathology and translational research

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Abstract: Primary chronic venous disease (CVD) is an inflammatory pathology involving an erratic structural remodeling in the venous well leading to vascular incompetence and the development of varicose vein, characterized by altered collagen and elastin content. In the early steps of varicose vein formation is crucial the role of MMP/TIMP balance, implicated in both ECM and vascular degradation during inflammation processes in early and late stages of venous diseases. Although several pharmacological and surgical strategies are being utilized in the management of varicose vein and CVD with variable success and recurrence rate, inhibition of MMP through glycosaminoglycans may represent a novel therapeutic intervention to limit the progression of varicose vein to CVD and leg ulceration, suggesting possible opportunity to prevent future morbidity and enhancing clinical benefits and quality of life.

Keywords: Matrix metalloproteinase, glycosaminoglycan, chronic venous disease, inflammation, venous ulcer, leukocyte, fibroblast, dermatan sulphate, varicose vein, tissue inhibitor of MMP

Introduction

Primary chronic venous disease (CVD), with well-known diversity of symptoms, clinical signs and prevalence, is a common worldwide pathology affecting mainly the adult population[1]. In general, "degeneration" of the peripheral veins leads to dilatation of the lumen and insufficient closure of the valves resulting in a backflow of blood from deep to the superficial venous system [2], leading to ambulatory hypertension in the superficial venous system and recirculation of large amounts of draining blood remaining in the affected leg of venous drainage blood in the affected leg [3]. Without treatment this results in the long term in variable degrees of decompensation of the recirculation pathways and an increasing secondary insufficiency of the deep venous system [4]: the result corresponds to the clinical symptoms of chronic venous insufficiency (CVI), an advanced form of CVD characterized by a sustained ambulatory venous hypertension and venous-specific skin changes (*e.g.,* leg edema, dermal hyperpigmentation, eczema, lipodermatosclerosis and finally ulceration) [5].

In patients with CVI, the lifetime of risk of chronic venous ulceration (CVU) is around 1% with approximately 10% of ulcers being open at any one time, and the incidence of skin changes disease is about 10 times greater (10%) [4,6,7]. However, many of the studies upon which these estimates are based are old and/or methodologically flawed, and there is reason to believe that the incidence, prevalence and characteristics of CVI/CVU may have changed considerably over the last 10-20 years and that future change in determining the actuarial numbers is likely [8].

As schematized in **Figure 1**, CVI culminating in CVU is primarily the result of sustained ambulatory venous hypertension, which in turn arises from superficial and/or deep venous reflux with

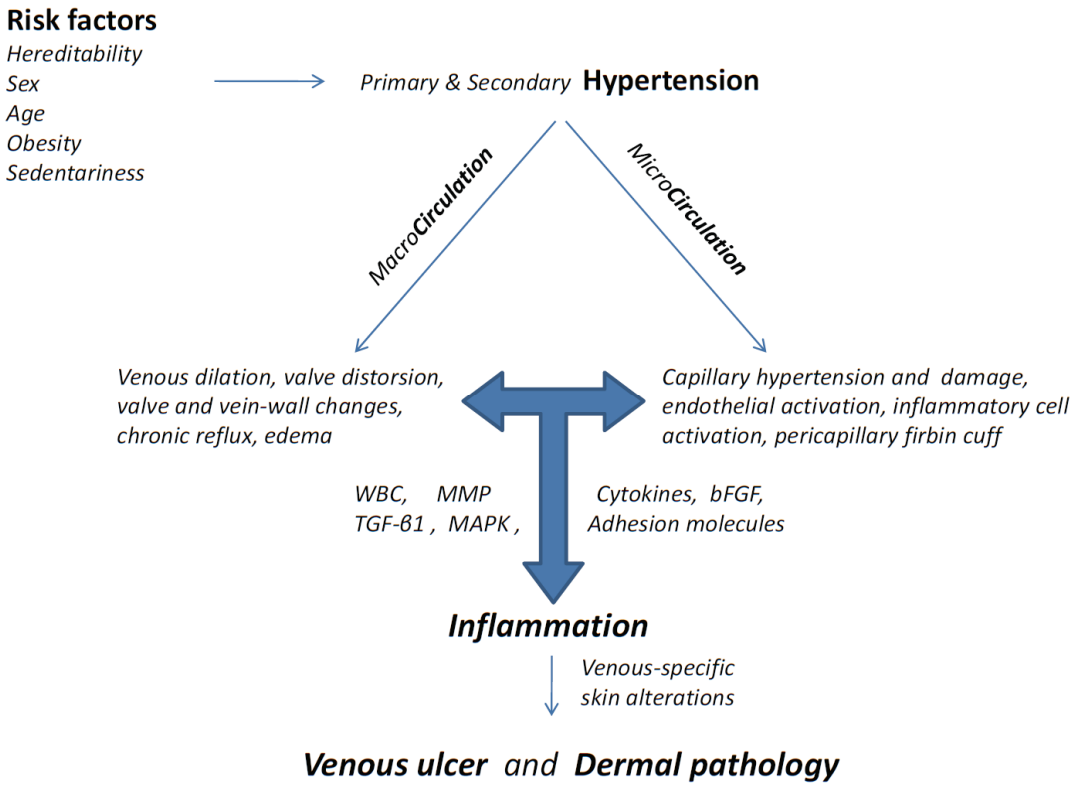

Figure 1. Schematic diagram of the pathophysiology of chronic venous disease and its progression leading to skin alterations. The cascade emphasizes the presence of predisposing factors leading to venous hypertension, causing changes in both the macrocirculation and microcirculation, and the central role of inflammatory cell activation, resulting in cytokine, growth factor, and MMP production and the unabated inflammatory and proteolytic environment that eventually leads to dermal skin changes and venous ulcer formation. WBC, white blood cells; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; TGFβ1, transforming growth factor β1; bFGF, basic fibroblast growth factor; MAPK, mitogen activated protein kinase.

or without deep vein obstruction. However, there are many other elements to this complex condition, for example, microvascular dysfunction; congenital and acquired thrombophilia; obesity and diet; muscle pump efficiency; dermal inflammation; recruitment of white blood cells; production and secretion of cytokines; stimulation of adhesion molecules and metalloproteinases; disordered fibroblast function and matrix rearrangement; bacterial colonization; failure of epithelialization; acute and chronic wound; ulcer and necrosis [1, 7].

Among the many proposed mechanisms linking venous hypertension to CVD, CVI and finally to CVU the complex interplay between inflammatory responses by leukocytes, cytokine cascade, stimulation of adhesion molecules and matrix metalloproteinase (MMP) activity may lead to cell dysfunction resulting in dermal changes observed clinically in patients [5].

Although we are beginning to understand the pathophysiologic mechanisms in CVD, several areas of our knowledge need to be addressed in order to perform translational studies reducing the risk of CVD progression. In particular, in this review we will focus attention on: 1) how glycosaminoglycan components of the extracellular matrix (ECM) may be involved in the unrestrained MMP activity; 2) how the MMPs may contribute to the venous dilation, and during inflammation to the breakdown of ECM components promoting then the ulcer formation and impairing healing; 3) how pharmacologic agents based on glycosaminoglycans could attenuate various elements of both the inflammatory cascade and inhibit the crucial proteolytic step,

offering through translational researche a greater opportunity to prevent future morbidity that is associated with CVD.

Valve and Vein-wall structural changes in CVD

CVD has been estimated to account for 1-3 % of the total health care budgets in countries with developed health care systems [8]. CVD is associated with a reduced quality of life, particularly in relation to symptom ascribed to aching, pain, heaviness, depression, cramps, itching, tingling and restless legs and ulcers [3]. In the absence of trophic skin changes, the two major mechanisms are 1) *Hypoxia* of the tunica media of the venous wall due to alteration of the vasa vasorum, and 2) *Venous wall tension* resulting from both dilatation of the vein due to hypertension and valvular insufficiency, and causing superficial and/or deep venous reflux [4]. Despite the diversity of signs and symptoms associated with CVD, it seems likely that all are related to *Venous Hypertension*, linked to altered hydrostatic and hydrodynamic components (e.g., elevated leg venous pressure for prolonged periods) and significantly influenced by the action of venous valves (due to structural changes in valve vein-wall changes) [1].

In this respect, ultrastructural morphology and biochemical studies of varicose veins have found hypertrophy of the vein wall with altered collagen and elastin content [9-12], together with disruption of the glycosaminoglycan rearrangements [13-20]. In particular, disturbed collagen synthesis with elevated ratios of type I to type III collagen, may significantly contribute to the weakness and reduced elasticity of varicose veins [21-23]. A complicating factor is the heterogeneity of the varicose vein wall; in fact, in CVD patients hypertrophic segments can alternate with thinner atrophic segments with reduced ECM and increased number of inflammatory cells (e.g., mast cells, macrophages, and neutrophils) [4], suggesting that both inflammation and degradation of ECM proteins would be crucial in the early etiopathogenesis of CVD [24].

Interestingly, the role of different glycosaminoglycan (GAG) species from the vessel walls has been extensively studied, suggesting that 1) the saphenous vein (the more frequent locus of CVD) contains high percentage of collagen and dermatan sulfate (DS) [17,18,20]; 2) the content of sulfated GAG was significantly increased in varicose vein, in particular in the presence of thrombophlebitis [19]; 3) the DS may have anticoagulant and antithrombotic activity [13]; 4) the disorders of vascular DS metabolism may contribute to vascular pathology and remodeling [14].

ECM proteolytic degradation and CVD initiation/progression

The ECM is an important structural and functional scaffolding made up of proteins (such as collagen, elastin, fibronectin, growth factors, proteoglycans, and glycosaminoglycans) that are necessary for a variety of cell functions, including cell differentiation and signaling, cellular migration, angiogenesis, blood vessel support, epithelialization, and wound repair [25]. Degradation of ECM is mainly caused by an array of proteolytic enzymes, including matrix metalloproteinases (MMPs) and serine proteases, which are produced by both vascular (endothelial cells, fibroblasts) and white blood cells [26,27], in particular during inflammation [28,29]. MMPs are released as inactive proenzymes that are finely regulated [30,31,27] in order to achieve the appropriate function during physio-pathologic conditions, such as activation by other proteinases, formation of complexes with regulatory proteins, inactivation by endogenous tissue inhibitors TIMP, and reciprocal interactions/controls with ECM peptidoglycans [32,33].

The involvement of MMP and TIMP in vascular diseases is a matter of a strong and continuous scientific interest, especially in the study of effective MMP modulators that would be important in the management of patients with arterial and venous diseases [34,35]. In particular, for what concerns CVD it has been suggested that the balance between MMP and TIMP play a crucial role in early steps of varicose vein formation in the lower extremities as well as in their progression to thrombophlebitis and venous leg ulcers [5,36].

It has been widely documented that the effects of MMP and TIMP on ECM degradation may result in a significant venous tissue remodeling [37,38], degenerative and structural changes in the vein wall [9,39], leading to venous dilation and valve dysfunction [2,40]. Taken together, increased MMP activity and altered MMP/TIMP balance [41,42] may also induce early modifications in the endothelium and venous

smooth muscle function in the absence of significant ECM degradation or structural changes in the vein wall. In addition, evidence suggests that increased activity of MMP is also present in the advanced stages of CVD encompassing skin changes and CVU [43-46], as well as in the wound fluid microenvironment [47,48].

Studies have highlighted that chronic wound fluid contained up to tenfold increased levels of gelatinase MMP [49-52], suggesting a high tissue turnover in ulcers and an imbalance of MMP/TIMP ratio; moreover, the inhibition of MMP activity by TIMP in ECM (by fibroblast and probably also glycosaminoglycans) may cause impaired ability to reorganize the ECM in chronic wounds leading to delayed healing [5,29,53-58]. These studies should indicate that although there is significantly increased MMP proteolytic activity in the venous ulcer and wound fluid, both cellular and extracellular components may be compensating by altering their expression of MMP and TIMP [5,54,59].

The altered MMP/TIMP balance may be crucial in CVD initiation and progression to CVI and finally CVU, due to the increased secretion of proteolytic enzymes from vascular cells and inflammatory cells (like macrophages, neutrophils and mast cells) [4,39,60,61]. The abnormalities in structure and healing processes seen in inflammatory and lipodermatosclerotic skin have also been attributed to MMP-mediated pathophysiology [62]; in fact, in lipodermatosclerotic and chronically inflamed skin, which are precursors to venous ulcer formation there is an unrestrained MMP activity [45,63], an imbalance of MMP/TIMP ratio [46,48] and an excessive ECM turnover [18-20], with an altered glycosaminoglycan accumulation/ degradation in vein wall [13,14,64,65].

For all these reasons, MMPs have been directly implicated in the pathophysiology of many arterial and venous disorders (in particular in CVD) and remain an important potential therapeutic target [66], even though more studies are needed to further demonstrate the clinical benefits of MMP modulating agents [67].

Future perspective in therapeutic interventions

Several pharmacological therapies and surgical strategies are being utilized in the management of varicose veins and CVU, with variable success and recurrence rates, and have been recently reviewed [1,3-5,7,8,68].

Although the causal and temporal sequences of events occurring in the development and progression of CVD (as well as suggested also by the diversity of signs and symptoms during the variable rates of disease evolution) have not been fully ascertained, the emerging themes of disturbed venous-flow patterns, chronic inflammation and in situ proteolysis may underlie all the clinical manifestations of the CVD (**Figure 1**), leading nevertheless to a blunted perception of cause and effect among clinicians [3].

In this respect, early treatments aimed at preventing venous hypertension and reflux, as well as treatments focused to inhibit the proinflammatory process, could offer an even larger opportunity to prevent future morbidity, alleviating symptoms of CVD and reducing the risk of CVI and ulcers, both of which reduce the quality of life and are expensive to treat. Rapidly advancing understanding of both cellular and biomolecular mechanisms involved in CVD initiation and progression has allowed the identification of new targets for possible future pharmacologic intervention, which are mainly related to the cellular and biomolecular aspects of disease, like collagen type I and III, transforming growth factor β1, basic fibroblast growth factor, vascular endothelial growth factor, cytokines, mast and neutrophils cells, fibroblast, integrin CD11b, iron, ICAM1, L-selectin, TIMP, and MMPs [3]. Even though currently and future available drugs/agents deserve more detailed studies, it therefore seems reasonable to speculate that the treatment of such targets could reduce the risk of ulcers if administered early in the course of CVD, especially inhibiting inflammation and preventing disease-related complications [4].

A proactive approach to the treatment of the early and late stages of CVD may be focused on the inflammation-related and MMP-dependent proteolysis [59]. In fact, actually the inhibition of MMPs may represent a realistic, novel and possible therapeutic intervention to limit the progression of varicose vein to CVI and leg ulceration [5]. The therapeutic hypothesis is based on the well known role of glycosaminoglycans (especially dermatan sulfate) in health and disease, in wound healing and vein remodeling

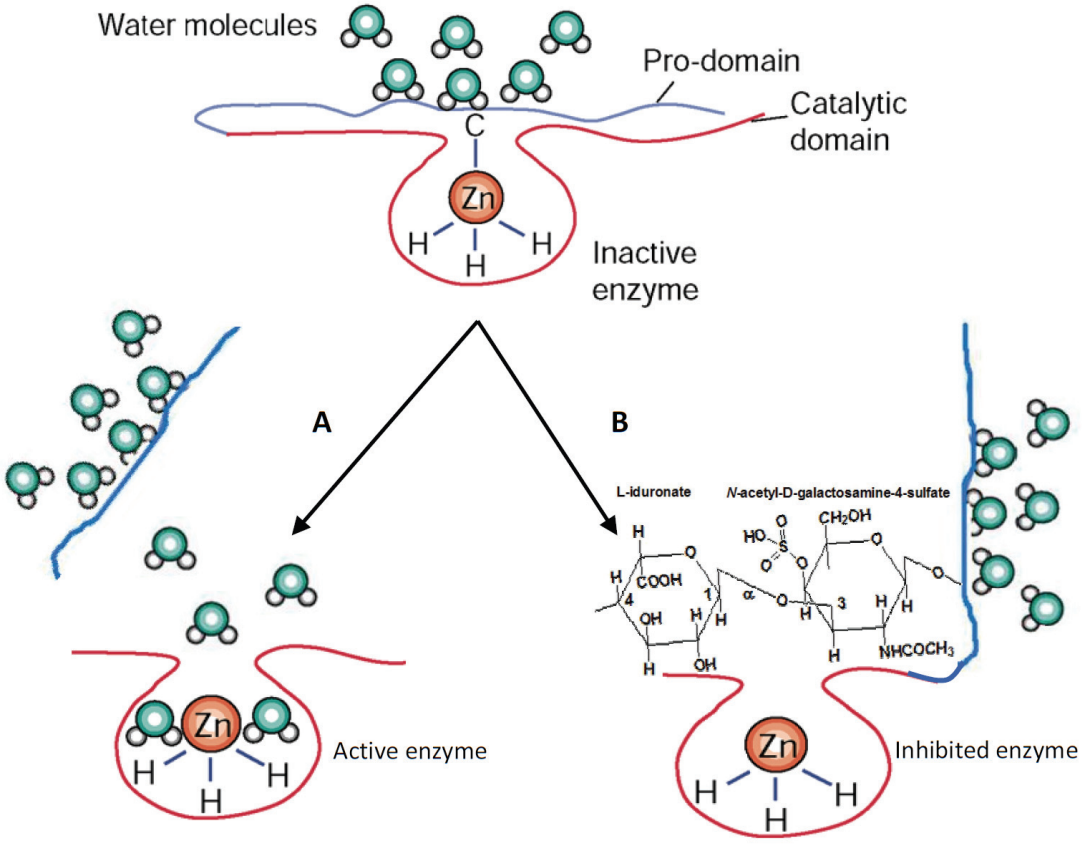

Figure 2. Scheme of MMP-9 modulation mechanisms. A) The classical chemical or proteolytic multistep removal of pro-domain, resulting in gelatinase B activated form with lower molecular weight. In the zymogen enzyme, the sulfhydryl group of the cysteine (-C) present in the pro-domain is coordinated to the zinc (Zn) atom of the catalytic site in a manner that covers the active site and renders the enzyme latent. Note that the zinc atom is bound by three histidine molecules (-H), which is characteristic of the conserved zinc-binding motif in the catalytic site of MMPs. The cysteine residue of the pro-domain is fully dissociated on removal of the zinc atom by EDTA (a divalent ion chelator) and orthophenanthroline (an inhibitor of MMPs). It is also released by proteolytic loss of pro-domain after autolytic activation, or activation by trypsin or other proteinases. Finally, the enzyme lacking the pro-domain results in fully activated gelatinase B form with lower molecular weight. B) In our hypothetical model of MMP-9 inhibition induced by the dermatan sulphate sulodexide, the pro-domain is only opened and not detached (e.g., via hinge-like opening), allowing the interaction between hydrated form of the dermatan sulfate and the zinc atom at the catalytic site. By this interaction, sulodexide stabilizes gelatinase B conformation and inhibits the enzyme activity without loss of the pro-domain.

[13,69,70]. In particular, it has been found that the amounts of collagen and dermatan sulfate were higher in the saphenous vein than in the mammary artery [17], suggesting also the involvement of vessel glycosaminoglycans in the process of atherosclerosis [18]. Moreover, it has been found that the most abundant glycosaminoglycan in human veins is dermatan sulfate whereas chondroitin 4/6-sulfate is preponderant in arteries [20], and that normal and varicose saphenous veins differed in their glycosaminoglycan contents [19].

Given the importance of GAG in vessel biology and disease, it is noteworthy to highlight the role of GAG (in particular dermatan sulfate) in the proteinases activity regulation [65,71], especially for MMP in fibroblasts dermal explants [72] and TIMP-3 [73]. We have recently studied the in vitro effects on MMP circulating in blood treated with the glycosaminoglycan sulodexide [74], to evaluate the possible modulation of MMP activity/secretion by the highly purified glycosaminoglycan sulodexide, a drug useful in venous ulcer treatment [75-77]. Sulodexide is a

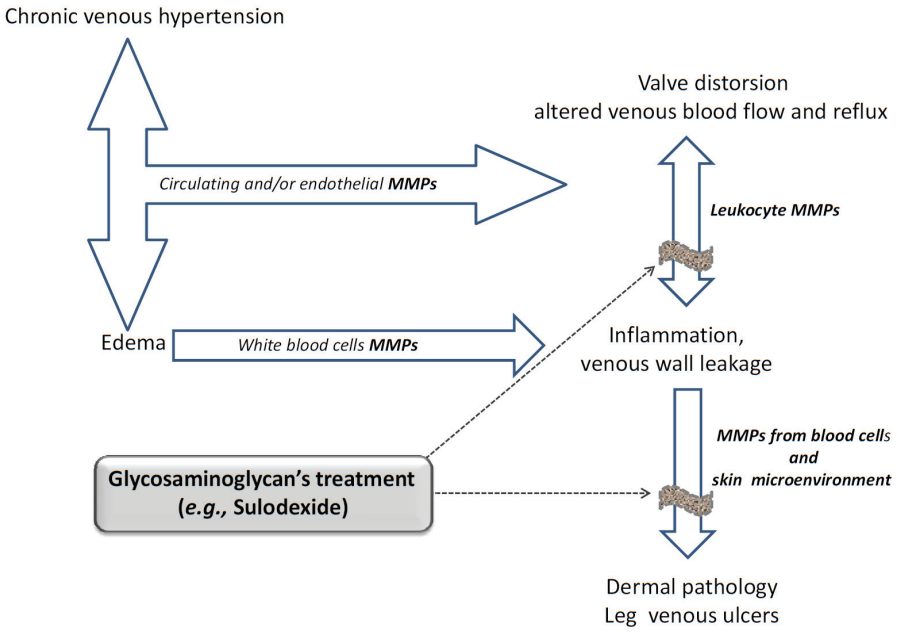

Figure 3. Risk factors for Chronic Venous Disease: involvement of Matrix Metalloproteinases and possible therapeutic application of glycosaminoglycans (e.g., Sulodexide).

highly purified mixture of glycosaminoglycans composed of 80% low molecular weight heparin and 20% dermatan sulfate, which exhibits antithrombotic and pro-fibrinolytic properties. Sulodexide main pharmacologic effects are characterized by a prolonged half-life and involving effects on global coagulation (e.g., increasing the bleeding parameters INR, PTT, and bleeding time) [78].

Sulodexide decreases blood viscosity and enhances fibrinolytic and lipolytic activities, which is useful in vascular-related pathologies of an aging population (e.g., diabetic nephropathy, peripheral vascular diseases, and post-thrombotic syndrome) [79,80].

Studying the proteolytic activity, enzyme levels, and active secretion of both MMP-2 and MMP-9 in peripheral blood collected from healthy subjects, as well as in cultured leukemia cells, we found that increasing amounts of sulodexide may modulate both proenzyme and complexed forms of MMP-9, with significant decrease of MMP-9 secretion from white blood cells in a dose-dependent fashion[74], without any displacement of the MMP prodomains, indicating that sulodexide possible mechanism is by directly inhibiting the active zinc binding site of the proMMP-9 molecule (**Figure 2**). Our suggested model of sulodexide-induced MMP-9 inhibition is in agreement with the well-known mechanism of inhibition of MMP latent enzyme, which can also be brought about by several agents, such as synthetic collagen peptidomimetic compounds, and tetracycline derivative [81,82], which inhibit the enzyme activity by stabilizing the conformation of the gelatinase B protein where the cysteine is spatially displaced from the active site zinc atom without loss of pro-domain and change of molecular weight. Understanding the mechanism of reduced release of MMP-9 forms from leukocytes and inhibition of proteolytic activity due to sulodexide treatment may provide novel therapeutic applications in chronic inflammatory diseases (e.g., chronic venous diseases associated with MMP activation in blood and limbs), according to the well-established clinical effects, but not the biomolecular mechanisms of sulodexide in the treatment of venous diseases and leg venous ulcers [75,77,80], and the established involvement of MMP-9 in venous tissue remodeling and pathology [59,5].

Although the possible mechanism(s) of the reduction/inhibition of MMP-9 release from blood cells through sulodexide treatment is not actually known, pharmacological agents that could attenuate MMP release from leukocytes and into the venous diseases microenvironment may offer a further opportunity to limit the progression of vein pathology, preventing future morbidity (**Figure 3**). In fact, previous evidence

showed that sulodexide, administered topically as a local treatment in addition to compression bandaging [77], was associated with more frequent and faster venous ulcer healing without clarifying the mechanism of sulodexide-dependent ulcer healing.

Based on our in vitro preclinical results [74], and clinical studies that are actually in progress (Raffetto and Mannello, personal communications), we are investigating which component of sulodexide is responsible for the best MMP-modulation, in order to set the basis for future randomized controlled studies evaluating the safety and efficacy of new formulations in patients with venous diseases. These studies will improve the understanding of venous pathophysiology by allowing the following: 1) translating the research into clinical medicine; 2) elucidate the sulodexide-dependent mechanism of MMP-9 release/inhibition; 3) provide therapeutic benefit in reducing the symptoms of chronic venous disease, a worldwide pathology with high economic implication that accounts for 3-5 % of the national health expenditures in many western countries [59,5,3].

Finally, although these studies deserve more detailed investigations, in the long term the improved understanding of the cellular and biomolecular mechanisms involved in CVD and CVU and the clinical evaluation of possible agents as sulodexide that attenuate crucial inflammatory-linked MMP proteolysis, could enhance the translational research to slow or prevent disease progression from the earliest stages, improving the quality of life and reducing expenditure of intractable ulcer treatment.

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